Studies into the *in vivo* interactions between human immunodeficiency virus and human herpesvirus 8

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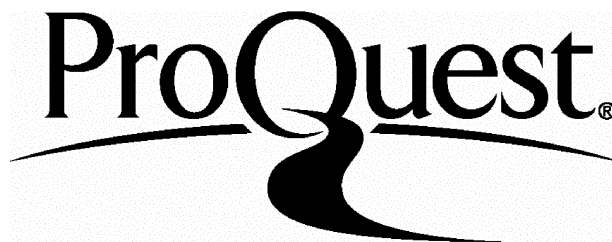
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This thesis is dedicated to my beloved wife Ana Claudia and my

precious children Jair Filho & Ana Catarina.

DECLARATION

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

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ABSTRACT

In HIV-coinfected people unaffected by Kaposi sarcoma (KS), circulating HHV-8 DNA and anti-HHV-8 antibodies are detected particularly frequently and at high levels. Studies were conducted to determine whether HHV-8 hyperactivity in such individuals could be the consequence of immunosuppression-associated reactivation, transactivation by HIV or the propensity of the host to multiple HHV-8 infection.

HHV-8 subgenomic DNA was amplified by PCR from peripheral blood of HIV-infected patients, health care workers and patients who had undergone bone marrow transplantation (BMT), and from oral lesional tissues of patients with AIDS-associated KS. Clones derived from amplicons originating from DNA fragments in open reading frame (ORF) 26 and ORF K1 were isolated. For each ORF, intra-specimen nucleotide sequence differences were determined. The extent of HHV-8 variation in clones derived from blood of HIV-positive patients was significantly higher than in blood from health care workers or post-BMT patients or in AIDS-KS lesional tissue. Among the clones derived from the latter three categories of specimens, sequence variations were not significantly different.

To investigate if HIV plays a role in inducing HHV-8 hyperactivity, the frequency of circulating HHV-8 DNA, HIV load, anti-HHV-8 antibody level and CD4+ T cell counts was studied before and after therapy with protease inhibitors. While there was an increase in the CD4+ T cell counts and a

decrease in HHV-8 DNA, no significant differences in the other variables were observed.

The findings of this study suggest that HIV-infected individuals tend to be multiply-infected with HHV-8, that each AIDS-KS lesion is associated with infection by a single HHV-8 variant or a small group of related variants, and that HIV may influence, or be associated with the replication of HHV-8.

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Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
APS	ammonium persulphate
BCBL	body cavity based lymphoma
BMT	bone marrow transplant
bp	base pair
CIs	confidence intervals
CMV	cytomegalovirus
CPHL	Central Public Health Laboratory
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
ds	double stranded
EBV	Epstein-Barr virus
EDTA	disodium ethylenediaminetetra-acetate
ELISA	enzyme linked immunosorbant assay
GRC	G protein coupled receptor
HAART	highly active antiretroviral therapy
HCW	health care worker
HHV-6	human herpesvirus 6
HHV-7	human herpesvirus 7
HHV-8	human herpesvirus 8
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSV-1	herpes simplex virus 1
HSV-2	herpes simplex virus 2
HVS	herpesvirus saimiri
IFA	indirect immunofluorescence assay
IL-6	interleukin-6
IL-8	interleukin-8

IgE	immunoglobulin class E
IgG	immunoglobulin class G
IgM	immunoglobulin class M
IPTG	isopropylthiogalactoside
kb	kilobase pair
LANA	latency-associated nuclear antigen
KS	Kaposi's sarcoma
MCD	multicentric Castleman's disease
MGD	mean genetic distance
MIP	macrophage inflammatory protein
MGUS	monoclonal gammopathy of undetermined significance
μL	microlitre
ml	millilitre
MM	multiple myeloma
mRNA	messenger RNA
nt	nucleotide(s)
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEFF	paraffin embedded formalin fixed
PEL	primary effusion lymphoma
PrI	protease inhibitor
RE	restriction enzyme
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT	reverse transcription
RTI	reverse transcriptase inhibitor
SSCP	single stranded conformation polymorphism
ss	single stranded
sVCA	small viral capsid antigen
Taq	Thermus aquaticus
TBE	tris-borate-EDTA

TE	tris-EDTA
TEMED	tetramethylethylenediamine
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase

Chapter 1 - Review of the literature; hypotheses and aims of the study

1.1 Kaposi's sarcoma (KS)

1.1.1 Historical aspects

Moritz Kaposi first described five patients with an unusual vascular tumour of the skin (Kaposi, 1872). Similar cases of this malignancy - ultimately termed Kaposi's sarcoma (KS) - were subsequently reported from several parts of Europe and North America. Until the 1950's KS was known to be an extremely rare malignancy, except in southern Europe, where elderly patients of Mediterranean and of Ashkenazi Jewish origin were particularly affected (Oettle, 1962). These early cases were the first reported of classic or Mediterranean KS (CKS/MKS), a disease that usually occurs in elderly men, clinically characterised by the development of multiple haemorrhagic lesions of the skin, especially the hand and feet. The lesions are nodular or plaque-like, and the disease follows a benign course, few patients developing systemic involvement.

During the 1950s it became apparent that Kaposi's sarcoma was prevalent in indigenous populations of sub-Saharan Africa, where it was estimated that KS accounted for more than 4% of all malignancies (Martin *et al*, 1993). By the 1980s, it was also reported that KS was the fifth most common malignancy in male patients in Zaire and Uganda. This endemic form of KS occurs in a younger age group than the classic disease of Europe and often has a more aggressive course. The skin lesions in patients with

endemic KS are often florid with local lymph node metastases. A particular feature of this aggressive form is the presence of multiple plaques or nodules in the mucosa of the gastro-intestinal tract. Death is normally due to widespread systemic involvement, when nearly all organs may be affected.

In the 1970s, reports appeared of KS arising in patients receiving prolonged immunosuppression, particularly those who had undergone renal transplantation (Myers *et al*, 1974). This form of KS is now termed immunosuppression-associated, or iatrogenic KS. These patients present with clinical features different from the two aforementioned forms of KS, nodular forms predominating and early visceral involvement being frequent. The occurrence of KS in immunosuppression and its resolution after withdrawal of immunosuppression therapy (Montagnino *et al*, 1994) suggest that an infectious agent might underlie its pathogenesis.

In 1981, several cases of Kaposi's sarcoma were described in the United States in young homosexual men who were subsequently found to have acquired immunodeficiency syndrome. An epidemic of KS associated with the development of *Pneumocystis carinii* pneumonia in a restricted population of young sexually-active homosexual men signalled the appearance of AIDS (Siegal *et al*, 1981; Masur *et al*, 1981; Gottlieb *et al*, 1981). In these HIV-related (or epidemic KS) cases, the course of KS disease is aggressive. The early lesions, which consist of small patches, may not have the classical tumour histology (atypical spindle cells) but appear as bizarre vascular spaces within the dermis. Table 1.1 summarises the various forms of KS.

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Before the emergence of HIV disease, KS was a rare tumour in North America and Europe, with annual incidences of 0.02-0.06 per 100,000 (Oettle, 1962). However, with the significant increase in number of individuals infected with HIV in the 1980s, the number of patients with KS increased substantially in these countries, KS being observed in 15-20% of European patients with severe HIV disease (Hermans *et al*, 1996). However, since the beginning of the 1990s the incidence of HIV-related KS has started to decline, and the current introduction of therapy using protease inhibitors against HIV is accelerating this decline. Thus, the annual incidence was 59/1,000 patient-years in 1984 vs 21.2/1,000 patient-years in 1990, 12.5/1,000 patient-years in 1996, and 3.2/1,000 patient-years in 1997 (Hermans *et al*, 1998).

1.1.2 Genetic predisposition

The role of genetic factors in Kaposi's sarcoma is suggested from the observation that HLA-DR5 is found frequently in the classical form of KS. In one study, 7 of 8 family members from Greece with classical KS possessed HLA-DR5. In addition 57% of 54 unrelated Greek patients with CKS were HLA-DR5 positive compared with 37% in the control group (Kaloterakis *et al*, 1995). Besides, two young heterosexual HIV-negative men of Greek origin who had KS in their third decade were also HLA-DR5 positive (Potouridou *et al*, 1998). In another study, the prevalence of HLA-DR5 subtypes in 44 patients with HIV-related KS was compared with that of 83 HIV-1-seropositive (disease-free) and 87 seronegative homosexual men. KS patients had higher frequencies of HLA-B35, -C4, -DR1, and -DQ1 and lower frequencies of HLA-

C5 and -DR3 compared to the KS-negative populations (Mann *et al*, 1990). In contrast, no statistical significance was found in a cohort of 49 patients with CKS of Jewish origin who were tested serologically for HLA class I and class II antigens (Strichman-Almashanu *et al*, 1995). Furthermore, 23 histologically confirmed endemic KS from central Africa showed no association with any of the HLA antigens, including DR5 and DR3, when compared with a local gender and tribe matched control group (Melbye *et al*, 1987).

1.1.3 The search for the aetiology of Kaposi's sarcoma

Epidemiological studies suggest that an infectious agent is implicated in the aetiology of all forms of KS (Wahman *et al*, 1991). In the early phase of the HIV epidemic, the frequency of KS was observed to be much higher in HIV-infected homosexual or bisexual males than HIV individuals who had received infected blood or blood products, or who were injecting drug users (Hermans *et al*, 1996, Beral *et al*, 1990). Kaposi's sarcoma was also more common in HIV-infected female sexual partners of bisexual men than those of heterosexual injecting drug users (Beral *et al*, 1990). Furthermore, HIV-related KS in homosexual males occurred in distinct geographic areas suggesting a clustering of disease (Beral *et al*, 1990), and disease similar to classic KS was observed to be more common in non-HIV-infected homosexual men than the general population. These all suggest transmission of an infectious aetiological agent possibly via sexual routes or close faecal contact (Beral *et al*, 1992).

The rise in the frequency of classic KS in Sweden in the 25 years prior to 1982 (Dictor and Attewell, 1988), and the fall in the prevalence of HIV-related KS in the US (Katz *et al*, 1994) and Europe over the last decade (Hermans *et al*, 1996), also point towards an infectious agent causing KS. The reasons for these observed changes possibly reflect differences in sexual behaviour, moderation in sexual activity, and increased use of barrier-type contraception, which may reduce transmissibility of an infectious causative agent of KS (Fife and Bower, 1996).

Several candidates had been proposed as aetiological agents, including cytomegalovirus (CMV) (Giraldo *et al*, 1980), HIV itself (Nakamura *et al*, 1988) and human T-cell lymphotrophic virus type 1 (HTLV-1) (Lebbe *et al*, 1997b; Warmuth *et al*, 1997). When representational difference analysis was employed to examine for pathogen-specific genomic nucleotide sequences in AIDS-associated KS lesions (Chang *et al*, 1994), sequences homologous to Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS) of the gamma-herpesviridae subfamily (Roizman, 1995) were identified. The virus that bears the unique sequences was ultimately termed Kaposi's sarcoma herpesvirus (KSHV) or human herpesvirus 8 (HHV-8). Sequencing of a 20.7-kb clone from a KS library provided evidence that this virus should be classified as a gamma-2 herpesvirus of the Rhadinovirus genus (Moore *et al*, 1996a). Human herpesvirus 8 is therefore the first member of Rhadinovirus known to infect humans.

1.1.4 Clinical features

Mucocutaneous KS may precede or follow other manifestations of HIV disease. In 50% or more of patients with mucocutaneous KS, lesions are oral or perioral and these are often early manifestations of severe HIV disease (Scully *et al*, 1990; Epstein and Scully, 1991). Early oral lesions present in the palate (lateral aspects) as pigmented macules which later become nodular. KS may thus present as red, bluish or purple patches, nodules or ulcers. There are occasional reports of non-discoloured oral KS (Daly *et al*, 1989; Reichart and Schiodt, 1989). Sometimes the tongue, gingiva (resembling an epulis) and other mucosal sites are affected (Table 1.2). The lesions may be painful, bleed and interfere with speech, eating or oral hygiene.

The differential diagnosis of oral KS includes, for macular lesions: atrophic candidosis, median rhomboid glossitis, erythroplakia, amalgam tattoo, macular melanosis, and ecchymosis; and for elevated KS: gingival epulis/pyogenic granuloma, giant cell lesions, minor salivary gland tumour, bacillary angiomatosis, vascular lesions, haematoma, melanoma, Wegener's granulomatosis and lymphoma {Flaitz *et al*, 1995a). Drugs such as ketoconazole and zidovudine, and HIV itself, can cause oral pigmentation, which although usually brown, may be confused with the reddish or purple colour of KS lesions.

A high index of suspicion is required in order not to overlook early KS lesions, biopsy and histopathological examination often being indicated. KS may occasionally mimic periodontal infection (Chapple *et al*, 1992), present

as cervical lymph node or salivary gland enlargement (Yeh *et al*, 1989), or

give rise to an intraosseus lesion in the mandible (Langford *et al*, 1991) or the

external auditory canal (Delbrouck *et al*, 1998). Moreover, congenital KS was

reported in a two week old infant born to a mother with AIDS-KS (McCarty

and Bungu, 1995).

The prognosis of HIV-infected patients with KS depends on the degree

of immunosuppression, rather than on the neoplastic proliferation and tumour

load (Errante *et al*, 1991). There is a suggestion that oral mucosal lesions of

KS are associated with a lower peripheral CD4+ T cell count than those with

skin lesions alone (Orfanos *et al*, 1995). Currently, the majority of patients

with AIDS-associated KS die of other opportunistic infections rather than KS.

1.1.5 Histopathology

Kaposi's sarcoma is an endothelial cell multicentric malignant

neoplasm or proliferative disorder, characterised by increased capillary growth

and prominent spindle-shaped cells with surprisingly few mitoses,

extravasation of erythrocytes, and the appearance of haemosiderin (Table

1.3) (Green *et al*, 1984). Histologically, KS patterns may be defined as

occurring in two stages. The early stage, clinically corresponding to the flat

presentation, consists of focal proliferation of thin-walled vessels that often

show plump endothelial cells. In the late, or neoplastic stage, the endothelial

cells group as prominent nodules with numerous extravasated erythrocytes

and siderophages. The spindle cells show various degrees of pleomorphism,

but they never involve the epithelium. The presence of eosinophilic bodies is another important diagnostic feature (Green *et al*, 1984).

It is of interest that even clinically normal oral mucosa from HIV-positive persons may show vascular changes histologically similar to those seen in early KS (Zhang *et al*, 1989). Endothelial proliferation culminating in KS may be related to various angiogenic or other factors (Ensoli *et al*, 1994).

The precise histogenesis of KS is still unknown. Some studies have indicated that KS may have a blood vascular endothelial origin (Mitsuyasu *et al*, 1984); other studies have suggested a lymphatic derivation (Beckstead *et al*, 1985).

Whether Kaposi's sarcoma is a clonal neoplasm (Rabkin *et al*, 1997), polyclonal (Delabesse *et al*, 1997; Gill *et al*, 1998) or represents hyperplastic growth (Costa and Rabson, 1983) remains unclear. Several lines of evidence indicate that KS proliferative cells are not neoplastic. The three histological features of KS (angiogenesis, inflammation and proliferation), the absence of a histologically discernible neoplastic cell (Mitsuyasu *et al*, 1984; Beckstead *et al*, 1985), the sporadic spontaneous regression, the lack of chromosomal abnormalities and the variable clonality of KS (monoclonal lesions in some cases, oligo or even polyclonal in others) (Gill *et al*, 1998) all point to KS as not being a "true" malignancy (Gallo *et al*, 1998).

1.1.6 Therapeutic aspects

1.1.6.1 Head and neck Kaposi's sarcoma

There are no data showing that local treatment of KS improves patient survival. The primary goal of treatment of HIV-related KS is palliation. Relief of pain, reduction of oedema, associated lymphadenopathy and functional and cosmetic factors remain the main factors to be considered. Occasional exophytic lesions may be considered appropriate for excision. Lesions with a pedicular attachment may be easily removed by carbon dioxide or argon laser (Schweitzer and Visscher, 1990) or surgical excision, and if the lesion is resected as far as to where the surrounding oedema extends, it generally does not recur at the same site. Cryosurgery has been used, but HIV-related KS often persists beneath the cryolesion (Tappero *et al*, 1991).

Intra-lesional therapy with vinblastine has been shown to provide effective palliation in HIV-related oral KS (Epstein and Scully, 1989; Nichols *et al*, 1993, Moyle *et al*, 1993; Flaitz *et al*, 1995b; McCormick, 1996), although it produces pain for 1-3 days (Epstein and Scully, 1989). Some patients may develop numbness at the site of palatal lesions, which may persist for 2-4 weeks, and is probably related to local effects of the drug. The use of sclerosing agents, such as 3% sodium tetradecyl sulphate, has been shown to be effective (Lucatorto and Sapp, 1993), but can induce considerable mucosal ulceration and discomfort, which limit their use. Phototherapy was attempted in a pilot study involving 3 patients with plaque-type HIV-related KS during which indocyanine green (ICG) was used as photosensitizer in

combination with diode laser. Biopsies revealed tumour necrosis after 24 hours, and complete remission was achieved in 4 weeks. No significant systemic side-effects were noted (Abels *et al*, 1998). Interferon-alpha has also been reported to be beneficial clinically, not only in AIDS-KS but also in classical KS (Tur and Brenner, 1998). The disadvantages of interferon are the requirement for repeated injection (twice weekly) and the expense (Sulis *et al*, 1989; Safai *et al*, 1990).

When lesions involve a more extensive area, resulting in regional involvement, such as the entire hard palate and/or oropharynx, regional treatment provided by radiotherapy may be appropriate. While radiation therapy has been reported to result in a severe, painful and persisting mucositis, and may require hospitalization of the patient, increased numbers of fractions rather than single fraction radiation may reduce the severity of the oral complications (Epstein and Silverman, 1992). Alternatively, intracavitary contact X-ray therapy (ICRT) may be beneficial in avoiding such side-effects (Caccialanza *et al*, 1997).

1.1.6.2 Kaposi's sarcoma involving other body sites

Single agent chemotherapy controls the disease in approximately 30% . of patients, while combination chemotherapy can lead to transient responses in up to 93% of selected patients (Boshoff *et al*, 1997). Drugs include vinblastine, vincristine, doxorubicin, liposomal-encapsulated doxorubicin, pegylated-liposomal doxorubicin, bleomycin and interferon-alpha. Therapy always results in further suppression of the immune system, and interference

or interaction with other medications (Boshoff *et al*, 1997; Gottlieb *et al*, 1997; Northfelt *et al*, 1997; Grunaug *et al*, 1998; Stewart *et al*, 1998). The decision to treat with systemic chemotherapy depends upon the extent of systemic involvement, the general health of the patient, potential systemic side-effects of therapy, and the risk of additional immunosuppression (Epstein and Scully, 1992).

More recently, based upon the knowledge that KS is more common in men than in women, the role of human chorionic gonadotrophin (hCG) has been investigated. Early results show that hCG induces growth inhibition and apoptosis of KS cells (Albini *et al*, 1997). In addition, in a double-blind study, intralesional or subcutaneous injection of hCG induced regression of HIV-related KS (Gill *et al*, 1996; Gill *et al*, 1997; Witzke *et al*, 1997). HIV-related KS may also regress with zidovudine alone (Langford *et al*, 1989; Joffe *et al*, 1997), in combination with interferon alpha (Shepherd *et al*, 1998) or with HIV-1 protease inhibitors (Murphy *et al*, 1997; Lebbe *et al*, 1998; Krischer *et al*, 1998). Thalidomide therapy has also been reported to induce the remission of HIV-related KS (Soler *et al*, 1996).

Overall, treatment of HIV-related KS does not significantly affect the prognosis or survival of AIDS patients. However, treatment can alleviate aerodigestive and/or respiratory dysfunction, allow for adequate nutritional intake, and improve the quality of life for these patients (Flaitz *et al*, 1995b).

1.2 Human herpesvirus 8 (HHV-8)

Since the first description of HHV-8 (Chang *et al*, 1994), knowledge of its virology, pathogenic mechanisms and clinical consequences of infection has risen exponentially. There is gathering evidence that infection with HHV-8 may be the causative agent of KS (Moore *et al*, 1996a), primary effusion lymphoma (PEL) (also termed body cavity based lymphomas (BCBL)) (Cesarman *et al*, 1995), and multicentric Castleman disease (MCD) (Soulier *et al*, 1995). HHV-8 has also been tenuously linked to multiple myeloma (Rettig *et al*, 1997) and non-neoplastic disorders such as sarcoidosis (Di Alberti *et al*, 1997a) and pemphigus vulgaris (Memar *et al*, 1997).

1.2.1 Transmission

Sexual transmission seems to be the major route by which HHV-8 is acquired. Anal intercourse, in particular, is the activity associated with transmission (Beral *et al*, 1990). Thus, male homosexuals and females infected with HIV by their bisexual male partners are at greater risk of developing KS than those who were infected from haemophiliacs, transfusion recipients or injecting drug users (Beral *et al*, 1990; Hermans *et al*, 1996). In a cohort of Danish homosexual men, HHV-8 seropositivity was correlated with the frequency of receptive anal intercourse (Melbye *et al*, 1998). Furthermore, the number of male intercourse partners is a stronger risk factor associated with HHV-8 infection than a history of blood or blood products receipt or injecting needle usage (Martin *et al*, 1998).

HHV-8 is present in semen, but at different frequencies according to risk groups. Human herpesvirus 8 DNA was detected by PCR in semen samples of 2 of 15 HIV negative and 8 of 15 HIV positive men living in Central Africa (Belec *et al*, 1998a). In contrast, HHV-8 was not detected in the semen of 4 HIV-infected American males with KS (Ambroziac *et al*, 1995), and only 2 of another cohort of 14 HIV-infected American homosexual men with KS were found to be HHV-8 DNA positive (Gupta *et al*, 1996). However, others failed to detect HHV-8 DNA in the semen of HIV-positive patients from the US (Diamond *et al*, 1997).

In situ hybridisation studies showed HHV-8 in the glandular epithelium of an elderly HIV negative male (Staskus *et al*, 1997). In addition, in a cohort of Italian patients of unknown HIV status who had no apparent clinical disease other than varicoceles, HHV-8 DNA was detected by PCR in 12% of tissue specimens from the urogenital tract, 44% of prostate tissues and 81% of ejaculates from immunocompetent persons were HHV-8 positive, suggesting that the viral DNA may be present in different parts of the urogenital tract (Monini *et al*, 1996). HHV-8 was detected in prostate tissue of men with KS by means of *in situ* hybridisation; between 1 to 5% of cells expressed viral transcripts associated with HHV-8 replication, while more than 90% expressed gene products associated with viral latency. These findings suggest that the prostate is a site for intermittent viral replication and shedding into semen (Diamond *et al*, 1998).

Sexual transmission is unlikely to be the only route of transmission of HHV-8, particularly in geographic areas where HHV-8 infection is endemic, and where KS is a commonly found tumour before the AIDS era, e.g. in Central and East Africa (Ziegler 1996). KS was commonly observed in Ugandan children before the AIDS epidemic (Olweny *et al*, 1976), and a recent study found HHV-8 infection in early childhood in Uganda, reaching an adult frequency before puberty. In this study, the presence of HHV-8 was not associated with antibodies to hepatitis A or C virus nor with the quality of water supply, which points to a horizontal mode of transmission that is different from western countries (Mayama *et al*, 1998).

Infectious HHV-8 has been found in saliva specimens of patients without oral KS. In one study, HHV-8 DNA was amplified from 25 of 76 HIV-infected individuals (32.9%) in unstimulated whole saliva (Vieira *et al*, 1997). Human herpesvirus 8 DNA was also found in the saliva of individuals without detectable viral sequences in their peripheral blood mononuclear cells (PBMCs) (Koelle *et al*, 1997). In addition HHV-8 DNA was detected in the saliva of 5 of 29 (17%) symptomatic HIV infected patients (only one had oral KS at the time of sampling) but not in 15 healthy controls (Lucht *et al*, 1998) nor in samples from 39 HIV-negative patients (Boldogh *et al*, 1996).

Transmission of HHV-8 in faeces was suggested by the findings that oro-anal contact was associated with a higher likelihood of KS in homosexuals (Beral *et al*, 1990). Nevertheless, supporting evidence is inconsistent as HHV-8 DNA was not detected in faeces of one cohort of HIV-

infected patients (Lin *et al*, 1995) but was in 24 of 51 duodenal and rectal biopsies of HIV-seropositive patients (Thomas *et al*, 1996).

Parenteral transmission may be another route of HHV-8 infection, as suggested by the findings of HHV-8 being present in CD19+ B cells separated from PBMCs of a healthy blood donor (Blackbourn *et al*, 1997). However, in a later study, 13 of the 14 patients were found to remain seronegative despite receiving cellular components from HHV-8 seropositive donors (Operskalski *et al*, 1997).

Data concerning vertical transmission is also contradictory. In one study all 9 children born from HHV-8 positive Haitian and American mothers were HHV-8 seronegative (Goedert *et al*, 1997). More recently, 8 of 19 children who had HHV-8 seropositive mothers were also HHV-8 seropositive (Bourboulia *et al*, 1998). Apart from vertical transmission, intrafamilial person-to-person spread of the virus has also been suggested. In families of KS patients, the seroprevalence of spouses, children and siblings may be higher than non-family controls, gender and age matched (Angeloni *et al*, 1998).

1.2.2 Epidemiology of human herpesvirus 8 infection

Epidemiological data based upon detection of antibodies to HHV-8 have been conflicting, owing to methodological differences and perhaps more importantly, geographical variations. Using an indirect immunofluorescence assay (IFA) based on preparation of nuclei of body cavity based lymphoma (BCBL-1) cells (to exclude the detection of cytoplasmic antigens) (Kedes *et al*,

1996) a seroprevalence of 1% was found in a group of HIV-negative blood donors and 8% for HIV-negative patients with syphilis. In another IFA study using an uninduced cell preparation and adopting with a high cut-off fluorescence reading to avoid non-specific background (Gao *et al*, 1996a), HHV-8 antibodies were not detected in a large cohort of US blood donors. In contrast, an IFA based upon induced cell preparations optimised for the expression of both nuclear and cytoplasmic antigens (Lennette *et al*, 1996) showed 25% of American adults and 2-8% of children to have IgG antibodies to HHV-8.

An IFA detecting antibodies to a latency-associated nuclear antigen (LANA) showed none of 195 exclusively heterosexual men from San Francisco had antibodies to HHV-8, while 12.5% of the men who admitted frequent but not exclusively homosexual activity, and 39.6% who were exclusively homosexual were HHV-8 positive (Martin *et al*, 1998). More recently, using uninduced and TPA-induced BCBL-1 cells, IFA antibody titres to HHV-8 were found to be much higher in HIV-infected Los Angeles patients with KS (449 geometric mean titre, range 40-10,240) in comparison with those HIV-infected without KS (84 geometric mean titre, range 40-1,280) (Chandran *et al*, 1998). Furthermore, the HHV-8 antibody positivity rate was 8% in healthy individuals, 56% in HIV-positive homosexual men, and 12% in age-matched HIV negative controls (Verbeek *et al*, 1998).

Furthermore, using an IFA assay based on both lytic and latent anti-HHV-8 detection, 28% of blood donors in Rome were HHV-8 seropositive

(Rezza *et al*, 1998). This prevalence rate is similar to another study where 23% of the control group comprised of healthy volunteers and patients with various dermatological diseases from central and southern regions of Italy were found to carry HHV-8 DNA in their PBMC (Cattani *et al*, 1998).

Using an IFA based on detection of LANA, HHV-8 antibodies were found in 16 of 16 French KS patients but in only 3 of 83 patients with non-KS dermatologic diseases and 2 of 100 healthy controls (Dupin *et al*, 1998). Likewise, only 5 of 169 (3%) HIV negative patients from Honduras were found to be HHV-8 positive when a cut-off dilution of 1:40 was used (Sosa *et al*, 1998).

Studies based on enzyme-linked immunosorbent assay (ELISA) utilizing recombinant capsid-related proteins of HHV-8 open reading frame (ORF) 65 (which is homologous to EBV-BFRF3, share 49% of sequence similarity, from which 27% are identical) (Simpson *et al*, 1996) found seroprevalence rates of 1.7% in British, 5% in North American and 12% in Mediterranean blood donors, and up to 47% in Ugandan HIV-negative individuals. Investigations using another ELISA test, which utilizes two regions of the ORF 26 differing substantially from EBV-BDLF1, found 6 of 30 (20%) US healthy blood donors group to be HHV-8 seropositive (Davis *et al*, 1997). Another study, using small viral capsid antigen (sVCA) found 3 of 28 (11%) US blood donors to be seropositive, although none of 25 haemophiliac patients nor 22 children (10 HIV-negative and 12 HIV-positive) were HHV-8 seropositive (Lin *et al*, 1997). In another study, none of the 52 patients tested

who were negative for HIV, hepatitis B and C virus had antibodies for HHV-8 (Smith *et al*, 1997). In addition, using a latency-associated nuclear antigen (LANA) IFA and an ELISA with plates coated with capsid related protein encoded by ORF 65, 24.1% of 779 Italian blood donors from different regions had antibodies to at least one antigen (Calabro *et al*, 1998).

An ELISA developed to detect antibody to ORF 65.2 antigen showed positivity in 24 of 26 (92.3%) of Swiss HIV positive patients with KS, 21 of 87 (24.1%) HIV positive patients without KS, 11 of 54 (20%) HIV negative homosexual men and 9 of 178 (5.1%) blood donors (Regamey *et al*, 1998). Another ELISA using whole virus lysate as antigen gave a seroprevalence rate of 11% (10/91) in US blood donors. In this study the average titre of different groups was also measured: blood donors had an average titre of 118, classic KS patients had a titre of 14,111 while HIV-associated KS patients a titre of 4,000 (Chatlynne *et al*, 1998).

In order to elucidate and standardize HHV-8 antibody assays to asymptomatic infection, a blinded comparison was recently performed between five laboratories using four IFA's and three ELISA's. It was found that while the HHV-8 antibody tests were adequate for epidemiological investigations, the poor specificity and sensitivity in detecting asymptomatic HHV-8 infection requires further confirmatory testing using nucleic acid detection methods (Rabkin *et al*, 1998).

It is particularly important to determine the seroprevalence of HHV-8 in the general healthy population as this can clarify the role of HHV-8 in the

pathogenesis of KS (Neipel *et al*, 1997a). A summary of the current data is shown on Table 1.4. Most studies show a global HHV-8 seroprevalence of 2 to 10%. Assuming a 5% seroprevalence of HHV-8 in the US and 1970s baseline incidence of KS in men in the US (about 0.3 cases per 100,000 men) the HHV-8 rate would be one case of KS for every 17,000 HHV-8 infections (Gallo *et al*, 1998).

1.2.3 Disease associations of human herpesvirus 8

1.2.3.1 Kaposi's sarcoma

$KS_{330}Bam$ and $KS_{631}Bam$ were the two small fragments of the HHV-8 genome isolated from KS tissues by representational difference analysis (Chang *et al*, 1994). Since then, other groups, using PCR primers derived from $KS_{330}Bam$ sequence, have detected HHV-8 DNA in lesional tissues of KS of all four epidemiological forms: HIV-related (Ambroziak *et al*, 1995; Huang *et al*, 1995; Lellis *et al*, 1995; Moore and Chang, 1995; Noel *et al*, 1996; Roizman, 1995; Schalling *et al*, 1995; Su *et al*, 1995; Cathomas *et al*, 1996; Corbellino *et al*, 1996; Ganem, 1996; O'Neill *et al*, 1996; Lebbe *et al*, 1997b); classic (Boshoff *et al*, 1995b; Dupin *et al*, 1995; Huang *et al*, 1995; Lebbe *et al*, 1995; Lellis *et al*, 1995; Rady *et al*, 1995; Buonaguro *et al*, 1996); endemic (Huang *et al*, 1995; Lebbe *et al*, 1995; Lellis *et al*, 1995; Schalling *et al*, 1995; Buonaguro *et al*, 1996; Cathomas 1996; Chang *et al*, 1996; Chuck *et al*, 1996; Eto *et al*, 1996); and immunosuppression related (Gluckman *et al*, 1995; Lebbe *et al*, 1995; Buonaguro *et al*, 1996; Alkan *et al*, 1997; Henghold *et al*, 1997).

HHV-8 DNA could be amplified from KS tissue in different clinical stages of the disease (Luppi *et al*, 1996). Furthermore, by semiquantitative analysis of HHV-8 DNA it was found that HHV-8 DNA load was higher in patients with multicentric and visceral involvement than in those with localised disease, and that the nodular stage has also higher viral load than patch and plaque stages, thereby showing a correlation between viral load and disease severity (Mendez *et al*, 1998).

The presence of HHV-8 DNA in PBMCs of HIV-infected individuals predicts the subsequent appearance of KS lesions (Whitby *et al*, 1995; Moore *et al*, 1996a). In addition, being seropositive for HHV-8 is also associated with increased risk of developing KS in HIV co-infected patients (Gao *et al*, 1996b; Verbeek *et al*, 1998).

1.2.3.2 Body cavity based lymphoma

Body cavity based lymphoma (BCBL) is a rare, rapidly fatal malignancy, first described in AIDS patients (Cesarman *et al*, 1995). The disease has a distinctive presentation, that of malignant peritoneal, pericardial, or pleural effusions in the absence of an identifiable tumour mass or nodal involvement. Carriage by BCBL cells of both EBV and HHV-8 infection has been observed but carriage of HHV-8 alone has also been reported (Cesarman *et al*, 1996; Said *et al*, 1996; Hermine *et al*, 1996; Jones *et al*, 1998). Of note, most cases of EBV-negative BCBL have occurred in patients who are not HIV-infected, whereas BCBL in HIV-infected patients are usually EBV-positive.

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1.2.3.3 Multicentric Castleman's disease

Multicentric Castleman's disease (MCD) is an atypical lymphoproliferative disorder mostly found in patients with HIV disease. A striking observation has linked MCD with KS, although HHV-8 has only been found infrequently in MCD of HIV negative patients (Soulier *et al*, 1995), making the association less strong in comparison with that of HHV-8 with KS or primary effusion lymphoma (PEL). Multicentric Castleman's disease was first described as a benign mediastinal lymphoid mass originally termed mediastinal lymph node hyperplasia. These lesions may occur in any part of the body that contain lymphoid tissue, although 70% are found in the anterior mediastinum. Most frequent is the localised hyaline vascular type, which appears as a solitary asymptomatic, tumour-like mass in the mediastinum. The rarer plasma-cell type of MCD occurs as localised and generalised forms. The localised form presents as a solitary tumour-like mass similar to the hyaline type; the generalised or multicentric form presents as widespread lymphadenopathy and as part of the POEMS syndrome (polyneuropathy, organomegaly, endocrine abnormalities, monoclonal gammopathy and skin rashes). All forms are accompanied by fever and anaemia.

1.2.3.4 Lymphoid diseases

HHV-8 sequences have also been proposed in some angioimmunoblastic lymphadenopathies in non-HIV-infected patients, in particular a distinct benign, non-HIV-related, lymphadenopathy histologically characterised by a predominantly follicular lesion with giant germinal centre

hyperplasia and increased vascularity (Luppi *et al,* 1996). A similar disease entity with an identical histological appearance has been reported in one HIV-infected patient (Soulier *et al,* 1995). Furthermore HHV-8 sequences have been detected in lung nodules of a non-HIV infected Italian patient with interstitial pneumonitis (Luppi and Torelli, 1996).

HHV-8 DNA has also been detected, albeit rarely, in other lymphoproliferative disorders, including non-Hodgkin's lymphoma, Hodgkin's disease, reactive lymphadenopathies (Bigoni *et al,* 1996) and cutaneous lymphoma in AIDS (Corbellino *et al,* 1996). The viral load is significantly higher in lymphoid tissue from HIV-infected persons as compared to HIV-seronegative individuals (Bigoni *et al,* 1996), although it is still lower than in splenic tissue or peripheral blood mononuclear cells from the same patients. This suggests that the presence of HHV-8 in these lesions may be a reflection of HHV-8 carriage by non-neoplastic B cells (Corbellino *et al,* 1996). Detection of HHV-8 in mature T-cell lymphoproliferative disorders has been reported (Sander *et al,* 1996), but this has not been confirmed by others (Cesarman *et al,* 1995; Pastore *et al,* 1995; Pawson *et al,* 1996).

1.2.3.5 Multiple myeloma

Human herpesvirus 8 has also been implicated in the aetiology of multiple myeloma (MM). HHV-8 was first reported in bone marrow dendritic cells of 25% of American patients with monoclonal gammopathy of undetermined significance, a condition that may progress to MM (Rettig *et al,* 1997). Subsequently, HHV-8 DNA was found by PCR in 6 of 7 fresh biopsy

samples, and by *in situ* hybridisation of bone marrow dendritic cells in 17 of 20 American patients with MM (Said *et al*, 1997). In addition, 20 of 27 (81%) of other group of US patients with MM were HHV-8 seropositive using an ELISA assay to a recombinant minor capsid protein as antigen, while only 22% of patients with other malignancies and 6% of blood donors were found to be HHV-8 seropositive (Gao *et al*, 1998). In contrast, using a nested PCR and a serological assay to detect HHV-8 latency-associated nuclear antigen (LANA) and ORF 65 (lytic) protein, only one of 20 Italian patients with MM was found to be HHV-8 seropositive (Parravicini *et al*, 1997). Moreover, none of 10 Swedish patients were found to be HHV-8 positive by PCR or serological assay (Yi *et al*, 1998). No statistical significant differences between MM patients and blood donors was observed in the frequency of HHV-8 seropositivity using an IFA assay (Whitby *et al*, 1997). Similar results have also been found by others (MacKenzie *et al*, 1997; Marcelin *et al*, 1997; Cull *et al*, 1998; Mitterer *et al*, 1998; Tarte *et al*, 1998). The significance of HHV-8 in the aetiology of MM remains thus unclear.

1.2.3.6 Other diseases with a possible association with HHV-8

Human herpes virus 8 DNA is present in normal skin of HIV-infected patients with KS (Dupin *et al*, 1995; Gaidano *et al*, 1996b; Lebbe *et al*, 1997b); and more rarely, in the normal skin of patients with classic KS, endemic KS (Lebbe *et al*, 1995; Ambroziak *et al*,. 1995; Uthman *et al*, 1995; Boshoff *et al*, 1996; Monini *et al*, 1996), or iatrogenic KS (Dictor *et al*, 1996) and lesional tissue of patients with squamous cell or basal cell carcinomas

(Rady *et al*, 1995; Inagi *et al*, 1996). The HHV-8 infection load appears to be lower in the normal skin and the other cutaneous lesions than in lesional KS tissue (Dupin *et al*, 1995; Rady *et al*, 1995).

HHV-8 sequences were detected in one sample of skin with scabies and in a glomerulonephritis lesion from immunosuppressed patients with KS (Noel *et al*, 1996), a patient with pemphigus vulgaris without HIV infection or KS, (Memar *et al*, 1997) and a patient with mycosis fungoides (Uccini *et al*, 1997). HHV-8 sequences were also detected in occasional angiosarcomas (Gyulai *et al*, 1996; McDonagh *et al*, 1996) and tissue affected by angiolymphoid hyperplasia with eosinophilia (Gyulai *et al*, 1996). However, HHV-8 DNA was not detected in immunosuppression-associated dermatofibromas, despite sharing many histologic similarities with HIV-related KS lesions (Foreman *et al*, 1997).

HHV-8 has been implicated with other non-neoplastic disorders such as sarcoidosis (Di Alberti *et al*, 1997a). This was based on the very frequent detection of HHV-8 DNA from ORF 25 and 26 from sarcoid tissue of Italian patients. However, HHV-8 ORF 26 DNA could not be amplified from sarcoid tissues of French patients (Belec *et al*, 1998b). Furthermore, a serological assay (ELISA) using ORF 65.2 as antigen detected antibodies in only 3 out 15 (20%) Swiss patients with sarcoidosis (Regamey *et al*, 1998). At present it is unclear if granulomatous inflammation activates latent HHV-8 or whether the virus is indeed an aetiological agent.

1.2.4 HHV-8 and BCBL cell lines

Cell lines derived from body cavity based lymphoma frequently contain HHV-8 genomes. Their study has provided significant insights into the biological properties of HHV-8. The BC-1 line harbours HHV-8 but not EBV DNA, while another line, BC-2, harbours both viruses (Cesarman *et al,* 1995). Treatment of BC-1 with phorbol esters rapidly induces lytic growth of HHV-8, and progeny virus are then shed into the supporting medium (Renne *et al,* 1996). The induced B cells can be observed to contain 110 nm intranuclear herpesvirus-like nucleocapsids and complete cytoplasmic virions (Said *et al,* 1996). The length of genome of the virus is estimated to be similar (e.g. 160-170 kb) to other gammaherpesviruses. The HHV-8 genome is, like that of EBV, maintained in latently-infected B cells as extrachromosomal, episomal, monomeric circles, with induction from latency leading to the selective accumulation of linear genomic forms (Renne *et al,* 1996). By contrast, only covalently closed, circular episomes of HHV-8 are identified in KS tissue, while linear forms, arising from viral replication, are additionally found in PBMCs of KS patients (Decker *et al,* 1996). While uninduced BCBL lines have not yet been shown to permit propagation of HHV-8, the virus can be cultured from skin lesions of patients with AIDS-associated KS using the human embryonal-kidney epithelioid line 293 (Foreman *et al,* 1997), thus providing evidence that the virus is able to replicate vegetatively *in vitro.*

Sequencing of a 12.3-kb HHV-8 clone obtained from a genomic library derived from BC-1 revealed homology between HHV-8 with parts of the EBV

and herpesvirus saimiri (HVS) genome. The sequences of some ORFs of HHV-8 are homologous to EBV-membrane antigen p140, HVS p160, cellular type D cyclins and cellular G protein coupled receptors (Cesarman *et al*, 1996). Furthermore, transcription of these 4 ORFs can be demonstrated in BC-1 (Moore *et al*, 1996b). A novel abundant 1.2-kb RNA, polyadenylated nuclear RNA (called PAN RNA) has also been identified from the BC-1 line; it appears speckled in the nuclei by immunofluorescence and may be a viral lytic early transcript (Zhong *et al*, 1996; Zhong and Ganem, 1997).

The BC-1 cell line was used to create a cosmid and phage genomic library, allowing full characterisation of the HHV-8 nucleotide sequence, except for a 3 kb region at the right end of the genome (Russo *et al*, 1996). The BC-1 HHV-8 genome is 140.5-kb long, with a unique coding region flanked by multiple 801-bp terminal repeat sequences. A genomic duplication that apparently arose in the parental tumour is present in this cell culture-derived strain. At least 81 ORFs and 5 internal repeat regions are present in the long unique region. In addition to viral structural and metabolic proteins, the virus encodes homologues to complement-binding proteins, three cytokines (two macrophage inflammatory proteins, MIP-1α, MIP-1β, and interleukin-6, IL-6), dihydrofolate reductase, *bcl-2*, interferon regulatory factors, interleukin 8 receptor, neural cell adhesion molecule-like adhesin and a D-type cyclin, (Russo *et al*, 1996). A subsequent study which sequenced a 17-kb segment of HHV-8 between ORFs 11 and 17 confirmed that the viral genome contains a single 13-kb divergent locus wherein are nine ORFs that are homologous or related to, cellular proteins (Nicholas *et al*, 1997). A fourth

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potential cytokine gene, BCK, was also identified, in addition to a viral thymidylate synthetase gene, the T1.1 abundant lytic cycle nuclear RNA gene and two genes related to the immediate-early protein of the gamma-2 class herpesvirus, bovine herpesvirus type 4: 1E1-A and 1E1-B (Nicholas *et al*, 1997).

The herpes viral-like particles produced from BC-1 cells can further infect PBMC-derived CD19+ B cells. This suggests that HHV-8 is transmissible and B-lymphotropic (Mesri *et al*, 1996).

More recently, other BCBL-derived cell lines have been established and characterised: BC-3, BCP-1 and CRO-AP/3 do not contain EBV DNA, while CRO-AP/5 and HBL-6 contains both EBV and HHV-8 (Arvanitakis *et al*, 1996; Boshoff *et al*, 1998; Carbone *et al*, 1998; Gaidano *et al*, 1996a). The structure of the HHV-8 genome in these cell lines has not yet been reported.

1.2.5 HHV-8 homology to other viral and cellular proteins

The nucleotide sequence of HHV-8 has been determined from viral sequences isolated from BCBL cell line BC-1. Of 81 ORFs (open reading frames), 66 have homology to those in herpesvirus saimiri (HVS), with 15 (ORFs K1 to K15) not homologous to HVS genes, these being unique to HHV-8 and related rhadinoviruses (Russo *et al*, 1996). In addition, analysis of the putative translation products of some ORFs revealed homology between a number of known human cellular receptors, cell cycle enzymes and chemokines, which are involved in a variety of cellular and immunological as

well as homeostatic and angiogenic mechanisms. A summary of the relevant data is provided in Table 1.5.

Human herpes virus 8 possesses three potential oncogenes that are able to tranform cell lines *in vitro*. ORF K1, for instance, encodes a class I transmembrane glycoprotein with transforming properties when expressed in Rat1 fibroblasts (Lee *et al*, 1998).

The putative translation product of ORF 74 is a G protein-coupled receptor (GCR) and the second HHV-8 potential oncogene. These receptors are important in cellular growth and differentiation, some GCRs being implicated in malignant transformation (Arvanitakis *et al*, 1997). Expression of HHV-8 GCRs stimulates proliferation and causes transformation of mouse fibroblasts. Hence, the HHV-8 GCR homologue may be a mediator of KS tumourogenesis (Bais *et al*, 1998). The closest cellular homologue to the putative HHV-8 GCR are the IL-8 receptors A and B and the closest viral homologue is the HVS *ECRF3* gene, which encodes a functional IL-8 receptor (Ahuja *et al*, 1993).

Human herpes virus 8 also contains a protein homologous with a cyclin D-type *v-cyclin*, which displays 53% homology to cyclin D2 (Li *et al*, 1997). Cyclins are required for cellular division and are involved in the control of the G_1S cell cycle (Peters, 1994). Cyclin D proteins are regulatory subunits that activate cellular kinases to phosphorylate checkpoint molecules. The cyclin protein of HHV-8 is reported to possess kinase activity, being able to phosphorylate (and thus inactivate) the retinoblastoma-tumour suppressor

protein. Hence HHV-8 has the potential to overcome cell-cycle arrest, thereby increasing the likelihood of tumour development (Chang and Moore, 1996; Murphy, 1997).

ORF 16 (vBcl-2) shares 15-20% amino acid identity to cellular members of the Bcl-2 family (Russo *et al*, 1996; Sarid *et al*, 1997; Cheng *et al*, 1997) - a group of genes known to prevent programmed cell death (apoptosis).

Other ORFs of HHV-8 have been found to encode proteins similar to two human macrophage inflammatory protein (MIP) chemokines (vMIP-1, from ORF K6, and vMIP-2, from ORF K4), interleukin-6 (from ORF K2) and interferon regulatory factor (vIRF) (from ORF K9) (Moore *et al*, 1996b). The HHV-8 ORF K6 protein (vMIP-1) inhibits non-syncytial-inducing HIV-1 entry via the CCR5 chemokine receptor, suggesting that vMIP-1 is functional in binding CCR5 and thus contributes to interactions between HHV-8 and HIV-1. Potentially, HHV-8 may exert some inhibitory action in HIV-1 infection, which might underlie the observation that patients with AIDS and KS have a better prognosis than patients with AIDS but not KS (Mocroft *et al*, 1997). However, a recent epidemiological study found that KS appears to accelerate the clinical course of HIV infection and that survival is shorter in patients with KS than in controls (Brodt *et al*, 1998). Furthermore, as vMIP-1 is only expressed by a small subpopulation of HHV-8 infected cells (productively, virus-replicating cells), the impact of vMIP-1 on KS angiogenesis may be limited (Sturzl *et al*, 1998). The virally-derived IL-6 (vIL-6) has been found to be

expressed in HHV-8 infected BCP-1 cells, ascitic lymphoma cells from HIV-negative BCBL and B-cell rich areas of lymph node tissue from a patient with HIV-related KS. vIL-6 has also been reported in KS lesions, in CD34+ (endothelial) or CD45+ (haematopoeitic) cells (Moore *et al*, 1996a), and after generation of a genomic library from KS tissue-derived DNA extract (Neipel *et al*, 1997a).

Human herpes virus 8 may have developed the means of overcoming typical cellular strategies against viral infection including cell cycle arrest, induction of apoptosis and induction of cell-mediated immunity. Thus HHV-8-derived cyclin D may prevent cell cycle arrest (Chang *et al*, 1996), vbcl-2 and vIL-6 may inhibit apoptosis, while vIRF may interfere with interferon-induced-MHC antigen presentation and cell-mediated responses. In addition, it is also possible that a synergistic effect between basic fibroblast growth factor (bFGF) and HIV-1 tat protein may play an important role in the pathogenesis of KS (Kelly *et al*, 1998) through induction of cellular genes that are pro-proliferative and proinflammatory, thereby enhancing the recruitment of leukocytes, themselves sources of other cytokines.

1.2.6 HHV-8 ORFs investigated in the present study and their functions

1.2.6.1 ORF 26

Three major patterns of HHV-8 gene expression have been identified. These are similar to those found for other herpesviruses. Gene expression

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patterns were categorised in BCBL cells according to whether expression is constitutive (latent, class I), requires induction with phorbol esters or butyrate (lytic, class III) or is constitutive but enhanced by inducting agents (class II).

ORF 26 and ORF 25, which putatively encode structural proteins, are class III genes. ORF 26 encodes a 300 - amino acid capsid protein, homologous to EBV BDLF1 and HSV UL18 proteins (Russo *et al*, 1996).

1.2.6.2 ORF 25

This ORF is predicted to encode a product of about 1370 amino acids, thought to be a major component of the viral capsid and homologous to EBV BcLF1 and HSV UL19 protein (Russo *et al*, 1996).

1.2.6.3 ORF K1

This ORF encodes a 279-289 amino acid transmembrane glycoprotein, with a cysteine-rich extracellular domain and some regional homology to immunoglobulin lambda light chains (Lee *et al*, 1998). There is significant sequence and length variation between isolates in its extracellular domain. The cytoplasmic domain, which contains several tyrosine residues, and the membrane-spanning region, appear more conserved (Russo *et al*, 1996; Neipel *et al*, 1997a). There is some evidence (Lee *et al*, 1998) that sequence variability in the extracellular domain may affect multimerization of the K1 protein, but it is still unknown whether this has any impact on its function.

As described earlier in this section, ORF K1 has transforming properties when expressed in Rat1 fibroblasts (Lee *et al*, 1998). When recombined into a C strain of herpesvirus saimiri, from which its transforming gene STP has been deleted, ORF K1 can immortalise common marmoset CD8 T cells *in vitro*, and cause lymphoproliferative disease of a CD8+ phenotype in common marmosets *in vivo* (Lee *et al*, 1998). However, ORF K1 is not expressed in latently-infected BCBL cell lines (Sarid *et al*, 1998) and whether it is expressed in KS spindle (endothelial tumour) cells has not yet been established. Its role in the pathogenesis of HHV-8 associated neoplasms thus remains to be established.

1.2.6.4 ORF K12

The ORF K12 transcript is expressed at high levels in BCBLs, but is further induced by phorbol ester treatment (Staskus *et al*, 1997). This ORF is predicted to encode a small 60 amino acid hydrophobic protein, "kaposin" (Zhong *et al*, 1996; Russo *et al*, 1996, Neipel *et al*, 1997a). It is translated from a 0.7 kb mRNA, which is abundantly expressed in persistently infected PEL cell lines and KS spindle (endothelial tumour) cells (Renne *et al*, 1996; Zhong *et al*, 1996; Staskus *et al*, 1997). The function of ORF K12 product is still unknown.

1.2.6.5 nut-1

Like some other herpesviruses, HHV-8 encodes a nuclear RNA which does not appear to encode any protein (Zhong *et al*, 1996). The nut-1 RNA is

polyadenylated, lacks a trimethylguanosine cap, is transcribed by RNA polymerase II and can associate with small ribonuclear proteins (Zhong and Ganern, 1997). In KS tumours it is expressed in a few tumour cells undergoing lytic replication (Staskus *et al*, 1997) and is inducible in PEL cell lines as an immediate early or delayed early transcript (Zhong and Ganem, 1997). In this respect it differs from non-coding nuclear RNAs encoded by EBV (EBERs), HVS (HSURs) and herpes simplex virus (HSV) (LATs), which are all expressed during latency (and at reduced levels during lytic replication). ORF nut-1 contains regions which are highly homologous or complementary to U1 and U12 small nuclear RNA and may therefore be involved in the control of splicing (Zhong and Ganem, 1997).

1.3 Genetic variability in herpesviruses

1.3.1 Introduction

DNA viruses are genetically more stable than RNA viruses, due to the proof-reading ability of their DNA polymerases, which permit excision and repair of mismatched nucleotides. While it is well recognised that inter-host variability in DNA viruses frequently occurs, whether variability occurs at the intra-host level has not been extensively studied. It is increasingly being recognised that individuals affected by AIDS and/or engaged in certain "high risk" sexual practices can be reinfected with multiple strains of herpesviruses, including HSV-1, EBV, CMV and HHV-6 (Rojas *et al*, 1995; Katz *et al*, 1988; Chou, 1986; van Loon *et al*, 1995). Multiple herpesvirus infections are briefly reviewed here.

1.3.1.1 Multiple HSV-1 infection

HSV-1 is a member of the alpha herpesviridae subfamily. HSV-1 strains vary widely in their ability to cause disease in animal models, indicating that the genetic makeup of the virus plays an important role in the disease process (Stulting *et al*, 1985). It has been shown that mixed infection with two relatively avirulent viral strains can result in a significant increase in disease severity (Brandt, 1991).

Intra-host genetic diversity of HSV-1 has been reported. In one study, genetic variability in thymidine kinase (TK) and glycoprotein B (gB) gene of HSV-1 from 2 patients showed that 4 HSV-1 isolates could be obtained from four serial recurrent lip lesions in one patient and 2 HSV-1 isolates could be recovered from different anatomic areas of the other patient (Rojas *et al*, 1995). In addition, studies with human volunteers and patients suffering from recurrent HSV infections have shown that reinfections with autologous or heterologous strains, occurring at sites distant from those of the recurrences, do occur in a variable proportion of the subjects (Klein, 1989).

1.3.1.2 Multiple EBV infection

EBV, a gamma herpesvirus widespread in human populations, is increasingly recognised as being involved in a wide range of disease processes, particularly involving the head and neck (Gratama *et al*, 1995). The virus is the cause of infectious mononucleosis (Henle *et al*, 1974), and is causally associated with African Burkitt lymphoma (Zur and Schulte-

Holthausen, 1970), nasopharyngeal carcinoma (Niedobitek *et al*, 1996), oral

hairy leukoplakia (OHL) (Greenspan *et al*, 1985), Hodgkin's disease (Weiss *et al*, 1991), peripheral T-cell lymphoma (Ho *et al*, 1998) and only rarely in

sporadic B-cell non-Hodgkin's lymphoma (Hummel *et al*, 1995) .

Multiple infection with distinct strains of EBV in different body sites or

even within the same compartment (blood) has also been reported (Katz *et al*, 1988). Sculley *et al*, (1990) found that 12% (3/26) of HIV infected patients

were infected with both EBV type A and B strains. Yao *et al*, (1998) found that

14 of 39 HIV positive haemophiliac patients carried more than one EBV

strain, suggesting that T-cell impairment does predispose individuals to

reinfection with new strains of exogenously transmitted virus. In contrast, only

3 of 157 healthy individuals shed both type A and B from throat washings

(Sixbey *et al*, 1989). It seems therefore that infection with more than one EBV

strain is more common in immunocompromised individuals than in healthy

carriers. Indeed, no mixed infection was found in a cohort of 76 healthy

individuals from whom sequential isolation (over 7 years) showed retention of

the same strain (Yao *et al*, 1991).

Another pattern was reported by Oosterveer *et al*, (1993) in healthy

subjects, where 2-4 type A strains, co-infecting each of these individuals,

could be distinguished from each other by using EBNA protein size

polymorphism assayed by Western blotting. It has been suggested that EBV

co-infection or recombination, or both, may play a pathogenic role in oral hairy

leukoplakia (OHL) (Walling *et al*, 1992; Walling *et al*, 1994).

1.3.1.3 Multiple CMV infection

CMV belongs to the beta herpesvirus subfamily. It can be classified into four distinct groups according to variability in the glycoprotein B (gB), a hypervariable gene that encodes a protein which is a major component of the virion envelope.

It is thought that infection with a defined strain of CMV does not necessarily protect against superinfection with a different strain. In one study, prior infection did not protect from reinfection with different CMV strains, as 19 CMV seropositive renal allograft recipients frequently became infected with donor CMV strains after having undergone transplantation (Chou, 1986).

In addition, women attending sexually transmitted diseases clinics and with presumably normal host defences were found to simultaneously harbour different CMV strains in different compartments (throat, urine and cervix). Two women shed different strains in serial isolates, and two shed different strains simultaneously from different body sites (Chandler *et al*, 1987). Moreover, an immunocompetent heterosexual man was found to be infected by at least two different CMV strains isolated concurrently from urine and semen (McFarlane and Koment, 1986). More recently, Numazaki *et al*, (1998) found that 2 of 33 immunocompetent children less than 2 years of age were infected simultaneously by more than one CMV strain. Reinfection with CMV may also occur in children sharing facilities in group child care centres, where the rate of reinfection can be as high as 19%; it was demonstrated that in a cohort of

37 children tested serially, at least 7 were infected with more than one CMV strain (Bale *et al*, 1996).

In another study, based on restriction fragment length polymorphism (RFLP) analysis of CMV gB region, four of 11 homosexual men shed more than one CMV strain over a follow up period of 24 months, three subjects having shed two different strains, and one having shed four variants (Collier *et al*, 1989). More recently, using RFLP analysis of multiple genome regions, HIV-infected immunocompromised patients were found to be infected concomitantly by unrelated CMV strains, while no strain variation was detected in immunocompetent individuals (Baldanti *et al*, 1998).

It has also been shown that in more than 50% of AIDS patients with CMV retinitis, the strains isolated from the eye were different from those found in the blood. Cloning of CMV subgenomic amplicons revealed that different strains from the same compartment could be detected (Verbraak *et al*, 1998). Moreover, simultaneous infection with multiple strains of CMV has been found in healthy individuals at different sites: in five of 25 patients, diverse CMV gB strains could be found in different tissues (blood, lung and brain) (Meyer-Konig *et al*, 1998).

Multiple CMV infection may be associated with progression to AIDS in HIV infected homosexual men, possibly via activation of HIV-1 infected CD4+ T cells. It is possible that antigen from newly-infecting CMV strains induces activation of CD4+ T cells already infected with HIV-1, resulting in replication

of additional HIV-1, subsequently increasing the HIV-1 burden (Leach *et al*, 1994).

Serial excretion of multiple CMV strains may be explained by several possible mechanisms, including recombination among coexisting strains (Chou, 1989), mutations in the initial infecting strain (Plotkin *et al*, 1987) or infection with multiple CMV strains acquired through sexual contact. It is also possible that in the same body compartment, one CMV strain replicates while the other remains latent.

1.3.1.4 Multiple HHV-6 infection

HHV-6 is a ubiquitous betaherpesvirus causing infection in early childhood, infecting almost everyone by the age of 3 years (Hall *et al*, 1994). Primary infection produces variable febrile illnesses and are caused almost exclusively by one of the two strain groups, called variant A (HHV-6A) and variant B (HHV-6B) (Dewhurst *et al*, 1993). In adults, however, the asymptomatic persistence of both HHV-6B and HHV-6A has been detected, suggesting that both variants can be converted to latent forms. Reactivation and disease due to HHV-6 infection have also been described, primarily in immunocompromised adults (Drobyski *et al*, 1993). Coinfection by both variants has been reported. Thus, lung tissue specimens from both normal adults and BMT recipients with pneumonia could be shown to contain a mixture of HHV-6A and HHV-6B (Cone *et al*, 1996).

Furthermore, HHV-6 and CMV coinfection was reported in a patient

who carried different strains, resulting in concurrent amplification from saliva and PBMCs (van Loon *et al*, 1995). More recently, in a study of a large cohort of 2716 children, PBMCs, saliva and cerebrospinal fluid (CSF) specimens were examined for HHV-6 DNA and variants, it was found that infection with HHV-6A can occur in individuals who had previous infection with HHV-6B (Hall *et al*, 1998).

1.3.2 Hypothesis

Immunodeficiency is associated with

HHV-8 intra-host genetic variability.

1.3.3 Aims

To date, only inter-host HHV-8 sequence variability has been reported (Zong *et al*, 1997; Di Alberti *et al*, 1997a; Luppi *et al*, 1997; Nicholas *et al*, 1998), with no studies published to investigate HHV-8 intra-host genetic variation. Hence the initial aim of the present study was to investigate intra-host genetic diversity in a variety of different patient study groups, with a view to evaluate the extent of mixed infection, whether by co-infection or superinfection.

1.4 HIV-1 protease inhibitors and HHV-8

1.4.1 Protease inhibitors: a brief review

Until recently, nucleoside inhibitors of the viral enzyme reverse transcriptase were the only specific treatment available for HIV-1 infection. Though initially promising, they exerted modest antiviral activity, and the benefits of treatment were limited by the appearance of drug resistance and dose-limiting side-effects (Richman *et al*, 1987; Larder *et al*, 1989).

Development of more potent drugs which target different stages of the virus cycle continued. Efforts to develop inhibitors of HIV-1 protease produced a potent new class of compounds that could suppress HIV-1 replication to an extent much greater than was previously possible. (Roberts *et al*, 1990; Alteri *et al*, 1993; Vacca *et al*, 1994; Vaillancourt *et al*, 1995; Budt *et al*, 1995; Billich *et al*, 1995)

The HIV protease is essential for this virus's replication as it cleaves both structural and functional proteins from precursor viral polyprotein strands. Inhibition of this process suppresses HIV replication, resulting in the production of immature non-infectious virions (Kohl *et al*, 1988). The main antiviral action of HIV-protease inhibitors is therefore to prevent subsequent emergence of infection; they have no effect on cells already harbouring integrated proviral DNA (Overton *et al*, 1990).

Protease inhibitors (PrI) when combined with reverse transcriptase inhibitors (RTI) have been found to be highly effective in suppressing viral replication, both *in vitro* and *in vivo*. The use of two RTIs in combination with a protease inhibitor (triple therapy) or a combination of two reverse transcriptase and two protease inhibitors (quadruple therapy) delays or prevents the onset of resistance to any single agent. Since protease inhibitors induce potent inhibitory effects on their own metabolism, specific combinations taking advantage of pharmacokinetic interactions to increase or prolong drug concentrations allow dose reductions to be made without sacrificing anti-HIV activity, thus reducing drug toxicity (Ho, 1996; Tamalet *et al*, 1997; Hammer *et al*, 1997; Hoetelmans *et al*, 1998; Notermans *et al*, 1998). For instance, the PrI Ritonavir is a potent inhibitor of CYP3A4, an important hepatic enzyme involved in the metabolism of Saquinavir; hence Ritonavir by inhibiting Saquinavir metabolism increases the bioavailability of the latter drug (Merry *et al*, 1997).

However, important side-effects have been observed following the use of PrI. These include hyperglycaemia (Ault, 1997; Paterson *et al*, 1998), hyperlipidaemia (Sullivan *et al*, 1997), peripheral fat redistribution (Lipsky, 1998; Wurtz, 1998; Miller *et al*, 1998; Stocker *et al*, 1998), paraesthesia (Danner *et al*, 1995; Kakuda *et al*, 1998) and others (Behrens *et al*, 1998; Gallet *et al*, 1998) (summarised in Table 1.6). In general, the occurrence of these adverse effects does not always mandate change in therapy when a good therapeutic response is achieved.

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The pharmacological properties, toxic profile, drug interactions, and resistance patterns differ among protease inhibitors, and all must be considered when selecting the drugs for therapeutic use in HIV infected patients. The best combination, sequence of use, durability of response are issues that need to be fully elucidated. Guidelines for their use in HIV-infected patients are frequently being updated (Carpenter *et al*, 1996; Carpenter *et al*, 1997; Gazzard *et al*, 1998; Carpenter *et al*, 1998).

More recently, other targets for antiviral agents have been reported. Integrase inhibitors in combination with RTI and PrI in particular are very promising and may become the mainstay therapy of HIV infection in the future (Robinson, 1998).

1.4.2 The effect of protease inhibitors on KS and HHV-8 replication

In vitro susceptibility of HHV-8 to currently available antiviral drugs has been studied. Using the BCBL-1 cell line latently infected with HHV-8, it was found that HHV-8 replication could be inhibited by ganciclovir, foscarnet and cidofovir, but not acyclovir. In addition a PrI (a ritonavir analogue) had no effect upon HHV-8 replication (Kedes and Ganem, 1997). In another study, where synthesis of linear virion DNA and extracellular virus production was induced by phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), none of the antiviral drugs tested (cidofovir, ganciclovir and acyclovir) had a significant effect on the replication of episomal HHV-8 DNA. However, lytic replication was inhibited by low dose of cidofovir. Thus, while no therapeutic benefit from cidofovir would be expected in HHV-8 related lymphomas

(because these cells carry latent HHV-8), some level of suppression of HHV-8 reactivation may be expected in conditions where tissues carry replicating virus, e.g., KS (Medveczky *et al*, 1997; Neyts *et al*, 1997).

Highly active antiretroviral therapy (HAART), combining RTI and PrI has been shown to be very effective in inhibiting HIV replication, increasing CD4+ cell counts and delaying, lessening or resolving AIDS-associated opportunistic infections (Li *et al*, 1998; Pialoux *et al*, 1998). In addition, preliminary data, consisting of case reports, suggest that antiretroviral therapy using PrI may have an important effect on the remission of HIV-related KS (Blum *et al*, 1997; Burdick *et al*, 1997; Murphy *et al*, 1997; Hammoud *et al*, 1998; Martinelli *et al*, 1998; Aboulafia *et al*, 1998; Lebbe *et al*, 1998) and, indeed, on HHV-8 DNA clearance from PBMCs (Rizzieri *et al*, 1997; Blum *et al*, 1997; Martinelli *et al*, 1998; Burdick *et al*, 1998; Lebbe *et al*, 1998; De Milito *et al*, 1999). Moreover, following HAART, two HIV-positive patients with BCBL resulted in HHV-8 DNA becoming undetectable in their pleural effusions (Spina *et al*, 1998). In contrast, PrI treatment produced no effect on clinical and virological progression of an HIV-positive patient with MCD (Dupin *et al*, 1997).

1.4.3 Hypothesis

Therapy with HIV-1 protease inhibitors

is associated with suppression of HHV-8 replication

1.4.4 Aims

The second aim of this study was to evaluate the effect of HIV-1 PrI on the frequency of HHV-8 DNA detection from peripheral blood of HIV-positive patients. A longitudinal study of patients given PrI therapy was adopted. The effects of PrI upon CD4+ T cell count and HIV-1 plasma viral load were also compared.

Figure 1.1 HHV-8 genome map. At least 81 ORFs are encoded by the 140.5 kb genome. (Reproduced from Russo *et al*, 1996).
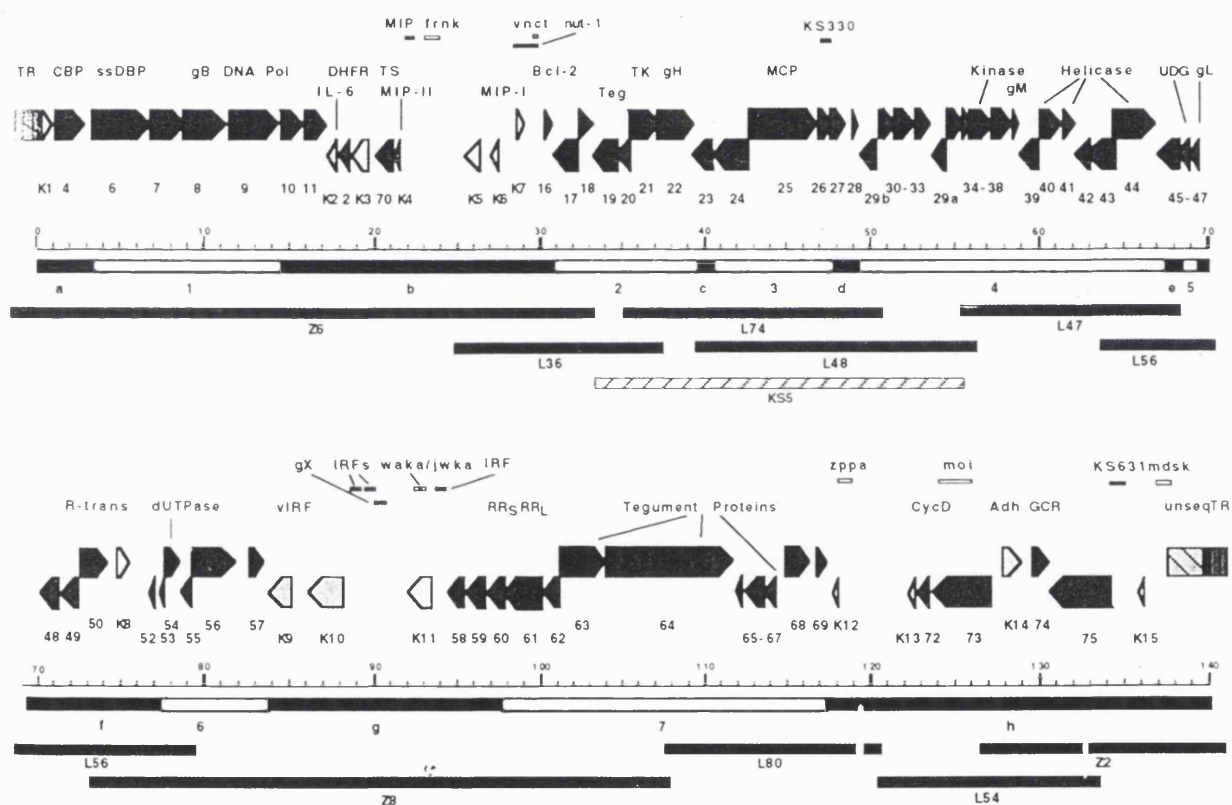
Table 1.1 - Summary of clinical types of KS - modified from Hermans *et al*, 1998.

	Classic	Endemic	Iatrogenic	Epidemic
Incidence	Rare	Endemic	Rare	Epidemic
M:F	10-15:1	10-15:1	-	20:1
Age (yr)	50-70	10-40	20-80	25-45
Sites of involvement	Legs (feet)	Legs, arms	Legs, arms, lymph nodes, visceral organs	Skin, oral, anal, visceral organs
Clinical behaviour	Indolent	Sometimes aggressive	Sometimes aggressive	Often aggressive
Oral lesions	Rare	Rare	Rare	Common
Prognosis and treatment response	Good	Good	Spontaneous remission after discontinuing treatment	Noncurative

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Table 1.2 - Clinical features of oral KS lesions in AIDS patients (Ficarra *et al*, 1988).

Location	Single Lesions	Multiple Lesions	Total
Palate	53 %	43%	95%
Gingiva	11%	12%	23%
Buccal mucosa	3%	7%	10%
Tongue (dorsum)	9%	0.7%	10%
Lips	4%	0%	4%

Table 1.3 - Comparative histologic features of KS and often clinically similar vascular lesions (Regezi *et al*, 1993).

Histologic feature	KS	BA*	PG**
Circumscribed tumour	-	+	+
Lobular tumour pattern	-	+	+
Oedematous stroma	-	-	+
Extravasated RBCs	+	-	-
Rounded blood vessels	-	+	+
Slit-like and bizarre vessels	+	-	-
Protuberant endothelial cells	-	+	+/-
Polygonal endothelial cells	-	+	+
Spindle-shaped endothelial cells	+	-	+
Spindle-shaped stromal cells	+	-	-
Mitotic figures	+/-	-	+/-
Nuclear atypia	+/-	+/-	+/-
Cytoplasmic bubbles	-	+	-
Eosinophils	-	-	-
Neutrophils, clusters	-	+	-
Neutrophils, diffuse	+/-	+/-	+
Basophilic granules (bacteria)	-	+	-
Hyaline globules	+	-	-
Haemosiderin	+	-	-

* Bacillary angiomatosis; **Pyogenic granuloma

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Table 1.4 - HHV-8 seroprevalence in general populations

Assay	Antigen	Seroprevalence in general population (%)	Reference
IFA	LANA	1-8 (US)	Kedes *et al*, 1996
WB	LANA	0 (US)	Gao *et al*, 1996a
IFA	LANA	25 (US)	Lennette *et al*, 1996
ELISA	ORF 65	1.7 (UK), 5 (US), 47 (Uganda)	Simpson *et al*, 1996
ELISA	ORF 26	11 (Germany)	Andre *et al*, 1997
ELISA	ORF 26	20 (US)	Davis *et al*, 1997
WB	SVCA	11 (US)	Lin *et al*, 1997
IFA	ORF 26/26	0 (US)	Smith *et al*, 1997
IFA	LANA	0 (US)	Martin *et al*, 1998
IFA/WB	LANA	8 (US)	Chandran *et al*, 1998
ELISA/IFA/WB	LANA/ORF 65	24 (Italy)	Calabro *et al*, 1998
IFA	LANA	2-3.6 (France)	Dupin *et al*, 1998
IFA	Latent/Lytic	12 (US)	Verbeek *et al*, 1998
IFA	LANA	2 (France)	Marcelin *et al*, 1998
ELISA	ORF 65.2	5 (Switzerland)	Regamey *et al*, 1998
ELISA	Whole virus	11 (US)	Chatlynne *et al*, 1998
IFA	Lytic/Latent	28/2 (Italy)	Rezza *et al*, 1998
IFA	Lytic	3-11 (Honduras)	Sosa *et al*, 1998

IFA = indirect immunofluorescence assay
ELISA = enzyme linked immunosorbant assay
WB = western blot
LANA = latency-associated nuclear antigen

Table 1.5 - HHV-8 homology to cellular proteins (modified from Whitby *et al*, 1998).

Host cell homologue	HHV-8 encoded protein	Possible function
D-Type cyclin	v-Cyc	Inactivation of pRB Promotes G1 to S phase transition
IL-8 GPCR	V-GPCR	Cellular growth signal
Interferon regulatory factor	v-IRF	Inhibits p21 and MHC class I expression
CC chemokines	v-MIP-I, vMIP-II, vMIP-1β	Chemoattraction, angiogenesis
IL-6	v-IL-6	Growth factor for KS cells
Bcl-2 family protein	v-Bcl-2	Inhibition of apoptosis
FLICE inhibitory protein	v-FLIP	Inhibition of CD95L and TNF-induced apoptosis
N-CAM family protein	v-Ox-2	Cellular adhesion molecule
CD21/CR2 complement binding protein	ORF 4	Escape from host immune response

Table 1.6 - Major side-effects of HIV-1 protease inhibitors (Behrens *et al*, 1998).

Side-effect	Protease inhibitors			
	Indinavir	**Nelfinavir**	**Ritonavir**	**Saquinavir**
Nausea	++	+	++	++
Vomiting	+	NR	++	+
Diarrhoea	+	++	++	++
Asthenia or fatigue	-	-	++	-
Hyperbilirubinemia	+	NR	-	+
Hyperglycaemia	+	+	+	+
Fat redistribution	+	+	+	+
Paraesthesias	NR	NR	++	-

NR = not reported

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Chapter 2 – Patients, materials and methods

2.1 Patients and materials for the HHV-8 genetic variability study

Peripheral blood specimens were obtained from serologically-proven HIV-infected patients from the Parasitic and Infectious Diseases unit of the Hospital das Clinicas (HC), Universidade Federal de Pernambuco (UFPE), Recife, Brazil and from the Department of Genito-Urinary Medicine of the Charing Cross Hospital, London, England. Blood was also obtained from patients who had undergone BMT at Hammersmith Hospital, London. KS tissues were paraffin embedded formalin fixed (PEFF) oral biopsies taken from HIV-infected patients from the Eastman Dental Hospital (patients details are listed in Table 2.1). As controls, blood from health care workers (HCW) assumed to be HIV-negative from HC in Recife were used, and the cell lines BC-1, BC-2 and BCP-1 (Cesarman *et al*, 1995; Boshoff *et al*, 1998) were ordered from the American Type Culture Collection. Informed consent (Appendix 2) and ethical committee approval were obtained prior to the study.

2.2 Patients and materials for the HIV-1 protease inhibitors and HHV-8 study

One hundred seventy six samples from 132 serologically proven HIV-infected male patients, aged 25 to 67 years, were studied over the period of 1996 until 1998. Of these, thirty three subjects were also longitudinally studied. The samples were collected from the Genito-Urinary Department of Charing Cross Hospital and processed at the Central Public Health

Laboratory (CPHL). Informed consent (Appendix 2) and ethical committee approval were received prior to the study.

2.3 Processing of blood specimens

Peripheral blood was drawn into EDTA-treated vacutainers and stored for no more than 7 days at 4^0 C. 1 ml of plasma was obtained after centrifugation for serological studies and the remaining blood was resuspended. 50 µl of Dynabeads (Dynal A.S., Oslo, Norway) was added, giving an approximate final concentration of 2×10^7 beads/ml of sample. Dynabeads are uniform, magnetizable beads coated with monoclonal antibody, specific in this case, for an epitope common to all CD45 (pan-leukocyte) isoforms. The mixture was then incubated for 20 min at 4^0 C, after which the Dynabeads were separated in a magnetic particle concentrator (MPC) (Dynal A.S., Oslo, Norway) and the supernatant pipetted off. The cells were washed three times using 2 ml of 2% foetal calf serum in phosphate buffered saline (PBS). Finally, the cells were eluted in 500 µl sterile water and stored at -20^0 C until use.

2.4 Deparaffinization of paraffin embedded formalin fixed (PEFF) tissues

PEFF tissues (30-50 µm thickness) were incubated at room temperature for 30 min with 1 ml of xylene in Eppendorf tubes to permit the paraffin to dissolve. Following vortexing and centrifugation in a fixed angled Eppendorf microfuge (30 s at 12,000g) the xylene was aspirated. A second

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xylene step of 15 min was taken to further ensure that the paraffin had dissolved. The samples were then incubated at room temperature for 10 min with 100% ethanol to remove the xylene, vortexed and centrifuged. This step was repeated twice. The pellets were left on the bench for 10 min to allow ethanol to evaporate.

2.5 DNA extraction

DNA was extracted from all the samples using Geneclean Kit III (BIO 101, La Jolla, CA). This procedure is based on the lysing and nuclease-inactivating properties of sodium iodide (NaI) together with the nucleic acid binding properties of silica particles (EZ-Glassmilk). The procedure was performed as follows: Cells eluted after the Dynabead cell separation procedure were thawed at room temperature and 500 μl of lysis buffer 6M NaI, and 20 μl of silica particles added. The test tubes were vortexed and incubated at room temperature for 5 min with frequent tube inversions. It is expected that during this period, all nucleic acids would have bound to the silica particles. The vessels were vortexed again, then centrifuged in a fixed angled Eppendorf microfuge (30 sec at 12,000g); the supernatant was removed by suction using an extended fine pipette, leaving the pellet intact. The pellets then underwent three washes with 500 μl of the NewWash (BIO 101, La Jolla, CA). After removal of NewWash the vessels were placed at 56^0 C with open lids in an Eppendorf heating block for 5 min to allow the pellets to dry. The DNA was eluted twice by resuspending the pellets in 40 μl of double-processed sterile and filtered water (Sigma, St. Louis, USA), then vortexed

and incubated at 56^0 C for a further 5 min followed by another vortexing and centrifugation step. The supernatant was removed for use as template in the first round of PCR. The remaining extract was then stored at -20^0 C until use.

2.6 A brief history of PCR and the *Taq* polymerase

Kary Mullis first conceived the idea for the polymerase chain reaction (Mullis, 1990) a technique used to amplify predefined segments of DNA. Amplification may be used either to raise the concentration of an unknown above detection threshold (as would be useful in the early detection of viral contaminants in blood) or to increase the amount of specific DNA (which is often precious) for a subsequent processing step, as in the case of *in vitro* gene construction. These two examples illustrate broad categories; one which uses PCR as its primary end and one which uses PCR merely as a process step. The first description of PCR was terse in its first publication on detection of the mutation causing sickle cell anaemia in whole genomic DNA (Saiki *et al*, 1985).

The DNA polymerase originally used for the PCR was extracted from the bacterium *E. coli*. However, after each cycle of DNA synthesis, the reaction must be heated to denature the double stranded DNA product. Unfortunately, heating also irreversibly inactivated this polymerase, so new enzyme had to be added at the start of each cycle. The bacterium *T. aquaticus* lives in hot springs, and produces a DNA polymerase which is not irreversibility inactivated at high temperature. David Gelfand and his associates (Saiki *et al*, 1988) purified and subsequently cloned this

polymerase (Lawyer *et al*, 1993) allowing a complete PCR amplification to be done without opening the reaction tube. However, the thermostable enzyme was found to be much more than just a convenience. The DNA synthesis step could now be done at a higher temperature than was possible with the *E. coli* enzyme, and it was discovered that the template DNA strand could be copied with high fidelity, eliminating the nonspecific products that had plagued earlier attempts at amplification.

2.6.1 PCR amplification of HHV-8 sub-genomic DNA and human human β-globin gene

A nested protocol was used in order to amplify subgenomic HHV-8 ORF 26 and K1 DNA and single round PCR for the amplification of the human β-globin gene, HHV-8 ORF 25, K12 and nut-1 region. The primers used are listed in Table 2.2

First round PCRs were carried out in a 25 μl reaction containing sterile water, *Taq* polymerase buffer (Gibco BRL, Paisley, Glasgow), 15 mM MgCl$_2$, 200 μM of each of the 4 deoxynucleotide triphosphates (dNTPs) (Boehringer Mannheim, Lewes, East Sussex), 20 pmol of each outer primer, 1 unit of *Taq* polymerase (Gibco BRL, Paisley, Glasgow) and 2.5 μl of HHV-8 DNA template. This PCR protocol has been published elsewhere (Di Alberti *et al*, 1997b). A PTC-100 thermal cycler (MJ Research, Inc., GRI Ltd, Dunmow, Essex) was programmed to perform 1 cycle of 5 min at 94^0 C followed by 34 cycles of 94^0 C for 1 min (denaturation), 60^0 C for 1 min (annealing) and 72^0 C

for 1 min (extension). Second-round reactions were also carried out in a volume of 25 μl, containing sterile water, *Taq* polymerase buffer, 15 mM MgCl₂, 200 μM dNTPs, 20 pmol of each inner primer, 1 unit of *Taq* polymerase and 2.5 μl of primary PCR product. The amplifications were carried out under the same cycling conditions as the primary reaction.

2.6.1.1 Detection of PCR products

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

2.6.1.2 Avoiding PCR contamination

The sensitivity of PCR occasionally results in problems with contamination from reagents or samples. This occurs when exogenous previously amplified sequences or positive control contaminate the reaction mixture resulting in a false-positive signal. A number of routine precautions

(Kwok and Higuchi, 1989) were taken in order to minimise PCR carry over.

2.7 T/A Cloning

This was done using the LigATor kit (R&D Systems, Minneapolis, MN). T/A cloning is based on the finding that PCR products generated by *Taq* polymerase possess a single adenosine overhang at each 3' end, which is caused by the template-independent terminal transferase activity of *Taq* polymerase (Holton *et al*, 1991; Marchuk *et al*, 1991). The procedure involves freshly amplified DNA fragments being ligated to a vector with single 5' thymidine overhangs (pTAg), transformation into competent *E. coli* cells (Stratagene, La Jolla, CA). One microlitre of plasmid DNA was added to the competent cells, mixed gently, and stored on ice for 30 min; the cells were then heat shocked at 42^0 C for 2 min and snap cooled on ice; finally, 80 µl of suspension organ culture (SOC) media was added to the cells followed by incubation at 37^0 C for 1 hour to allow the cells to start expressing antibiotic resistance. The cells were then plated out onto LB agar plates containing 50 mg/ml ampicillin (which allows the selection of bacteria that had taken up the plasmid/vector), 0.5 µM isopropylthiogalactoside (IPTG) and 80 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (x-gal). The cells were grown overnight in an incubator at 37^0 C following which 20 white colonies were picked. The presence of white colonies indicates that the cloning has been successful and that the PCR products are inserted into the vector. The plasmid contains the *Lac Z* gene which produces the enzyme ·β-galactosidase, which when induced by IPTG leads to breakdown of the

substrate x-gal, giving a blue product. Therefore, colonies without inserts are blue, being *Lac Z* positive. However, when DNA is inserted into *Lac Z*, the bacteria can no longer utilise x-gal, so the colony remains white.

The colonies were screened for inserts by PCR (Pampfer, 1993). This was done as follows: White colonies were labelled, picked, and transferred to 25 µl of second-round PCR master mix. The mixture was then submitted to PCR using the inner primers and the products visualised.

2.8 The PCR-single strand conformation polymorphism (SSCP) assay

Single strand conformation polymorphism was originally developed to screen for mutations in human genomes (Orita *et al*, 1989). The sequences of interest are first simultaneously amplified and radiolabelled. Radiolabelled PCR products, consisting of double-stranded DNAs, are denatured to produce single-stranded (ss) DNAs and electrophoresed through a non-denaturing gel. Electrophoretic mobility of ss DNA in such a gel is especially sensitive to both its size and conformation, since each ss DNA adopts a folded structure which is sequence-specific, being maintained by sequence-dependent local intramolecular interactions. In PCR-SSCP analysis, the sequence changes result in differences in mobility rate through the gel which are detectable after autoradiography as differential banding patterns. Although theoretically each PCR product is expected to produce two bands representing two ss DNAs, in practice, there may be more bands, indicating the presence of ss DNA molecules which adopt more than one conformation.

In rare cases, one or both strands are detected as one prominent band which might be due to the migration of ss DNA at similar rates. Because of its high resolving power, polyacrylamide gel electrophoresis can distinguish most conformational changes resulting from subtle sequence differences such as one base substitution in a several hundred base pair fragment. This mobility pattern cannot, however, determine either the precise position or the exact nature of the base changes.

For the PCR-SSCP assay, colonies were first amplified using the protocol previously described. Second-round PCR was then carried out in a 20 μl volume containing sterile water, *Taq* polymerase buffer, 15 mM $MgCl_2$, 70 μM dNTPs, 20pmol of each outer primer, 2 μCi of ^{32}P-deoxycytosine 5'-phosphate (Amersham International, Amersham, Bucks), 1 unit of *Taq* polymerase and 2 μl of primary PCR product.

2.8.1 Preparation of SSCP assay plates

Two plates were required for the SSCP electrophoresis procedure, a thermostatic plate (25 cm x 61 cm) and a glass plate (21 cm x 55 cm). The thermostatic plate was placed on a KB2010-100 macromould and wiped over with 100% ethanol. Then 10 ml of "repel-silane" (2% solution of dimethyldichlorosilane in 1,1,1-trichloroethane, BDH Limited, Poole, Dorset) was spread over the plate and left for 5 min. The remaining fluid was removed and the process repeated. Before using the plate it was wiped over again with ethanol and left to dry. The glass plate was wiped over with 100% ethanol and

then covered with 10 ml of "bind-silane" mix (20 ml absolute ethanol, 600 µl of 10% acetic acid, and 60 µl of gamma-methacryloxypropyltrimethoxy silane). After leaving for 5 min, the remaining fluid was removed and the procedure repeated. As with the thermostatic plate, it was wiped over with ethanol before use.

The spacers were placed on the thermal plate. Then the glass plate placed over it, making sure that the treated sides were facing inwards. The plates and spacers were clamped together, after which the gel was poured.

2.8.2 Preparation of SSCP assay gel

The gel mix contained 51.2 ml of distilled water, 20 ml of mutation detection enhancement (MDE) gel (Hydrolink, Flowgen), 4.8 ml of 10 x Tris-borate-EDTA-buffer (TBE) and 4.0 ml of glycerol were added and mixed thoroughly. Just before the gel was poured, 450 µl of 10% ammonium persulphate (APS) and 45 µl of N,N,N',N'-tetramethulethylenediamine (TEMED) (Gibco BRL) were added. The mixture was carefully transferred to a 50 ml syringe and the gel mixture introduced, by capillary action, in the space between the plates. The comb was then placed and firmly secured with a clamp into the top of the gel, and was left to set for a minimum of 1 hour.

2.8.2.1 Loading the gel

The PCR products were mixed in a 1:1 ratio with loading buffer (98% deionised formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and 20

mM EDTA). The diluted PCR products were denatured in a heating block at 95^0 C for 5 min to yield ssDNA. The samples were then snap cooled on ice and left until ready for loading on the gel. They were loaded and electrophoresed through the non-denaturing polyacryamide gel at 8 W for 18 hours at 15^0 C in a Macrophor sequencing apparatus, the temperature being maintained using a water jacketed thermostatic plate connected to a thermostatic circulator (Pharmacia LKB Biotechnology, Uppsala, Sweden). It is important that the temperature is maintained, as this method is sensitive to physical environment. A temperature rise during electrophoresis may affect the reproducibility of the results.

After electrophoresis, the gel was covered with Saran wrap and exposed to autoradiographic film (Hyperfilm-MP, Amersham International, Amersham, Bucks) for 18 hours.

2.8.3 Development of the autoradiograph

After 18 hours, the autoradiograph was placed in a tray of developer (4:1 ratio water to developer concentrate, Kodak, Hemel Hempstead, Herts) so that it was completely submerged, and agitated for 2 min, then rinsed thoroughly in water. The film was then immersed in fixative (4:1 ratio water to fixer concentrate, Kodak, Hemel Hempstead, Herts), rinsed thoroughly and left to dry. Once dry, it was examined on an X-ray viewer.

2.9 DNA sequencing

2.9.1 Purification of PCR products for sequencing

PCR products were purified using the Gelprep GP2 Kit (Helena BioSciences, Sunderland). Products were loaded onto a 2% composite agarose gel, as described earlier, in alternate wells to allow enough room for cutting out the product from the gel without risk of contamination from other products. The products were then electrophoresed and detected using ethidium bromide as described previously. The products were viewed using long wave uv light and each band cut with a scalpel and transferred to separate 1.5 ml Eppendorfs. The gel was then photographed in the presence of shortwave uv light to check that all the DNA had been removed. Each sample was weighed and TE added to ensure that the Eppendorfs were of roughly equal weight. The agarose was then melted at 56^0 C in the presence of Gel solution B (as our fragment was less than 500 bp) and gel additive (the latter reagent is required to optimise DNA binding to the gel resin, when TBE agarose gels were used). Once the agarose had melted, 10 µl of gel resin was added to each reaction vessel and vortexed. This was left on the bench for 1 min with frequent tube inversions. The tubes were vortexed once more, then spun for 30 sec at 12,000 g to pellet the gel resin. The supernatant was removed and the pellets underwent 1 wash with centrifugation (30 sec at 12,000 g) and removal of the supernatant. The tubes were then heated at 56^0 C with their lids open to allow evaporation of any residual alcohol for 1 min and the DNA from the gel resin then eluted. After elution the vessels were

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centrifuged (30 sec at 12,000 g) and the supernatant removed and placed in a 0.5 ml Eppendorf tube. To confirm that the cleaning had been successful and to roughly estimate the quantity of DNA recovered, 1 µl of this supernatant was taken and run on a 2% agarose gel, stained and visualised.

2.9.2 Automated sequencing of PCR products

Sequencing of the PCR products was done by cycle sequencing using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit with AmpliTaq™ FS DNA polymerase (Perkin Elmer, Forest City, CA). In each case, both strands of the PCR product were sequenced. The 20 µl sequencing reaction consisted of 8 µl of the sequencing mix, 3.2 pmol of one of the primers and purified DNA at approximately 100 ng for the HHV-8 fragment. The reaction vessels underwent 25 cycles of 30 sec at 95^0 C, 30 sec at 50^0 C and 4 min at 72^0 C in a PTC-100 thermal cycler. These were held at 4^0 C for a further 10 min. The reaction vessels were then placed on ice and the DNA precipitated. The sequencing pellets were then electrophoresed by the sequencing laboratory facilities in the CPHL site. Following this, the raw sequence data was processed and analysed using the programmes "Analysis", "SeqEd" (Applied Biosystems, Foster City, CA), and "Lasergene Navigator" (DNASTAR, Madison) (Appendix 1).

2.9.2.1 Estimation of amount of ds DNA required for cycle sequencing

The optimal conditions for cycle sequencing are a 4:1 molar ratio of primer to purified DNA i.e. 3.2 pmol of primers are used per sequencing reaction. Therefore, 0.8 pmol of DNA is required.

The relationship between size of DNA, picomoles and mass is:

$$\frac{2 \times 10^6}{(660 \times \text{number of bp})} = \text{pmol ends/}\mu\text{g}$$

[2×10^6 adjusts for 2 ends and μg; 660 is the mean molecular weight of a bp]

In practice:

$$\frac{0.8 \times (660 \times \text{number of bp})}{1000} = \text{ng of DNA required for sequencing}$$

2.9.2.2 Precipitation of sequenced PCR products

After cycling the sequencing reaction was placed in 50 µl of 95% ethanol containing 2 µl of 3 M sodium acetate (pH 4.5). This was then kept at -20^0 C for a minimum of 10 mins. The reaction vessels were then spun in a microfuge for 20 mins at maximum speed, following which the supernatant was removed and the pellets washed with 250 µl of 75% ethanol, then respun for a further 5 mins. The supernatant was aspirated and the pellets dried (these pellets are stable for 3 months if kept in the dark at -20^0 C).

2.10 HHV-8 IgG detection by indirect immunofluorescence assay

An indirect immunofluorescence assay (IFA) kit was used (ABI, Maryland). The assay utilises the indirect method of fluorescent antibody staining. The procedure was carried out in two basic reaction steps: in step one, the human serum or plasma to be tested was brought into contact with fixed infected cells. Antibody, if present in the test sample, will complex with the antigen in the cellular substrate. If the sample being examined contains no antibody for this particular antigen, no complex would be formed, and all the serum components would be washed away in the rinse step. The second step involves adding fluorescein-labeled anti-human antibody. If the antibody to HHV-8 was present (a positive reaction), bright apple-green fluorescence would be seen with the aid of a fluorescence microscope. Positive and negative controls were used to ensure the test was working. A well containing wash buffer was also included. There was a conjugate control to ensure the conjugate was not reacting with cell substrate.

One millilitre of each plasma was diluted 1:20 in washing buffer, and 20 µl aliquots were applied to each well according to manufacturer instructions. The slides were then incubated in a moist chamber for 30 min at 37^0 C. After the incubation step, the slides were removed from the moist chamber and washed for 5 min in wash buffer, while slowly stirring on a magnetic stir plate. The surface of the slide was dried with the provided blotter and care was taken not to touch the wells with the blotter. 20 µl of FITC anti-human IgG conjugate was dispensed on each well and the slides were placed

in the moist chamber for another incubation for 30 min at 37^0 C. The washing step was repeated and approximately 10 µl of mounting solution per well was carefully applied. The slides were finally examined under a fluorescence microscope.

2.10.1 Interpretation of anti-HHV-8 IgG antibody detection

A strong positive result as judged when using a known positive control was scored as 3+ while samples without any reactivity were scored as negative. Weak and medium positives were scored respectively as 1+ and 2+ respectively.

2.11 Evaluation of CD4+ T cell count and HIV-1 plasma viral load

The absolute CD4+ lymphocyte counts were determined by flow cytometry (Ortho Cytoron Absolute (Ortho-Clinical Diagnostics, Chalfont St. Giles, UK). HIV-1 plasma viral load was primarily measured using the Amplicor HIV-1 Monitor test (Roche Diagnostics, Welwyn, UK), although on occasions the Quantiplex assay (Chiron Diagnostics, Halstead, UK) was also used. These methods are comparable in sensitivity and performance (Giles *et al*, 1998).

2.12 Statistical analysis

2.12.1 Statistical analysis for HHV-8 genetic variability study

Comparison between two groups was done using the non-parametric procedure, Wilcoxon test. For comparing more than two groups, the Kruskal-Wallis test was employed.

2.12.1.1 Normalised entropy value

The Shannon-Wiener index, here expressed as entropy value, was used to further assess the degree of variability within clinical samples (Stewart *et al*, 1997). This index takes into consideration the number and frequency of minor variants within a single sample. Normalised entropy value is calculated by dividing the number and frequency of sequence variants by the number of clones amplified in each sample. The value can range from zero, when only one variant sequence is present, to one, when each variant sequence occurs once. Therefore, higher normalised entropy values indicate greater sequence variability. The index is calculated according to the following formula, where p is the frequency of clones within a sample and N is the total number of clones amplified in each sample (Stewart *et al*, 1997):

$$\sum \left(\frac{(p1 \times \ln p1) + (p2 \times \ln p2) \ldots\ldots\ldots}{\ln N} \right)$$

2.12.1.2 Mean genetic distance (MGD)

The mean genetic distance was also used to evaluate variability within clinical samples. It is generated by Clustal which gives the percentage of differences between sequences, ranging from 0-100%, the higher values representing greater variability (Stewart *et al*, 1997).

2.12.2 Statistical analysis for HIV-1 protease inhibitors and HHV-8 study

The Student t-test procedure was used to compare the means of two variables for a single group. It computed the differences between values of the two variables for each case and tested whether the average differs from 0. In the longitudinal study, the CD4+ T cell count and HIV-1 plasma viral load were measured prior to, and during PrI therapy. Thus, each subject had two measures, termed the "before" and "after" measures. .

The McNemar test was also applied. This is a non-parametric statistical test useful for detecting changes in responses due to experimental intervention in "before and after" designs, using the chi-square distribution.

Table 2.1 Patients details for studying genetic variability in HHV-8.

Subject number	Type of tissue	Country residency	Gender and age	Clinical details
Patient 01	HIV + blood	Brazil	M : 23	CDC C3; homosexual; CD4+ cell count 55 cells/μl
Patient 02	HIV + blood	Brazil	F : 28	CDC C3; bisexual partner; CD4+ cell count 20 cells/μl
Patient 03	HIV + blood	Brazil	F : 39	CDC B3; bisexual partner; CD4+ cell count 120 cells/μl
Patient 04	HIV + blood	England	M : 40	CDC B2; homosexual; CD4+ cell count 283 cells/μl
Patient 05	HIV + blood	England	M : 60	CDC B2; homosexual; CD4+ cell count 378 cells/μl
Patient 06	HIV + blood	England	M : 34	CDC B2; homosexual; CD4+ cell count 377 cells/μl
HCW PT 07	HCW blood	Brazil	F : 22	healthy volunteer
HCW PT 08	HCW blood	Brazil	M : 35	healthy volunteer
HCW PT 09	HCW blood	Brazil	M : 49	healthy volunteer
HCW PT 10	HCW blood	Brazil	M : 42	healthy volunteer
Patient 11	AIDS KS PEFF	England	M : 25	Not available
Patient 12	AIDS KS PEFF	England	M : 34	Not available
Patient 13	AIDS KS PEFF	England	M : 56	Not available
Patient 14	AIDS KS PEFF	England	M : 37	Not available
Patient 15	AIDS KS PEFF	England	M : 28	Not available

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Patient 16	BMT blood	England	F : 24	Not available
Patient 17	BMT blood	England	M : 39	Not available
Patient 18	BMT blood	England	M : 21	Not available
Patient 19	BMT blood	England	F : 15	Not available

F = female
M = male

Table 2.2 Primer sequences for amplifying different regions of HHV-8 genome

HHV-8 ORF 26

Sense primer	KS1	5'AGCCGAAAGGATTCCACCAT3'
Anti-sense primer	KS2	5'TCCGTGTTGTCTACGTCCAG3'
Sense primer	KSinn1	5'TTCCACCATTGTGCTCGAAT3'
Anti-sense primer	KSinn2	5'TACGTCCAGACGATATGTGC3'

HHV-8 ORF 25

Sense primer	KS25.1	5'CATGGATACATTGTCAGGACCTC3'
Anti-sense primer	KS25.2	5'GTCACATCTGACGTTGCCT3'

HHV-8 ORF K1

Sense primer	K1-1	5'CCTTTCTCTGCTATCGTCTC3'
Anti-sense primer	K1-2	5'GGTAGATGCCAAACGGTAAC3'
Sense primer	K1inn5	5'CCCTGGAGTGATTTCAACGC3'
Anti-sense primer	K1inn6	5'ACATGCTGACCACAAGTGAC3'

HHV-8 K12

Sense Primer	Latout1	5'ATGGATAGAGGCTTAACGGTG3'
Anti-sense primer	Latout2	5'GCTTTAATGCGGAGAGGAATG3'

HHV-8 nut-1

Sense primer	Lytout1	5'CACCTATGGATTTTGTGCTCG3'
Anti-sense primer	Lytout2	5'GCGCTGCTTTCCTTTCACATT3'

Figure 2.1 Flow chart of the methodology employed for studying genetic variability in HHV-8. Starting materials for the study specimens were either fresh blood or PEFF tissue, and cell lines as controls. Peripheral blood cells were then isolated using immunomagnetic separation, whereas tissues were first deparaffinised. DNA was extracted using Geneclean kit and PCR wasthen applied. Clones were generated using T/A cloning system and SSCP was performed. Heterogeneous clones were then sequenced.

Fresh blood   **PEFF tissue**   **Cell lines**

Dynabeads CD45   **Deparaffinization**   **Centrifugation**

DNA extraction (Geneclean)

PCR amplification

T/A cloning

PCR-radiolabelling

SSCP assay

Sequencing heterogeneous clones

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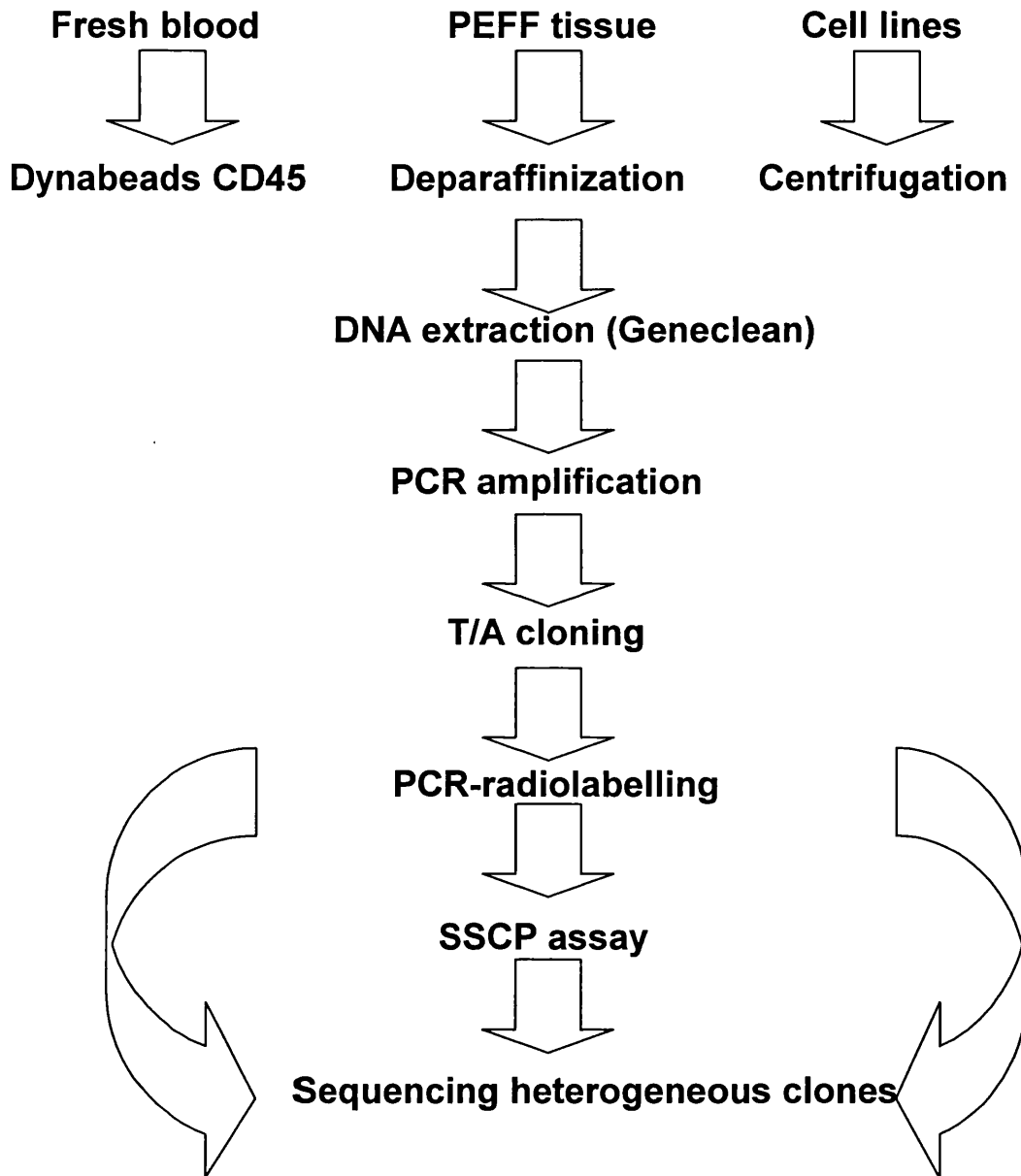
Chapter 3 – Results: Relative restriction of human herpesvirus 8 in KS tissues, peripheral blood of health care workers and bone marrow transplant patients compared to peripheral blood of HIV infected patients

3.1 Results

3.1.1 HHV-8 genome comparative sequence variability

In order to sample which regions of the HHV-8 genome shows more genetic variability, PCR amplicons were amplified from the cell lines BC-1, BC-2, BC-3 and BCP-1 encompassing segments within ORF 26 (minor capsid protein), ORF 25 (major capsid protein), ORF K1 (transmembrane glycoprotein), ORF K12 (kaposin or T0.7), and the nut-1 region which encodes T1.1, an RNA like transcript (Russo *et al*, 1996).

The amplicons derived from the cell lines show that the segments of the ORF K12 region were identical; this was also the case with the nut-1 region (Figure 3.1). By contrast, the segment within ORF K1, which corresponds to the region known as variable region I (VR I) (Nicholas *et al*, 1998) was the most hypervariable. Segments within ORF 25 and 26 showed intermediate variability. For subsequent studies, because amplification with K1 primers from fresh clinical specimens did not consistently yield amplicons, the primer set amplifying a segment within ORF 26, $KS_{330}Bam$ was used, to examine clinical specimens for HHV-8 heterogeneity. Only those samples from which the ORF K1 sequence was amplified were tested for K1 sequence variability. The reasons for not consistently amplify K1 from fresh samples are likely to be as result of poor PCR sensitivity. Many PCR optimisation steps were tried, including changing in the annealing temperature using the Robocycler Gradient 96 (Stratagene, La Jolla, CA); alteration in the PCR mix,

changing the pH through a change in the PCR buffer; increasing magnesium chloride, dNTPs and *Taq* concentration; redesign of primers, avoiding palidromic sequences and therefore formation of "hairpins" (a group of complimentary deoxyribonucleotides forming a bridge like linkage) and avoiding a rich G + C content.

Figure 3.1 Comparative sequence diversity of 5 subgenomic regions of HHV-8 in 4 BCBL cell lines. This experiment was performed in order to elucidate what is the most informative region of the HHV-8 genome. It is seen that the segments within ORF K12 and nut-1 are well conserved. In contrast, ORF K1 is the most hypervariable, all 4 cell lines showing different banding patterns. ORF 25 and 26 show intermediate variability: BC-1 and BCP-1 giving the same SSCP banding pattern, and BC-2 and BC-3 giving another banding pattern.

97
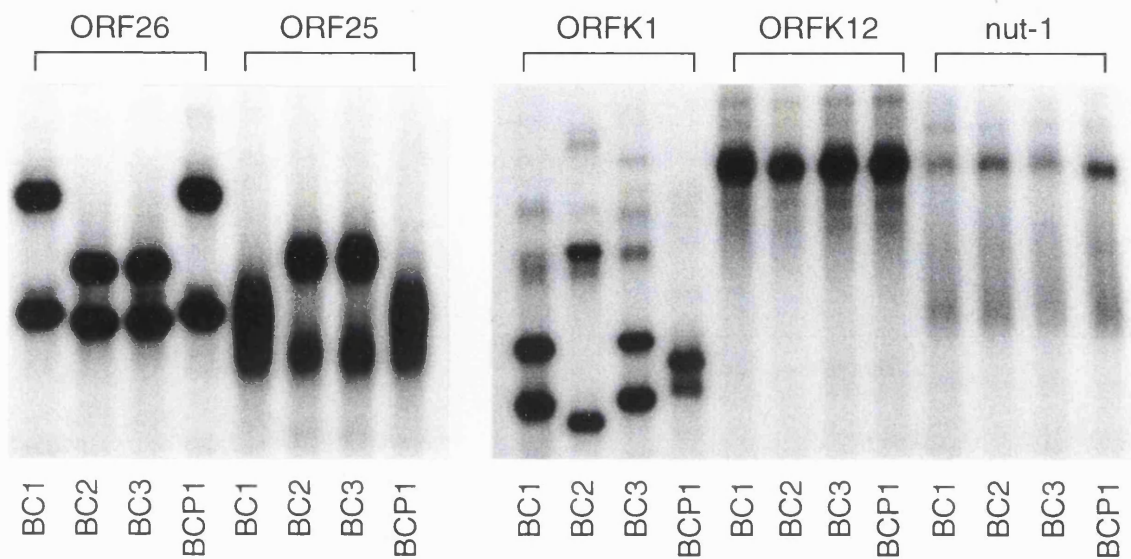
3.1.2 ORF 26 sequence diversity

3.1.2.1 Blood from HIV positive patients

This group comprised six HIV positive individuals, aged 23 to 60 years, median age 36 years, of whom three were resident in Brazil and three resident in England (Table 2.1). Seventeen to twenty clones were amplified from each patient.

Figure 3.2 shows the SSCP profile and sequences of clones from the blood of patient 01. It is seen that there were 6 variant sequences (I to VI), sequence II differing from sequence I (the predominant sequence) by one nucleotide (at position 90), sequence III differing from sequence I by one nucleotide (at position 123), sequence IV differing from sequence I by two nucleotides (positions 94 and 164), sequence V differing from sequence I by two nucleotides (positions 121 and 162), and sequence VI differing from sequence I by one nucleotide (at position 44).

Figure 3.3 shows the SSCP profile and sequences of clones from the blood of patient 02. There were 5 variant sequences (I to V), sequence II differing from sequence I by two nucleotides (positions 20 and 153), sequence III differing from sequence I by three nucleotides (positions 14, 20 and 128), sequence IV differing from sequence I by one nucleotide (at position 37) and sequence V differing from sequence I by one nucleotide (at position 115).

Figure 3.4 shows the SSCP profile and sequences of clones from the blood of patient 03. There were 6 variant sequences (I to VI), sequence II differing from sequence I by two nucleotides (positions 20 and 148), sequence III differing from sequence I by one nucleotide (at position 148), sequence IV differing from sequence I by three nucleotides (positions 6, 20 and 148), sequence V differing from sequence I by three nucleotides (positions 20, 115 and 148) and sequence VI differing from sequence I by one nucleotide (at position 20).

Figure 3.5 shows the SSCP profile and sequences of clones from the blood of patient 04. There were 3 variant sequences (I to III). Sequence II differing from sequence I by one nucleotide (at position 106) and sequence III differing from sequence I by two nucleotides (positions 47 and 108).

Figure 3.6 shows the SSCP profile and sequences of clones from the blood of patient 05. There were 2 sequence variants (I and II), sequence II differing from sequence I by two nucleotides (positions 20 and 137).

Figure 3.7 shows the SSCP profile and sequences of clones from the blood of patient 06. There were 5 sequence variants (I to V), sequence II differing from sequence I by one nucleotide (at position 65), sequence III differing from sequence I by two nucleotides (positions 53 and 114), sequence IV differing from sequence I by one nucleotide (at position 138) and sequence VI differing from sequence I by one nucleotide (at position 63). The results are summarised in Table 3.1.

Figure 3.2 SSCP profile and sequences of HHV-8 ORF 26 clones derived from blood of patient 01 (HIV positive). The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence which is represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.


```
    A A C G G A T T T G A C C T C G T G T T C C C C A T G G T C G T G C C G C A G C A A C T G G G G C A C G C T A T T C T G  Majority
                10              20              30              40              50              60
  1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT01-I
  1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT01-II
  1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT01-III
  1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT01-IV
  1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT01-V
  1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . . . . . . . . . . . . . .  PT01-VI

    C A G C A G C T G T T G G T G T A C C A C A T C T A C T C C A A A A T A T C G G C C G G G G C C C C G G A T G A T G T A  Majority
                70              80              90              100             110             120
 61 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT01-I
 61 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT01-II
 61 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT01-III
 61 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT01-IV
 61 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT01-V
 61 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT01-VI

    A A T A T G G C G G A A C T T G A T C T A T A T A C C A C C A A T G T G T C A T T T A T G G G G C G C              Majority
                130             140             150             160             170
121 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .              PT01-I
121 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .              PT01-II
121 . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .              PT01-III
121 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . . .              PT01-IV
121 G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . . . .              PT01-V
121 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .              PT01-VI
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100

Figure 3.3 SSCP profile and sequences of HHV-8 ORF 26 clones derived from blood of patient 02 (HIV positive). The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence which is represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.
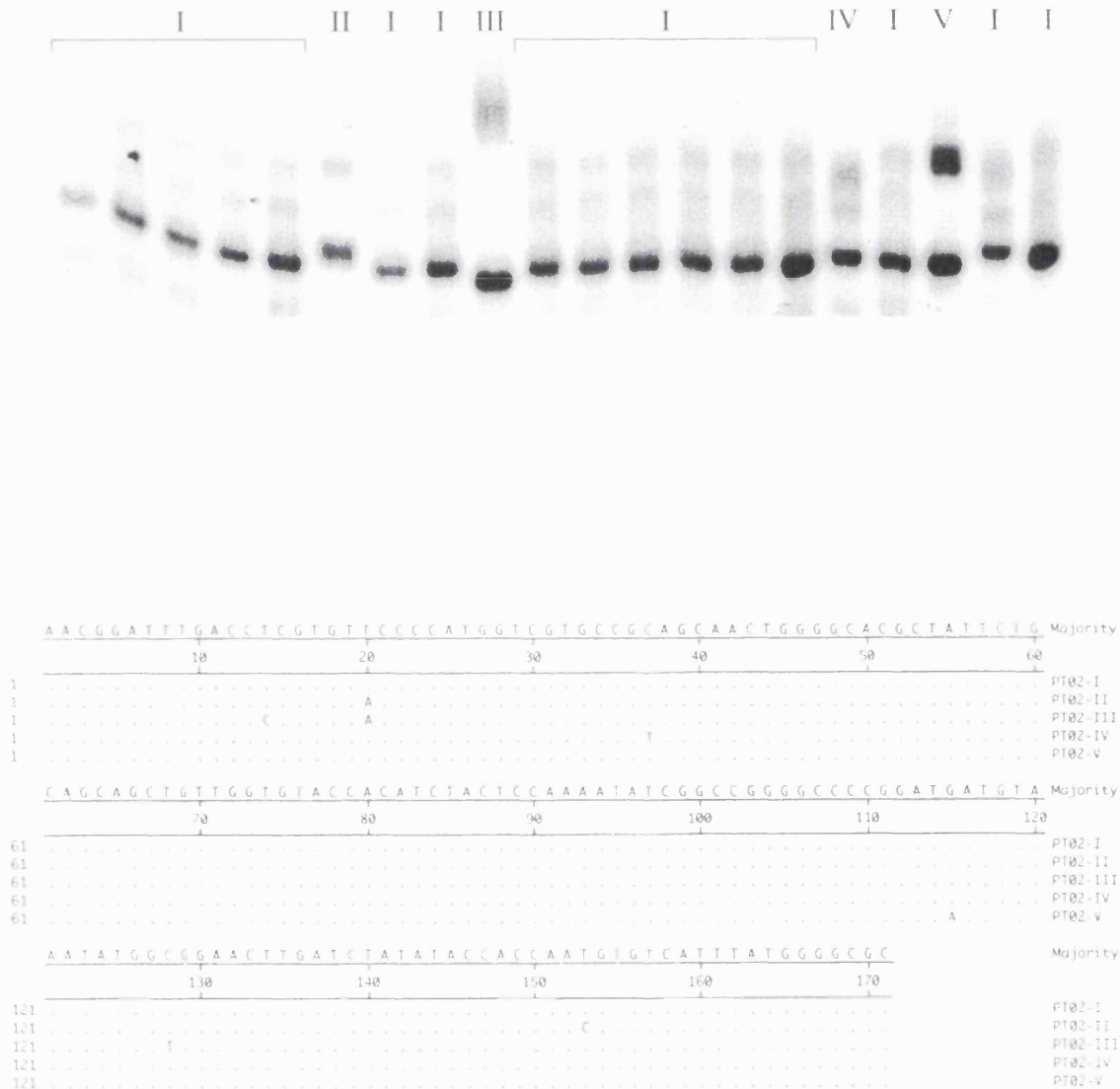
Figure 3.4 SSCP profile and sequences of HHV-8 ORF 26 clones derived from blood of patient 03 (HIV positive). The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence which is represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.


```
     A A C G G A T T T G A C A T C G T G T T C C C C A T G G T C G T G C C T C A G C A A C T G G G G C A C G C T A T T C T G  Majority
                   10                  20                  30                  40                  50                  60
  1  . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT03-I
  1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT03-II
  1  . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT03-III
  1  . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT03-IV
  1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT03-V
  1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT03-VI


     C A G C A G C T G T T G G T G T A C C A C A T C T A C T C C A A A A T A T C G G C C G G G G C C C C G G G T G A T G T C  Majority
                   70                  80                  90                  100                 110                 120
 61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT03-I
 61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT03-II
 61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT03-III
 61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT03-IV
 61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . .  PT03-V
 61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT03-VI


     A A T A T G G C G G A A C T T G A T C T A T A T A C C A C C A A T G T G T C A T T T A T G G G G C G C                    Majority
                   130                 140                 150                 160                 170
121  . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . .                    PT03-I
121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                    PT03-II
121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                    PT03-III
121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                    PT03-IV
121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                    PT03-V
121  . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . .                    PT03-VI
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Figure 3.5 SSCP profile and sequences of HHV-8 ORF 26 clones derived from blood of patient 04 (HIV positive). The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence which is represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.


```
A A C G G A T T T G A C C T C G T G T T C C C C A T G G T C G T G C C G C A G C A A C T G G G G C A C G C T A T T C T G  Majority
              10              20              30              40              50              60
1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT04-I
1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT04-II
1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . . . . . . . . . . . . . . .  PT04-III


C A G C A G C T G T T G G T G T A C C A C A T C T A C T C C A A A A T A T C G G C C G G G G C C C C G G A T G A T G T A  Majority
              70              80              90             100             110             120
61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT04-I
61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT04-II
61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . .  PT04-III


A A T A T G G C G G A A C T T G A T C T A T A T A C C A C C A A T G T G T C A T T T A T G G G G C G C  Majority
              130             140             150             160             170
121 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT04-I
121 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT04-II
121 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT04-III
```

Figure 3.6 SSCP profile and sequences of HHV-8 ORF 26 clones derived from blood of patient 05 (HIV positive). The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence which is represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.
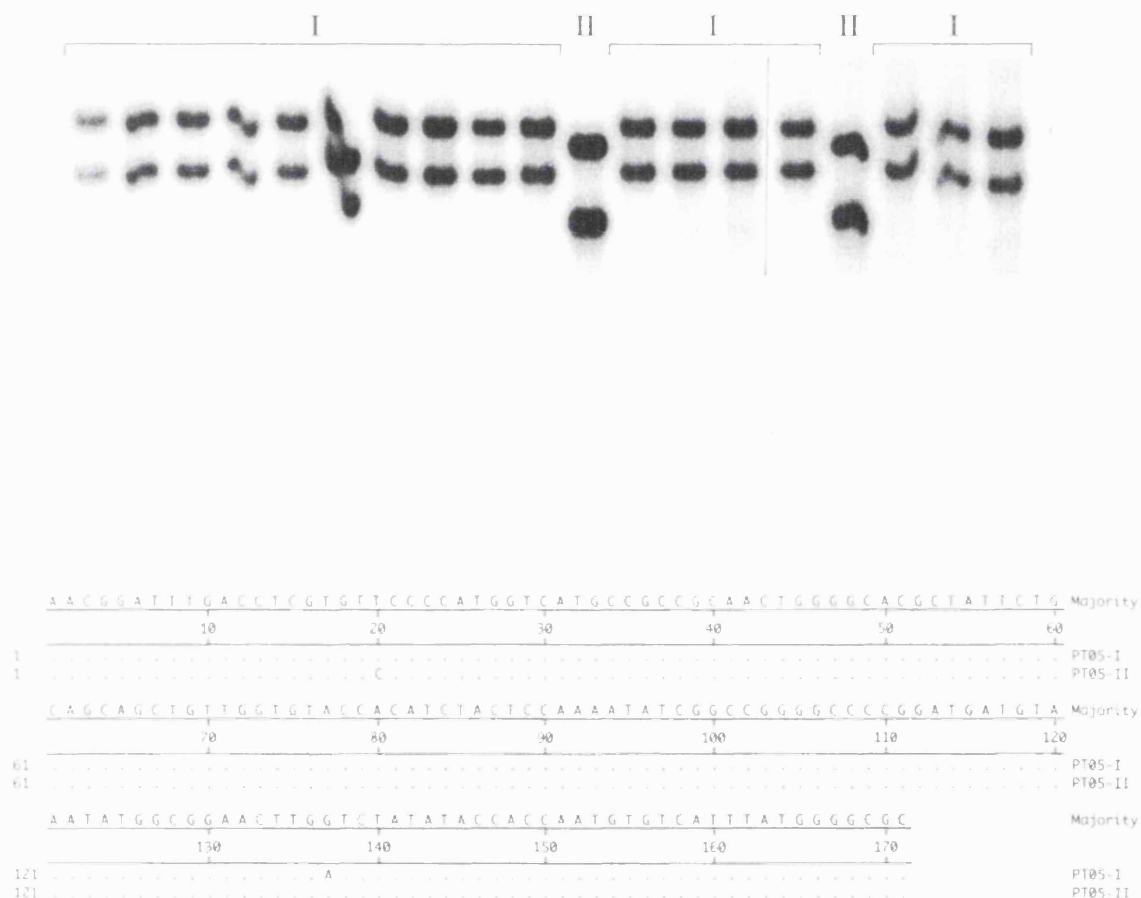
Figure 3.7 SSCP profile and sequences of HHV-8 ORF 26 clones derived from blood of patient 06 (HIV positive). The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence which is represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.
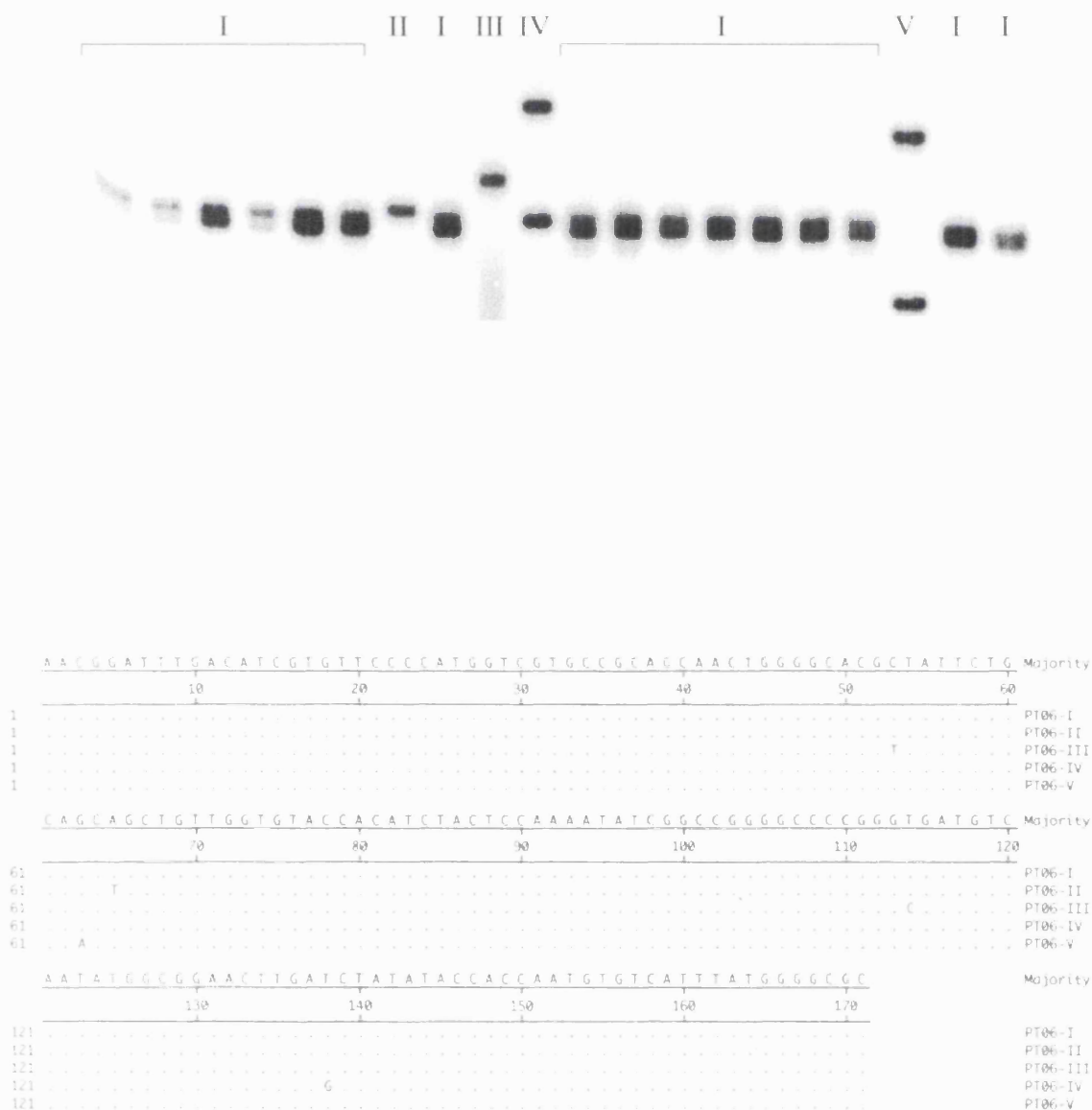
3.1.2.2 Blood of health care workers

This group was comprised of four healthy individuals assumed to be HIV negative, aged 22 to 49 years, median age 38 years. These individuals were health care workers at Hospital das Clinicas (HC) from whom HHV-8 ORF 26 DNA was amplified from their blood. Nineteen to twenty clones were amplified from each specimen.

Figure 3.8 shows the SSCP profile and sequences of clones from the blood of health care worker (HCW) - PT07. There were 3 variant sequences (I to III), sequence II differing from sequence I by one nucleotide (at position 139) and sequence III differing from sequence I by two nucleotides (positions 54 and 139).

Figure 3.9 shows the SSCP profile and sequences of clones from the blood of HCW - PT08. There were 3 sequence variants (I to III), sequence II differing from sequence I by one nucleotide (at position 11) and sequence III differing from sequence I by one nucleotide (at position 85).

Figure 3.10 shows the SSCP profile and sequence of clones from the blood of HCW - PT09. All 19 clones were identical in sequence.

Figure 3.11 shows the SSCP profile and sequences of clones from the blood of HCW - PT10. There were only 2 variant sequences (I and II), sequence II differing from sequence I by one nucleotide (at position 33).

Figure 3.8 SSCP profile and sequences of HHV-8 ORF 26 clones derived from blood of HCW - PT07. The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence which is represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.
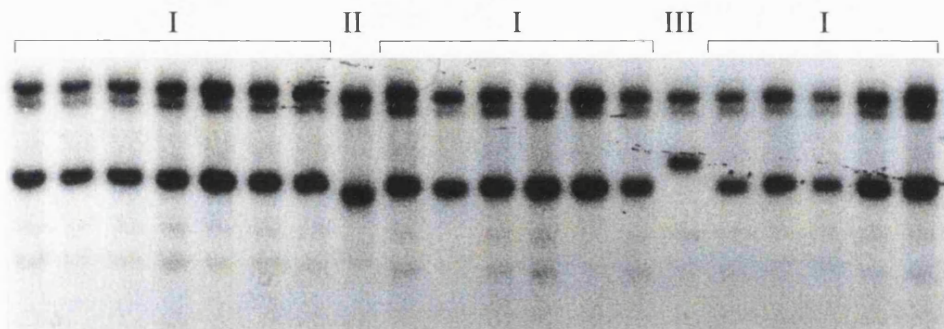

```
A A C G G A T T T G A C C T C G T G T T C C C C A T G G T C G T G C C A C A G C A A C T G G G G C A C G C T A T T C T G  Majority
              10              20              30              40              50              60
1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT07-I
1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT07-II
1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . .  PT07-III


C A G C A G C T G T T G G T G T A C C A C A T C T A C T C C A A A A T A T C G G C C G G G G C C C C G G A T G A T G T A  Majority
              70              80              90              100             110             120
61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT07-I
61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT07-II
61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT07-III


A A T A T G G C G G A A C T T G A T T T A T A T A C C A C C A A T G T G T C A T T T A T G G G G C G C  Majority
              130             140             150             160             170
121 . . . . . . . . . . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT07-I
121 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT07-II
121 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT07-III
```

Figure 3.9 SSCP profile and sequences of HHV-8 ORF 26 clones derived from blood of HCW - PT08. The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence which is represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.


```
     A A C G G A T T T G A C A T C G T G T T C C C C A T G G T C G T G C C T C A G C A A C T G G G G C A C G C T A T T C T G  Majority
                  10          20          30          40          50          60
  1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT08-I
  1  . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT08-II
  1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT08-III


     C A G C A G C T G T T G G T G T A C C A C A T C T A C T C C A A A A T A T C G G C C G G G G C C C C G G G T G A T G T C  Majority
                  70          80          90          100         110         120
 61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT08-I
 61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT08-II
 61  . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT08-III


     A A T A T G G C G G A A C T T G A T C T A T A T A C C A C C A A T G T G T C A T T T A T G G G G C G C  Majority
                  130         140         150         160         170
121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT08-I
121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT08-II
121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT08-III
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Figure 3.10 SSCP profile and sequence of an HHV-8 ORF 26 clone derived from HCW - PT09. All nineteen clones appear homogeneous following the SSCP assay.
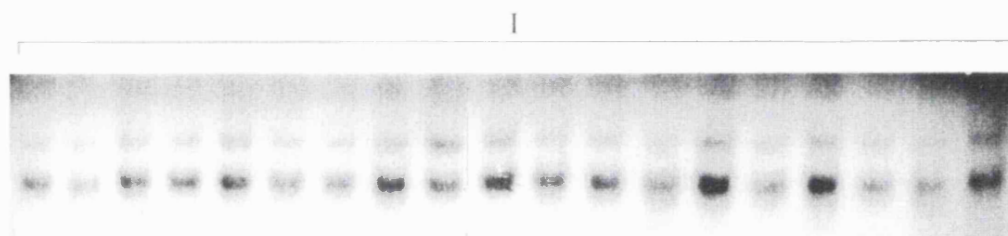
AACGGATTTGACCTCGTGTTCCCCATGGTCGTGCCTCAGCAACTGGGGCACGCTATTCTGCAGCAGCTGTTGGTGTACCACATCTACTCCAAAATATCGGCCGGGGCCCC
        10   20   30   40   50   60   70   80   90   100   110

PT09-I SEQ   AACGGATTTGACCTCGTGTTCCCCATGGTCGTGCCTCAGCAACTGGGGCACGCTATTCTGCAGCAGCTGTTGGTGTACCACATCTACTCCAAAATATCGGCCGGGGCCCC 110

GGGTGATGTCAATATGGCGGAACTTGATCTATGTACCACCAATGTGTCATTTATGGGGCGC
       120   130   140   150   160   170

PT09-I SEQ   GGGTGATGTCAATATGGCGGAACTTGATCTATGTACCACCAATGTGTCATTTATGGGGCGC   171

Figure 3.11 SSCP profile and sequences of HHV-8 ORF 26 clones derived from blood of HCW - PT10. The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence which is represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.


```
      AACGGATTTGACCTCGTGTTCCCCATGGTCGTGCCGCAGCAACTGGGGCACGCTATTCTG Majority
               10        20        30        40        50        60
  1   ............................................................ PT10-I
  1   ...........................................C................ PT10-II

      CAGCAGCTGTTGGTGTACCACATCTACTCCAAAATATCGGCCGGGGCCCCGGATGATGTA Majority
               70        80        90       100       110       120
 61   ............................................................ PT10-I
 61   ............................................................ PT10-II

      AATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATGGGGCGC          Majority
               130       140       150       160       170
121   ...................................................          PT10-I
121   ...................................................          PT10-II
```

3.1.2.3 Oral KS lesional tissue of HIV-positive patients

This group comprised 5 individuals aged 25 to 56 years, median age 34 years. Twenty clones were amplified from each specimen.

Figure 3.12 shows the SSCP profile and sequence of clones from lesional tissue of patient 11. All the 20 clones were identical in sequence.

Figure 3.13 shows the SSCP profile and sequences of clones from the lesional tissue of patient 12. There were 2 variant sequences (I and II), sequence II differing from sequence I by one nucleotide (at position 84).

Figure 3.14 shows the SSCP profile and sequences of clones from the lesional tissue of patient 13. There were again only 2 sequence variants (I and II), sequence II differing from sequence I by one nucleotide at position 69. Sequence III did not differ from sequence I, despite a small shift in the banding pattern.

Figure 3.15 shows the SSCP profile and sequence of clones from the lesional tissue of patient 14. All the 20 clones were identical in sequence.

Figure 3.16 shows the SSCP profile and sequences of clones from the lesional tissue of patient 15. There were 3 variant sequences (I, II and III), sequece II differing from sequence I by one nucleotide (at position 30) and sequence III also differing from sequence I by one nucleotide (at position 35).
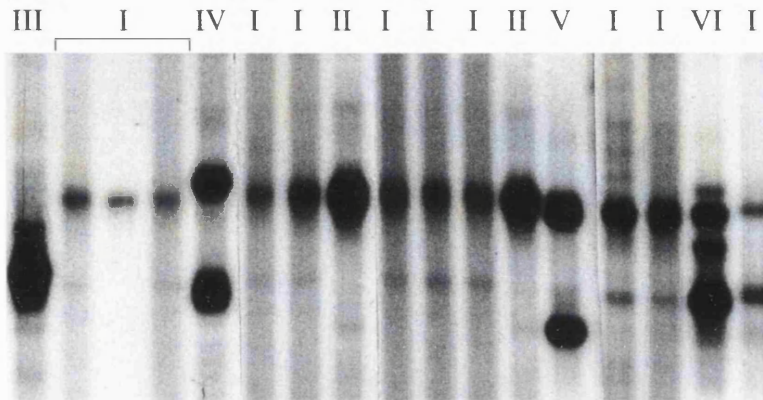
Figure 3.12 SSCP profile and sequence of an HHV-8 ORF 26 clone derived from KS lesional tissue of patient 11 (HIV-positive). All twenty clones appear homogeneous following the SSCP assay.

AACGGATTTGACATCGTGTTCCCCATGGTCGTGCCGCAGCAACTGGGGCACGCTATTCTGCAGCAGCTGTTGGTGTACCA˙CATCTACTCCAAAATATCGGCCGGGGCCC
```
       10        20        30        40        50        60        70        80        90       100       110
```
PT11-I.SEQ   AACGGATTTGACATCGTGTTCCCCATGGTCGTGCCGCAGCAACTGGGGCACGCTATTCTGCAGCAGCTGTTGGTGTACCA˙CATCTACTCCAAAATATCGGCCGGGGCCC 110

CGGGTGATGTCAATATGGCGGAACTTGATCTATATACCACCAATGTGTCAT˙TTATGGGGCGC
```
  120       130       140       150       160       170
```
PT11-I.SEQ   CGGGTGATGTCAATATGGCGGAACTTGATCTATATACCACCAATGTGTCAT˙TTATGGGGCGC   173

Figure 3.13 SSCP profile and sequences of HHV-8 ORF 26 clones derived from KS lesional tissue of patient 12 (HIV-positive). The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence which is represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.


```
A A C G G A T T T G A C C T C G T G T T C C C C A T G G T C G T G C C G C A G C A A C T G G G G C A C G C T A T T C T G  Majority
              10              20              30              40              50              60
  1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT12-I
  1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT12-II

C A G C A G C T G T T G G T G T A C C A C A T T T A C T C C A A A A T A T C G G C C G G G G C C C C G G A T G A T G T A  Majority
              70              80              90             100             110             120
 61  . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT12-I
 61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT12-II

A A T A T G G C G G A A C T T G A T C T A T A T A C C A C C A A T G T G T C A T T T A T G G G G C G C  Majority
              130             140             150             160             170
121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT12-I
121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT12-II
```

Figure 3.14 SSCP profile and sequences of HHV-8 ORF 26 clones derived from KS lesional tissue of patient 13 (HIV-positive). The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence which is represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.
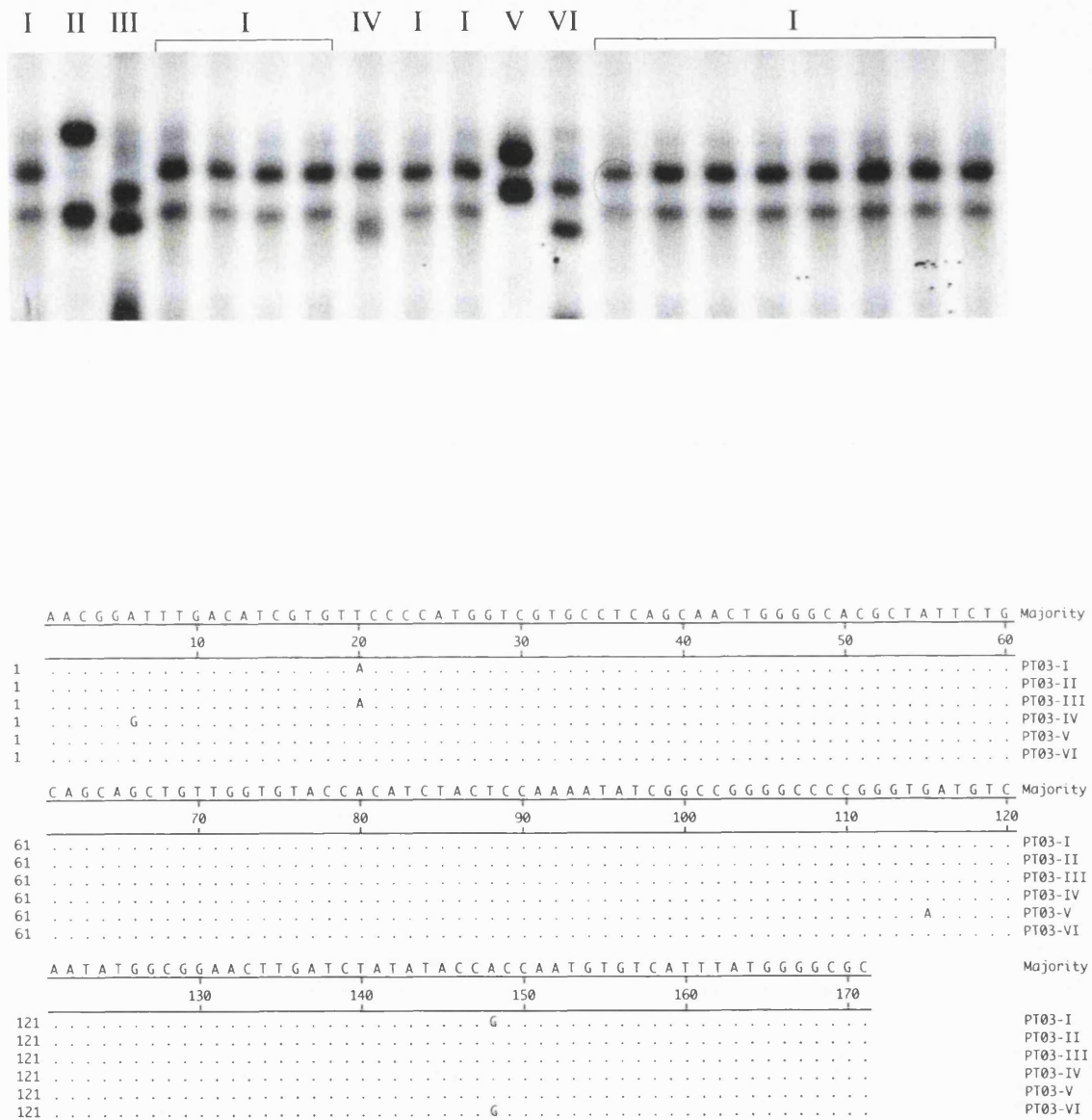


```
A A C G G A T T T G A C C T C G T G T T C C C C A T G G T C G T G C C G C A G C A A C T G G G G C A C G C T A T T C T G  Majority
              10              20              30              40              50              60
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT13-I
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT13-II
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT13-III

C A G C A G C T G T T G G T G T A C C A C A T C T A C T C C A A A A T A T C G G C C G G G G C C C C G G A T G A T G T A  Majority
              70              80              90             100             110             120
61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT13-I
61  . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT13-II
61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT13-III

A A T A T G G C G G A A C T T G A T C T A T A T A C C A C C A A T G T G T C A T T T A T G G G G C G C  Majority
              130             140             150             160             170
121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT13-I
121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT13-II
121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT13-III
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114

Figure 3.15 SSCP profile and sequence of an HHV-8 ORF 26 clone derived from KS lesional tissue of patient 14 (HIV-positive). All twenty clones appear homogeneous following the SSCP assay.
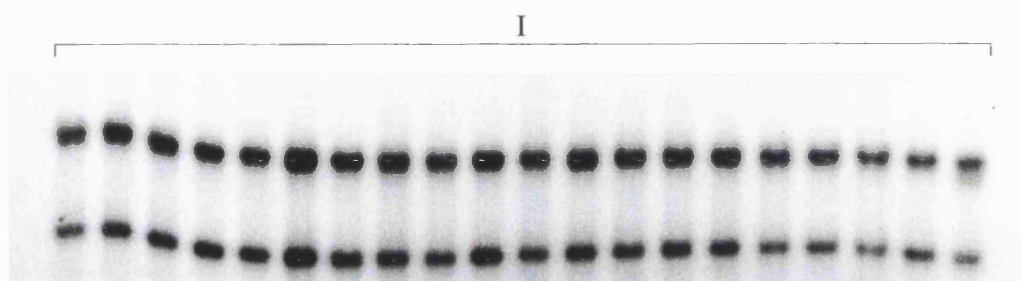
PT14-1.SEQ AACGGATTTGACATCGTGTTCCCCATGGTCGTGCCTCAGCAACTGGGGCACGCTATTCTGCAGCAGCTGTTGGTGTACCA·CATCTACTCCAAAATATCGGCCGGGGCCC 110

PT14-1.SEQ CGGGTGATGTCAATATGGCGGAACTTGATCTATATACCACCAATGTGTCAT·TTATGGGGCGC   173

Figure 3.16 SSCP profile and sequences of HHV-8 ORF 26 clones derived from KS lesional tissue of patient 15 (HIV-positive). The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence which is represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.


```
     A A C G G A T T T G A C C T C G T G T T C C C C A T G G T C G T G C C G C A G C A A C T G G G G C A C G C T A T T C T G  Majority
              10              20              30              40              50              60
1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT15-I
1    . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT15-II
1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT15-III

     C A G C A G T T G T T G G T G T A C C A C A T C T A C T C C A A A A T A T C G G C C G G G G C C C C G G A T G A T G T C  Majority
              70              80              90              100             110             120
61   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT15-I
61   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT15-II
61   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT15-III

     A A T A T G G C G G A A C T T G A T C T A T A T A C C A C C A A T G T G T C A T T T A T G G G G C G C  Majority
              130             140             150             160             170
121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT15-I
121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT15-II
121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT15-III
```

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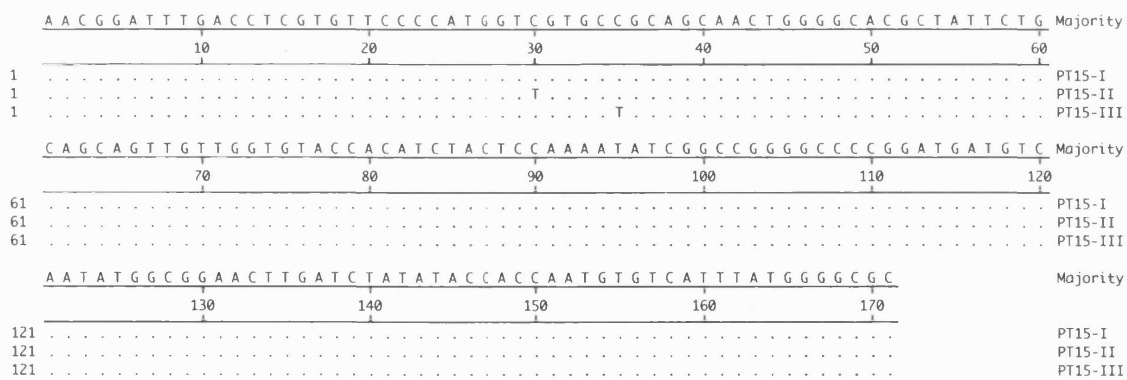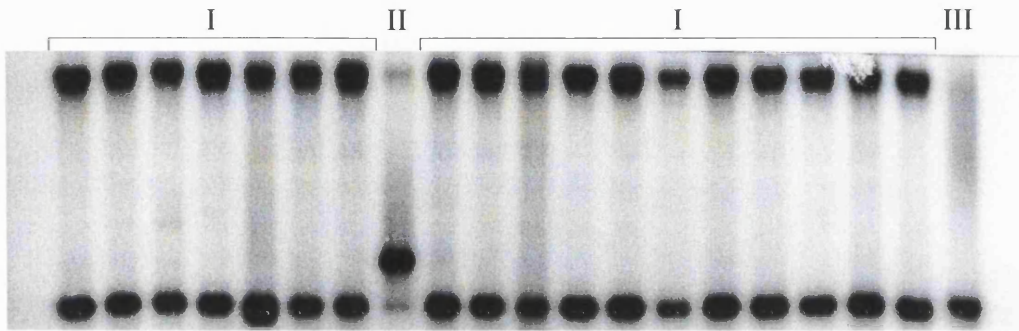
3.1.2.4 Bone marrow transplant (BMT) patients

The final group was comprised of 4 iatrogenically immunosuppressed BMT patients aged 15 to 39 years, median age 22. Nineteen to twenty clones were also amplified from each patient.

Figure 3.17 shows the SSCP profile and sequences of clones from the blood of patient 16. It is seen that there were only 2 variant sequences (I and II), sequence II differing from sequence I by one nucleotide (at position 118).

Figure 3.18 shows the SSCP profile and sequence of clones from the blood of patient 17. All the nineteen clones were identical in sequence.

Figure 3.19 interestingly shows the SSCP profile and sequences of clones from the blood of patient 18, displaying a pattern similar to what was observed in HIV-positive blood. It is seen that there were 4 variant sequences (I to IV), sequence II differing from sequence I by two nucleotides (positions 58 and 86), sequence III differing from sequence I by one nucleotide (at position 133) and sequence IV also differing from sequence I by one nucleotide (at position 117).

Figure 3.20 shows the SSCP profile and sequences of clones from the blood of patient 19. There were 2 variant sequences (I and II), sequence II differing from sequence I by a single nucleotide (at position 136). Table 4.2 shows a summary of the sequence diversity found.

Figure 3.17 SSCP profile and sequences of HHV-8 ORF 26 clones derived from blood of patient 16 (BMT). The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence is which represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.


```
         A A C G G A T T T G A C A T C G T G T T C C C C A T G G T C G T G C C T C A G C A A C T G G G G C A C G C T A T T C T G  Majority
                    10              20              30              40              50              60
   1     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT16-I
   1     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT16-II

         C A G C A G C T G T T G G T G T A C C A C A T C T A C T C C A A A A T A T C G G C C G G G G C C C C G G G T G A T G T C  Majority
                    70              80              90             100             110             120
  61     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT16-I
  61     . . . . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . .  PT16-II

         A A T A T G G C G G A A C T T G A T C T A T A T A C C A C C A A T G T G T C A T T T A T G G G G C G C                  Majority
                    130             140             150             160             170
 121     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                  PT16-I
 121     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                  PT16-II
```
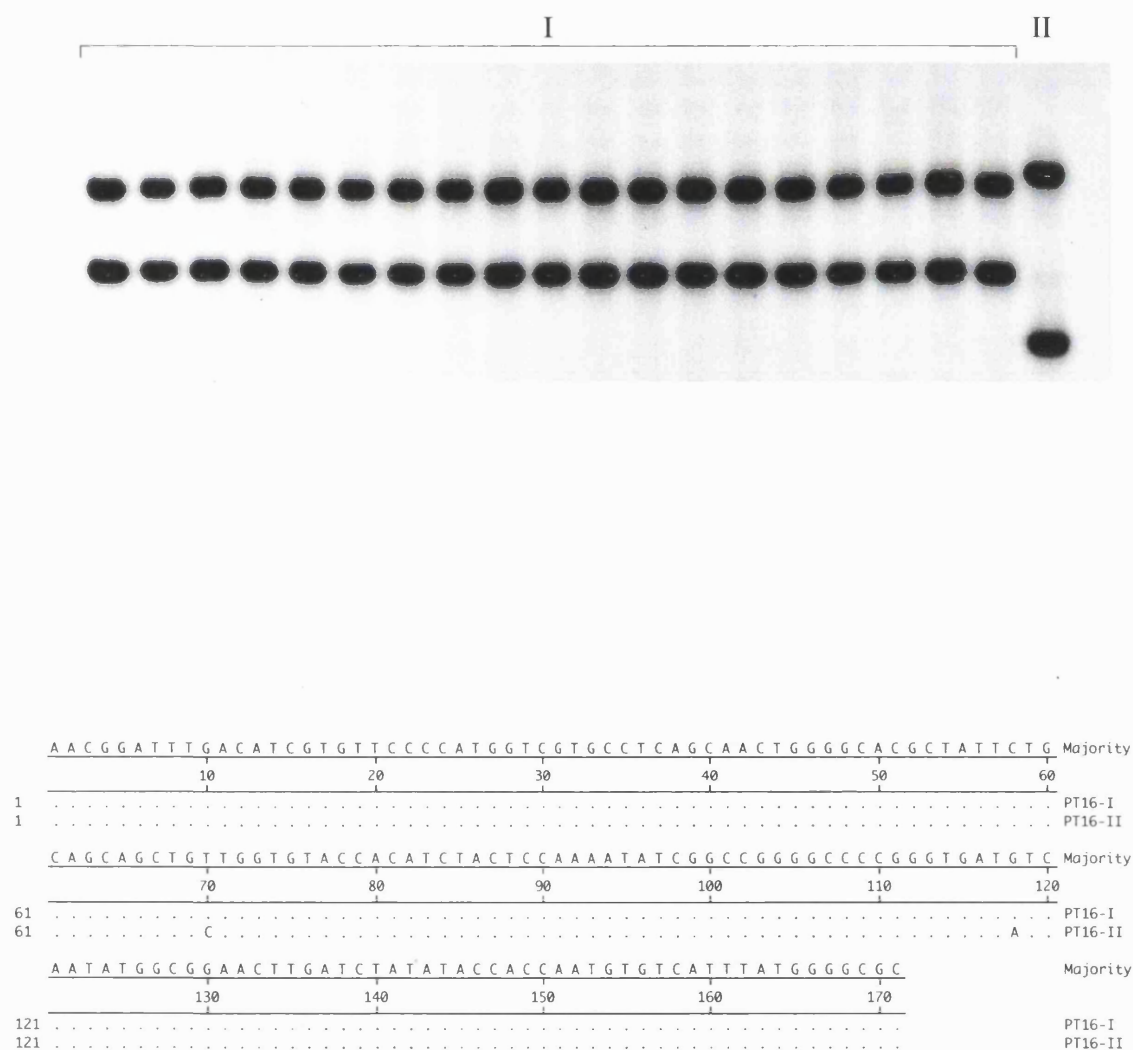
Figure 3.18 SSCP profile and sequence of an HHV-8 ORF 26 clone derived from blood of patient 17 (BMT). All nineteen clones appear homogeneous following the SSCP assay.
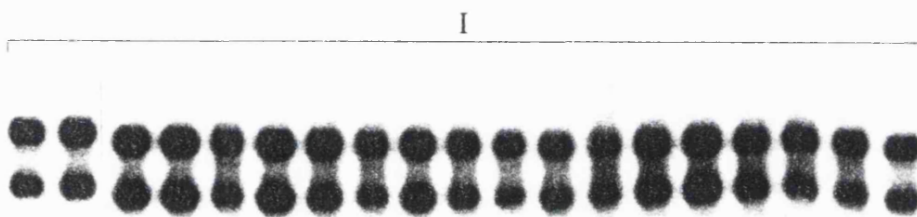

```
              AACGGATTTGACCTCGTGTTCCCCATGGTCGTGCCGCAGCAACTGGGGCACGCTATTCTGCAGCAGCTGTTGGTGTACCACATCTACTCCAAAATATCGGCCGGGGCCCC
              10        20        30        40        50        60        70        80        90        100       110
PT17-I.SEQ    AACGGATTTGACCTCGTGTTCCCCATGGTCGTGCCGCAGCAACTGGGGCACGCTATTCTGCAGCAGCTGTTGGTGTACCACATCTACTCCAAAATATCGGCCGGGGCCCC  110

              GGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATGGGGCGC
              120       130       140       150       160       170
PT17-I.SEQ    GGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATGGGGCGC                                                   171
```
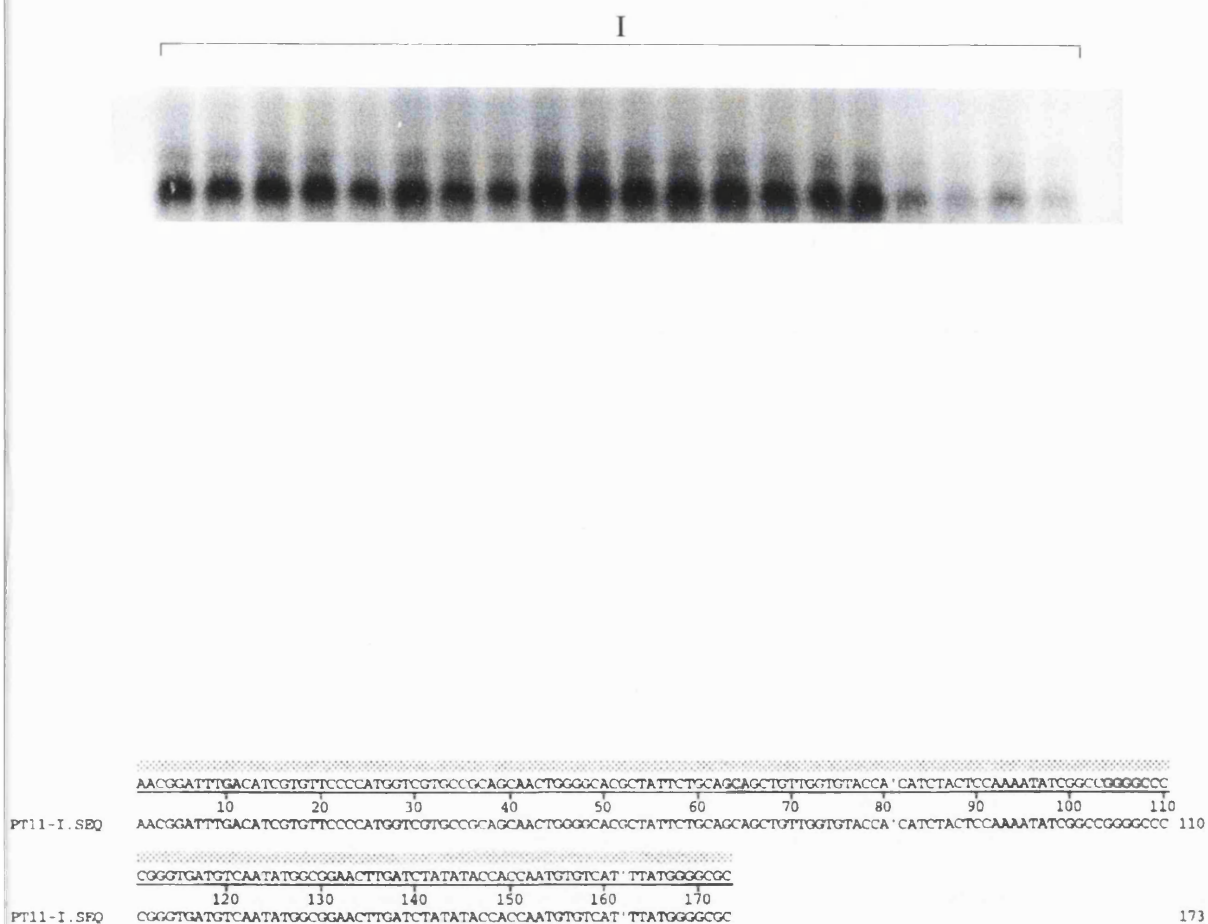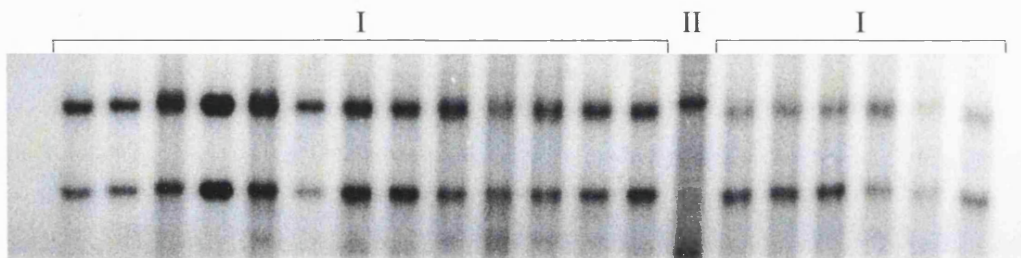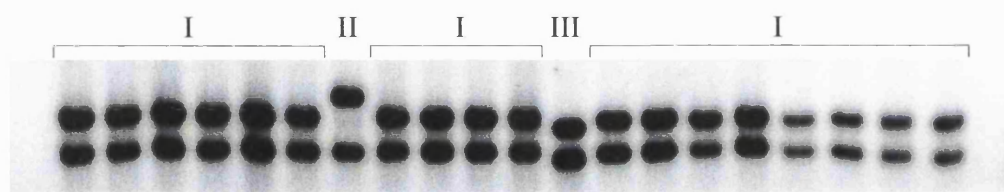
Figure 3.19 SSCP profile and sequences of HHV-8 ORF 26 clones derived from blood of patient 18 (BMT). The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence which is represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.


```
    A A C G G A T T T G A C C T C G T G T T C C C C A T G G T C G T G C C G C A G C A A C T G G G G C A C G C T A T T C T G  Majority
              10            20            30            40            50            60
1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT18-I
1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . .  PT18-II
1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT18-III
1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . .  PT18-IV

    C A G C A G C T G T T G G T G T A C C A C A T C T A C T C C A A A A T A T C G G C C G G G G C C C C G G A T G A T G T A  Majority
              70            80            90            100           110           120
61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT18-I
61  . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT18-II
61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT18-III
61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . .  PT18-IV

    A A T A T G G C G G A A C T T G A T C T A T A T A C C A C C A A T G T G T C A T T T A T G G G G C G C  Majority
              130           140           150           160           170
121 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT18-I
121 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT18-II
121 . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT18-III
121 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT18-IV
```

Figure 3.20 SSCP profile and sequences of HHV-8 ORF 26 clones derived from blood of patient 19 (BMT patient). The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence which is represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.



```
A A C G G A T T T G A C C T C G T G T T C C C C A T G G T C A T G C C G C C G C A A C T G G G G C A C G C T A T T C T G Majority
              10              20              30              40              50              60
  1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . PT19-I
  1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . PT19-II

C A G C A G C T G T T G G T G T A C C A C A T C T A C T C C A A A A T A T C G G C C G G G G C C C C G G A T G A T G T A Majority
              70              80              90              100             110             120
 61 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . PT19-I
 61 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . PT19-II

A A T A T G G C G G A A C T T G A T C T A T A T A C C A C C A A T G T G T C A T T T A T G G G G C G C Majority
              130             140             150             160             170
121 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . PT19-I
121 . . . . . . . . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . PT19-II
```
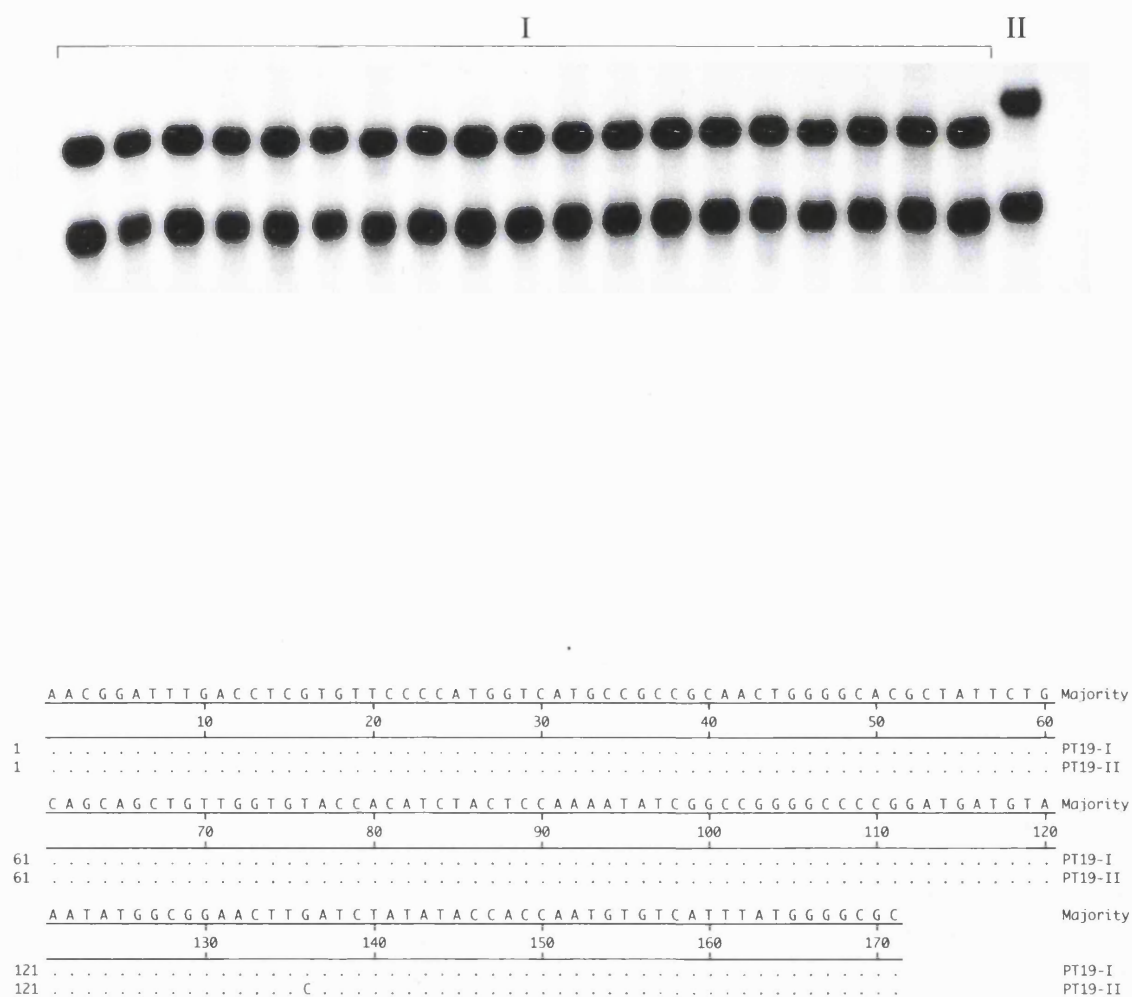
Table 3.1 ORF 26 sequence diversity. This table summarises all ORF 26 data from the 4 groups studied. It shows the frequency of predominant and minor sequence variants occurring at the intra-host level.

	type I predominant	type II	type III	type IV	type V	Type VI
HIV+Blood						
PT 01	11	2	1	1	1	1
PT 02	16	1	1	1	1	-
PT 03	15	1	1	1	1	1
PT 04	15	1	4	-	-	-
PT 05	17	2	-	-	-	-
PT 06	16	1	1	1	1	-
HCW-Blood						
HCW-PT 07	18	1	1	-	-	-
HCW-PT 08	17	1	1	-	-	-
HCW-PT 09	19	-	-	-	-	-
HCW-PT 10	19	1	-	-	-	-
HIV+/KS+						
PT 11	20	-	-	-	-	-
PT 12	19	1	-	-	-	-
PT 13	18	1	1	-	-	-
PT 14	20	-	-	-	-	-
PT 15	18	1	1	-	-	-
BMT						
PT 16	19	1	-	-	-	-
PT 17	19	-	-	-	-	-
PT 18	17	1	1	1	-	-
PT 19	19	1	-	-	-	-

3.1.3 Overall ORF 26 sequence diversity

3.1.3.1 Comparison of entropy value and MGD between individuals

and groups

Table 3.2 shows a comparison of the median normalised entropy values of the four study groups. Figure 3.21 is a graphic representation of the comparison. A comparison of the MGD of the study groups is also shown in Table 3.2, and Figure 3.22 is a graphic representation of the MGD comparison (see comments on section 2.12.1.1 for normalised entropy value and section 2.12.1.2 for mean genetic distance).

Table 3.2 ORF 26 normalised entropy value and mean genetic distances in individual patient specimens. This table shows normalised entropy values and MGDs for each subject tested. It also provides median values for the four groups studied and standard deviation values for the MGDs.

	Normalised entropy value	mean genetic distance	standard deviation
HIV+Blood			
PT 01	0.4535	0.012	(+/- 0.006)
PT 02	0.2596	0.016	(+/- 0.006)
PT 03	0.3220	0.010	(+/- 0.004)
PT 04	0.2295	0.012	(+/- 0.006)
PT 05	0.1143	0.012	(+/- 0.000)
PT 06	0.2596	0.012	(+/- 0.005)
Median	**0.260**	**0.012**	-
HCW-Blood			
HCW-PT 07	0.1317	0.008	(+/-0.003)
HCW-PT 08	0.1391	0.008	(+/-0.003)
HCW-PT 09	0.0000	0.000	(+/-0.000)
HCW-PT 10	0.0663	0.006	(+/-0.000)
Median	**0.099**	**0.007**	-
HIV+/KS+			
PT 11	0.0000	0.000	(+/-0.000)
PT 12	0.0663	0.006	(+/-0.000)
PT 13	0.1317	0.004	(+/-0.003)
PT 14	0.0000	0.000	(+/-0.000)
PT 15	0.1317	0.008	(+/-0.003)
Median	**0.066**	**0.004**	-
BMT			
PT 16	0.0663	0.012	(+/-0.000)
PT 17	0.0000	0.000	(+/-0.000)
PT 18	0.1961	0.015	(+/-0.006)
PT 19	0.0663	0.006	(+/-0.000)
Median	**0.066**	**0.009**	-

Figure 3.21 ORF 26 median normalised entropy values. This is a graphic representation of a comparison between median values of normalised entropy values obtained from each group studied. The Y axis is numbered with entropy values. It is seen that the blood of the HIV positive group has the highest entropy value; in contrast, blood from BMT patients and KS lesional tissues yield the lowest entropy values.

ORF 26 Median normalised entropy

Entropy values vs Study groups bar chart:
- HIV+Blood: ~0.26
- HCW Blood: ~0.1
- HIV+/KS+: ~0.065
- BMT: ~0.065
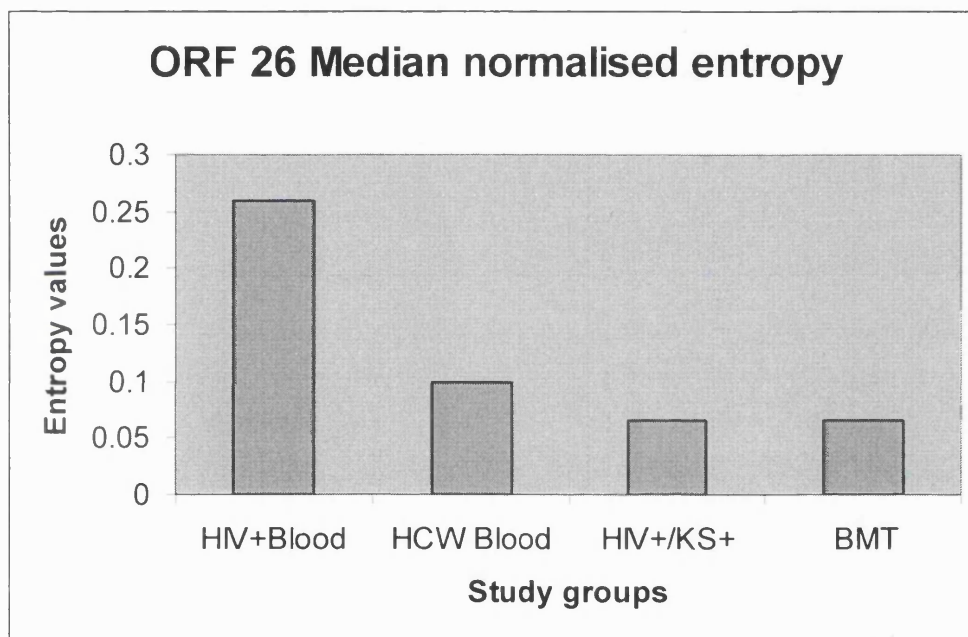
Figure 3.22 Median of the mean genetic distance (MGD) in the ORF 26 fragment between groups. This is a graphic representation comparing median values of MGDs obtained from the 4 groups studied. The Y axis shows MGD values. It is seen that the blood of the HIV positive group has the highest value, in contrast to that from KS lesional tissues.

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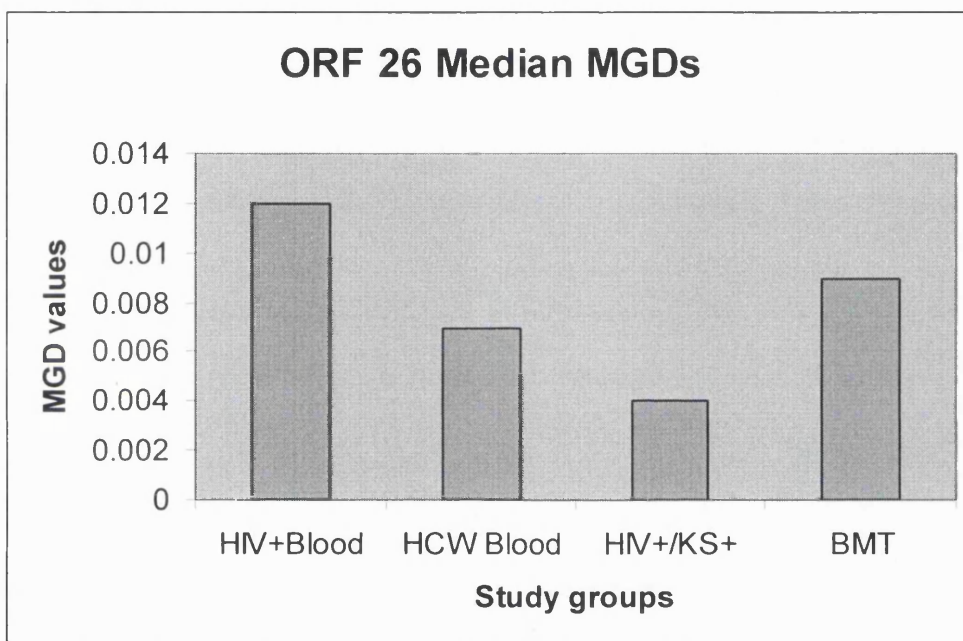
3.1.4 Phylogenetic analysis and alignment of ORF 26 sequences

To determine the genetic relatedness of the various sequences isolated from the study specimens, all sequences were subjected to analysis by Clustal method of multiple sequences alignment using the Lasergene Navigator software. The results are shown in Figure 3.23. It is seen that sequences derived from a given patient tended to group together. They thus form a cluster segregated from clusters derived from other patients. This suggests a quasispecies distribution of these minor variants. However, until more data regarding less conserved regions of the HHV-8 genome, i.e. ORF K1, becomes available, no conclusive mixed infection pattern can be reported.

Prototypes of sequences that is used to group HHV-8 based on ORF 26 (Di Alberti *et al,* 1997) were aligned with the sequences isolated from the study specimens and are shown on the phylogenetic analysis as A1, B1 to H4.

An alignment report is shown on Figure 3.24. This alignment was generated using Clustal from Lasergene Navigator. It is seen that grouping using ORF 26 can be performed as some of the changes occur in hot spot positions, although the majority of these changes seem to be randomly selected.

Figure 3.23 Phylogenetic analysis of HHV-8 ORF 26 DNA sequences
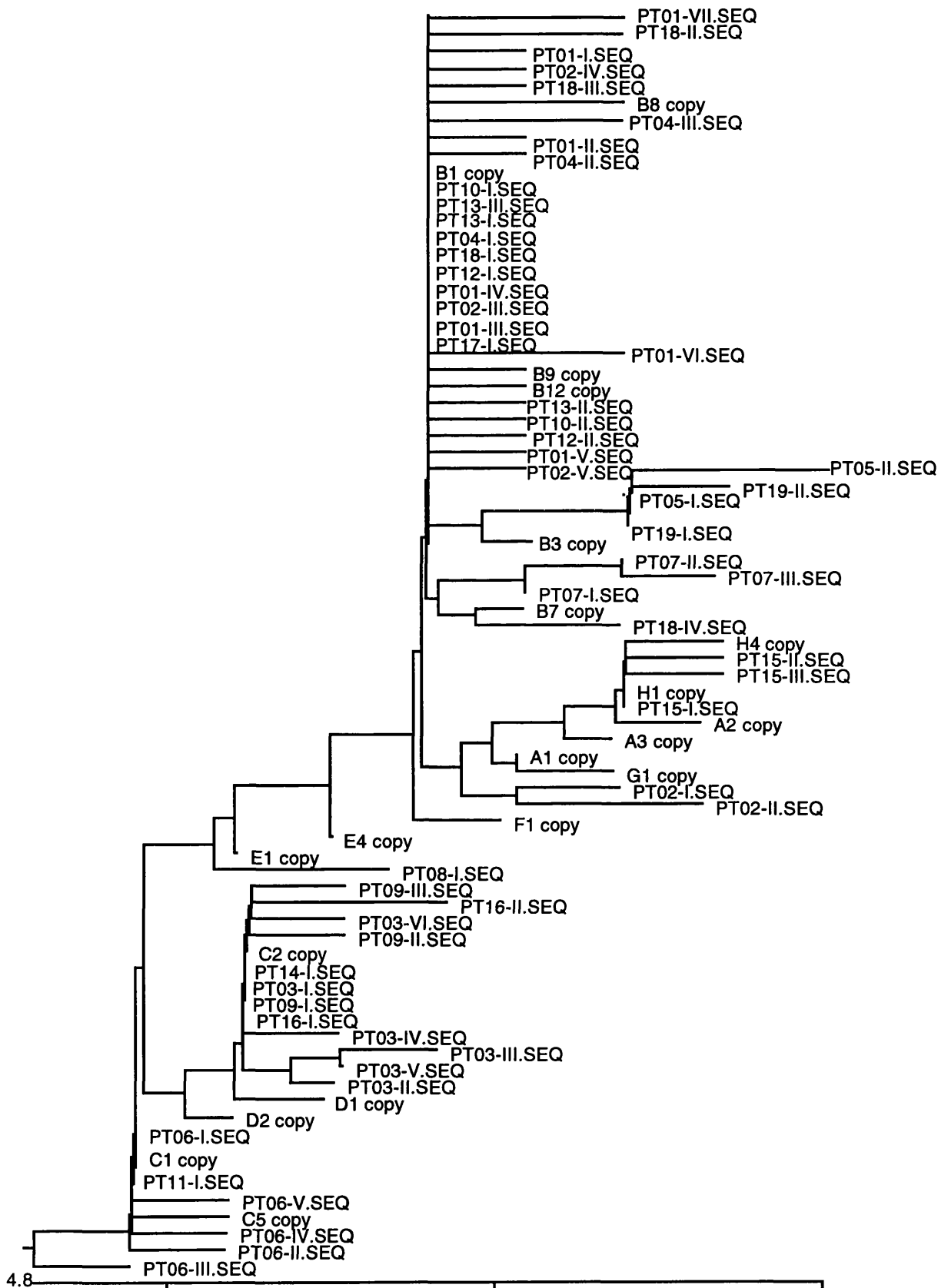
Figure 3.24 Alignment of ORF 26 sequences showing positions of the various mutations identified.
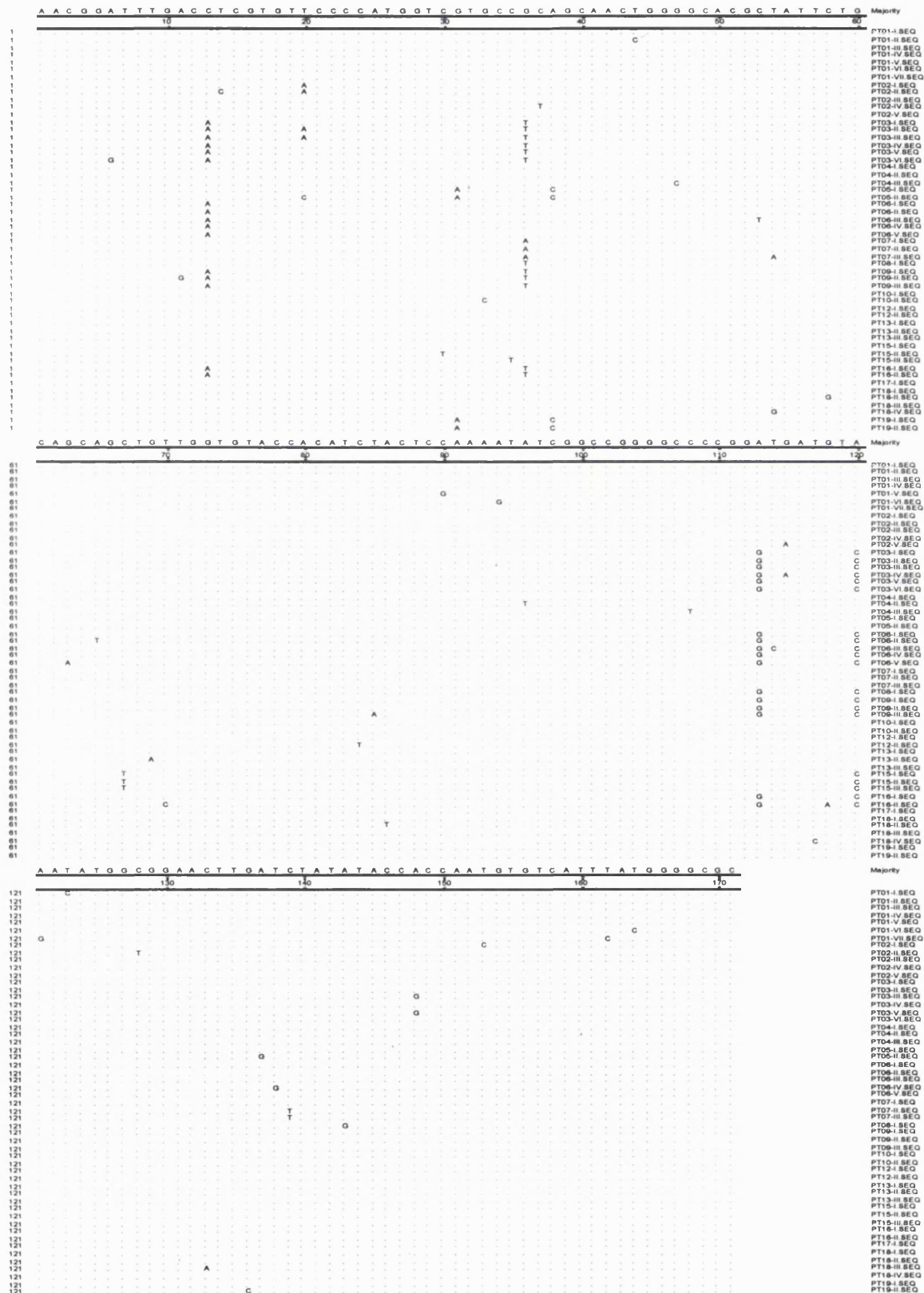
3.1.5 Overall ORF K1 sequence diversity

3.1.5.1 Blood of HIV positive patients

This analysis included only two individuals from whom ORF K1 could be amplified from their PBMCs.

From patient 04, twenty clones were studied, 14 representing the predominant variant (type I) plus 6 minor variants (II to VI). Unlike ORF 26, these minor variants differed from each other by up to eight nucleotides (Figure 3.25).

From patient 06, 20 clones were also studied, 16 representing the predominant variant and one of each minor variant type II, III, IV, V differing from each other by up to 4 nucleotides (Figure 3.26).

3.3.5.2 Oral KS lesional tissue of HIV-positive patients

ORF K1 DNA sequences could be amplified from all the five individuals from whom ORF 26 DNA was amplified. This high rate of amplification reflects the abundance of HHV-8 in the tissues. The variability found in this group was again much lower when compared with the ORF K1 HIV positive blood. Variants differed from each other by a maximum of two nucleotides.

From patient 13, 16 clones could be amplified, 15 representing the predominant variant and one as minor variant (type II), differing from type I by one nucleotide T→A at position 105 (Figure 3.29).

From patient 14, seventeen clones were amplified, 16 representing the predominant variant and one representing a minor variant (type II), differing from type I by one nucleotide G→C at position 22 (Figure 3.30).

A different pattern was observed for patients 11 and 15. In patient 11, 20 clones were amplified, 13 representing the predominant variant, 6 representing another variant (type II) and one of yet another variant (type III). They only differed from each other by only one nucleotide (Figure 3.27). Similarly, in patient 15, 15 clones could be amplified, 10 representing the predominant variant, 4 the minor variant type II and one of type III, but again they only differed from each other by one nucleotide (Figure 3.31). Finally, from patient 12, 15 clones could be amplified, 13 representing the predominant variant, one sequence type II and one type III, once more, differing from each other by a single nucleotide.

ORF K1 sequence variability is summarised in Table 3.3 and Table 3.4 summarises normalised entropy and MGD values. Figure 3.32 is a graphic representation of ORF K1 normalised entropy values and Figure 3.33 is a graphic representation of ORF K1 MGDs.

Figure 3.25 SSCP profile and sequences of HHV-8 ORF K1 clones derived from blood of patient 04 (HIV-positive). The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence which is represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.

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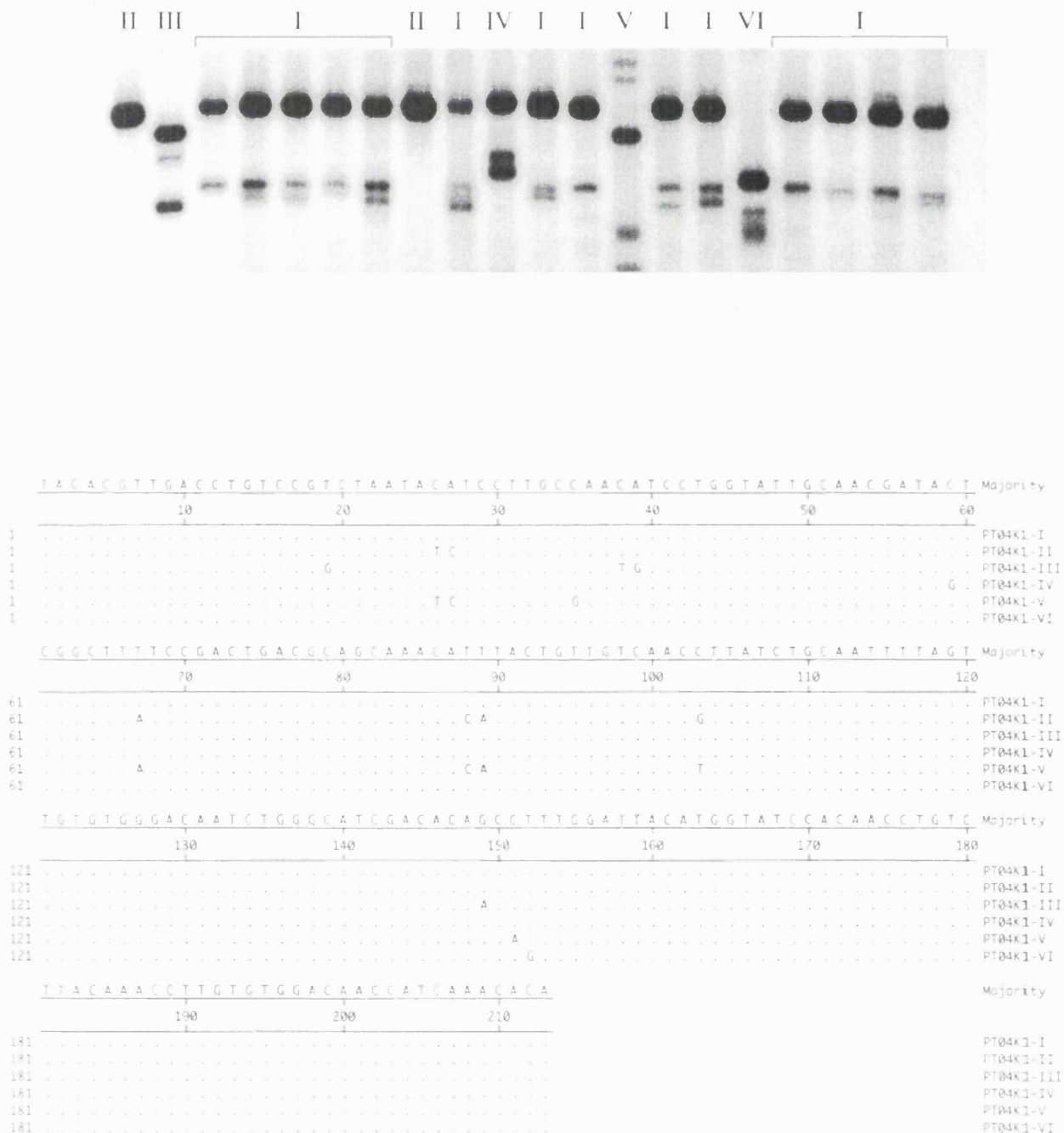
Figure 3.26 SSCP profile and sequences of HHV-8 ORF K1 clones derived from blood of patient 06 (HIV-positive). The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence which is represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.


```
        T A C A C G T T G A C C T G T C C G T C T A A T A C A T C C T T G C C A A C A T C C T G G T A T T G C A A C G A T A C T  Majority
                  10              20              30              40              50              60
  1     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT06K1-I
  1     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . .  PT06K1-II
  1     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT06K1-III
  1     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . .  PT06K1-IV
  1     . . . . . . . . . . . . . . . . . . . . . . T G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT06K1-V


        C G G C T T C T C C G A C T G A C G C A G C A A A C A T T C A C T G T T G T C A C C C T T A T C T G C A A T T T T A G T  Majority
                  70              80              90              100             110             120
 61     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT06K1-I
 61     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . .  PT06K1-II
 61     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT06K1-III
 61     . . . . . . . . . . . . . . . . . . . . . . . . . . . . A A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT06K1-IV
 61     . . . . . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT06K1-V


        T G T G T G G G A C A A T C T G G G C A T C G A C A C A G C C T T T G G A T T A C A T G G T A T C C A C A A C C T G T C  Majority
                  130             140             150             160             170             180
121     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT06K1-I
121     . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT06K1-II
121     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT06K1-III
121     . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT06K1-IV
121     . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT06K1-V


        T T A C A A A C C T T G T G T G G A C A A C C A T C A A A C A C A                                           Majority
                  190             200             210
181     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                                   PT06K1-I
181     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                                   PT06K1-II
181     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                                   PT06K1-III
181     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                                   PT06K1-IV
181     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                                   PT06K1-V
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Figure 3.27 SSCP profile and sequences of HHV-8 ORF K1 clones derived from KS lesional tissue of patient 11 (HIV-positive). The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence which is represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.
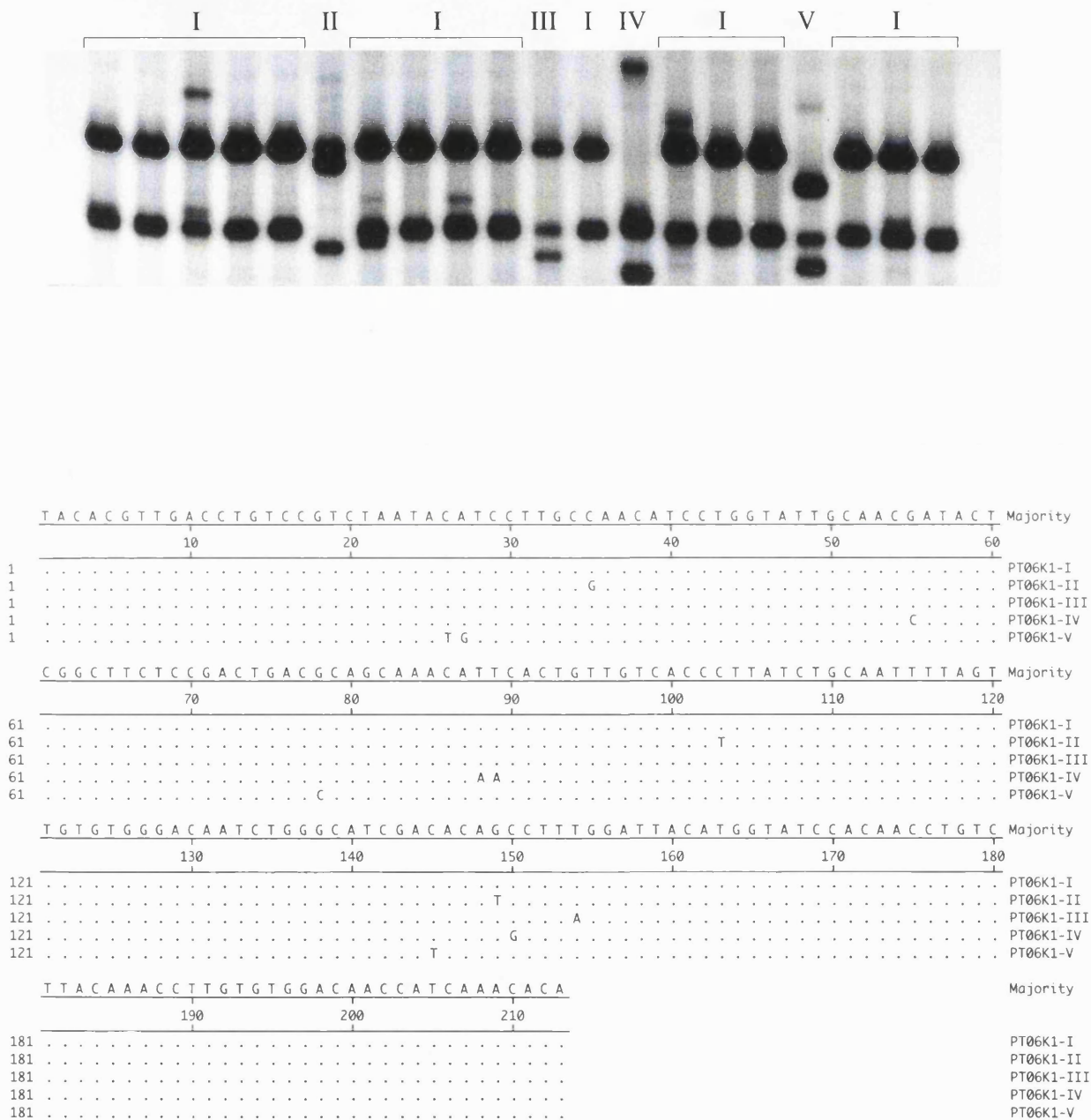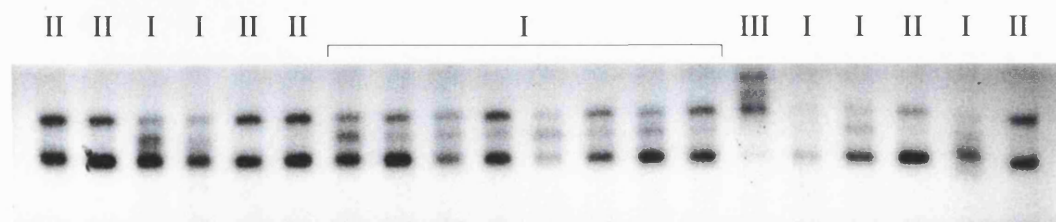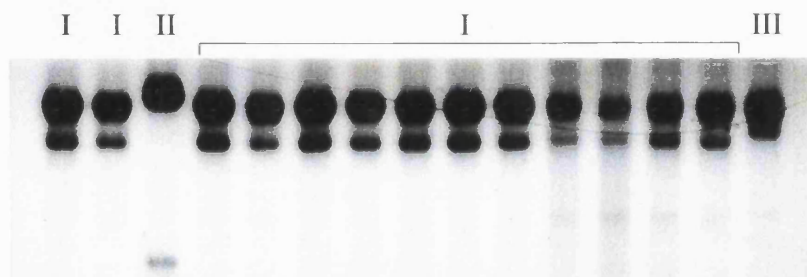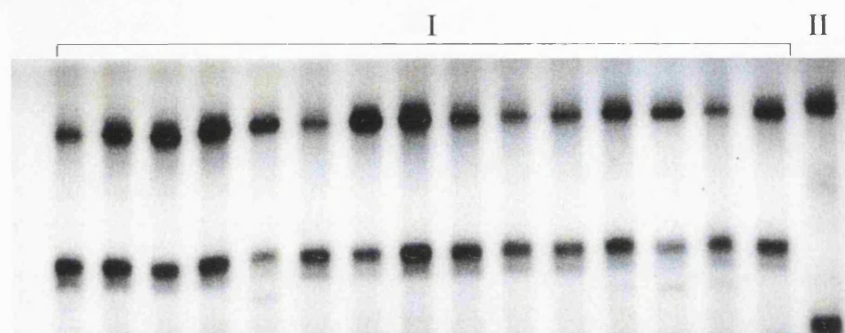


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CTTACACGTTGACCTGTCTGTCTAATGCATCCTTGCCAATATCCTGGTATTGCAACAATA Majority
         10        20        30        40        50        60
1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . PT11-I
1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . PT11-II
1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . PT11-III

CTCGGCTTCTCCGGCTCACGAAGACAACATTAGCTTCTGTCACCATTGCCTGCAATTTTA Majority
         70        80        90        100       110       120
61   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . PT11-I
61   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . PT11-II
61   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . '. . . . . . PT11-III

CTTGTGTGGAACAATCTGGGCATCGACAAAGCATTTCGATTACATGGCATGCACGACCTG Majority
         130       140       150       160       170       180
121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . PT11-I
121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . PT11-II
121  . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . PT11-III

TCTTACAAACCTTGTGTGCGCAGCCATCAAACACA                Majority
         190       200       210
181  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . PT11-I
181  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . PT11-II
181  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . PT11-III
```

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Figure 3.28 SSCP profile and sequences of HHV-8 ORF K1 clones derived from KS lesional tissue of patient 12 (HIV-positive). The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence which is represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.
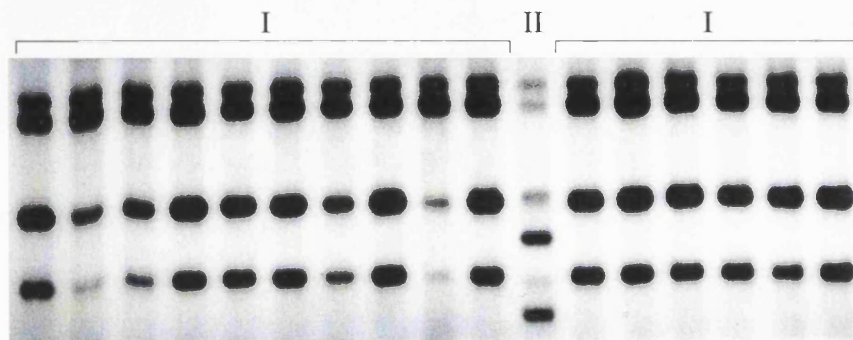


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C T T A C A A G T T G A C C T G T C T G T C T A A T G C A T C C T T G C C A A T A T C C T G G T A T T G C A A C A A G A  Majority
              10            20            30            40            50            60
1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT12-I
1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT12-II
1   . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . .  PT12-III

C T C G G C T T C T C C G A C T G A C G G A G A G A A C A C T T T T T C C T G T C A C C A T T C C C T G C A A T T T T A  Majority
              70            80            90           100           110           120
61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT12-I
61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT12-II
61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT12-III

C T T G T G T G G A A C A A T C T G G G C A T C G A C A G A G C A T T T G G A T T A C A T G G C A T G C A C A A C C T G  Majority
             130           140           150           160           170           180
121 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT12-I
121 . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT12-II
121 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT12-III

T C T T A C A A A C C T T G T G T G C A C A G C C A T C A A A C A C A                                        Majority
             190           200           210
181 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                                        PT12-I
181 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                                        PT12-II
181 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                                        PT12-III
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Figure 3.29 SSCP profile and sequences of HHV-8 ORF K1 clones derived from KS lesional tissue of patient 13 (HIV-positive). The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence which is represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.


```
        C T T A C A C G T T G A C C T G T C T G T C T G A T G C A T C C T T G C C A A T A T C C T G G T A T T G C A A C G A T A  Majority
                10              20              30              40              50              60
1       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT13K1-I
1       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT13K1-II

        C T C G G C T T T G G C G A C T G A G G A A G C C A A C A C T C A C T G T T G A C A C C T T T A C C T G C A A T T T T A  Majority
                70              80              90              100             110             120
61      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . .  PT13K1-I
61      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT13K1-II

        C T T G T G T G G A A C A A T C T G G G C A T C G A C A G A G C A T T T G G A T T A C A T G G T A T G C A C A A C C T G  Majority
                130             140             150             160             170             180
121     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT13K1-I
121     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT13K1-II

        T C T T A C A A A C C T T G T G T G C A C A G C C A T C A A A C A C A                                                    Majority
                190             200             210
181     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                                              PT13K1-I
181     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                                              PT13K1-II
```

Figure 3.30 SSCP profile and sequences of HHV-8 ORF K1 clones derived from KS lesional tissue of patient 14 (HIV-positive). The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence which is represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.


```
C T T A C A A C T T G A C C T G T C T G T G T A A T G C A T C C T T G C C A A T A T C C T G G T A T T G C A A C A A T A  Majority
           10              20              30              40              50              60
  1      . . . . . . . . . . . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT14K1-I
  1      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT14K1-II

C T C G G C T T T T C C G A C T G A C G G A G A G A A C A C T T T T T C T T G T C A C C A T T T C C T G C A A T T T T A  Majority
           70              80              90             100             110             120
 61      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT14K1-I
 61      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT14K1-II

C T T G T G T G G A A C A A T C T G G G C A T C G A C A G A G C A T T T G G A T T A C A T G G C A T G C A C A A C C T G  Majority
          130             140             150             160             170             180
121      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT14K1-I
121      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT14K1-II

T C T T A C A A A C C T T G T G T G C A C A G C C A T C A A A C A C A                                                    Majority
          190             200             210
181      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                                PT14K1-I
181      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                                PT14K1-II
```

Figure 3.31 SSCP profile and sequences of HHV-8 ORF K1 clones derived from KS lesional tissue of patient 15 (HIV-positive). The majority sequence, being the consensus sequence within each set of alignment and predominant (here designated as type I), is the sequence which is represented most frequently on the SSCP autoradiograph. Roman numerals designate different sequences. There was full concordance between SSCP banding patterns and sequences.


```
    C T T A C A C G T T G A C C T G T C C G T C T A A C A G A T C C T T G C C A A T A T C C T G G T A T T G C A A C G G G A  Majority
              10           20           30           40           50           60
1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT15K1-I
1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT15K1-II
1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT15K1-III

    C T C G G C T T T G G C G A G T A A C G G C G T C T A A C C T A A C T G T T T T T G G G T T A A C C T G C A A T T T T A  Majority
              70           80           90           100          110          120
61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT15K1-I
61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT15K1-II
61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT15K1-III

    C T T G T A T G A C A A C A T C T C G G C C T A C A C A C A G C A T T T G G A T T G A A T G G T A T A C A C A A C C T G  Majority
              130          140          150          160          170          180
121 . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT15K1-I
121 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PT15K1-II
121 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . .  PT15K1-III

    T C T T A C A A A C C T C G T G T G C A C A G C C A T C A A A C A C A                                                    Majority
              190          200          210
181 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                                                PT15K1-I
181 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                                                PT15K1-II
181 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                                                PT15K1-III
```

Table 3.3 ORF K1 sequence diversity in individual patient specimens.
This table summarises all patient samples from which ORF K1 could be
amplified. It shows the frequency of predominant and minor sequence
variants occurring at the intra-host level.

	type I predominant	type II	Type III	Type IV	type V	type VI
HIV+Blood						
PT 04	14	2	1	1	1	1
PT 06	16	1	1	1	1	-
HIV+/KS+						
PT 11	13	6	1	-	-	-
PT 12	13	1	1	-	-	-
PT 13	15	1	-	-	-	-
PT 14	16	1	-	-	-	-
PT 15	10	4	1	-	-	-

Table 3.4 ORF K1 normalised entropy value and mean genetic distances in individual patient specimens. This table shows normalised entropy values and MGDs of patient from which ORF K1 could be amplified. It also provides median values for the groups and standard deviation values for the MGDs.

	normalised entropy value	Mean genetic distance	standard deviation
HIV+Blood			
PT 04	0.3598	0.026	(+/-0.001)
PT 06	0.2596	0.021	(+/-0.001)
Median	**0.3097**	**0.024**	-
HIV+/KS+			
PT 11	0.2640	0.005	(+/-0.002)
PT 12	0.1560	0.005	(+/-0.002)
PT 13	0.0759	0.005	(+/-0.000)
PT 14	0.0730	0.005	(+/-0.000)
PT 15	0.2732	0.005	(+/-0.000)
Median	**0.1560**	**0.005**	-

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Figure 3.32 ORF K1 median normalised entropy values. This is a graphic representation comparing median values of normalised entropy values obtained from the two groups from which DNA from ORF K1 could be amplified. The Y axis is numbered with entropy values. It is seen that the blood of the HIV positive group has the higher entropy value, in contrast to the group comprising of KS lesional tissues.
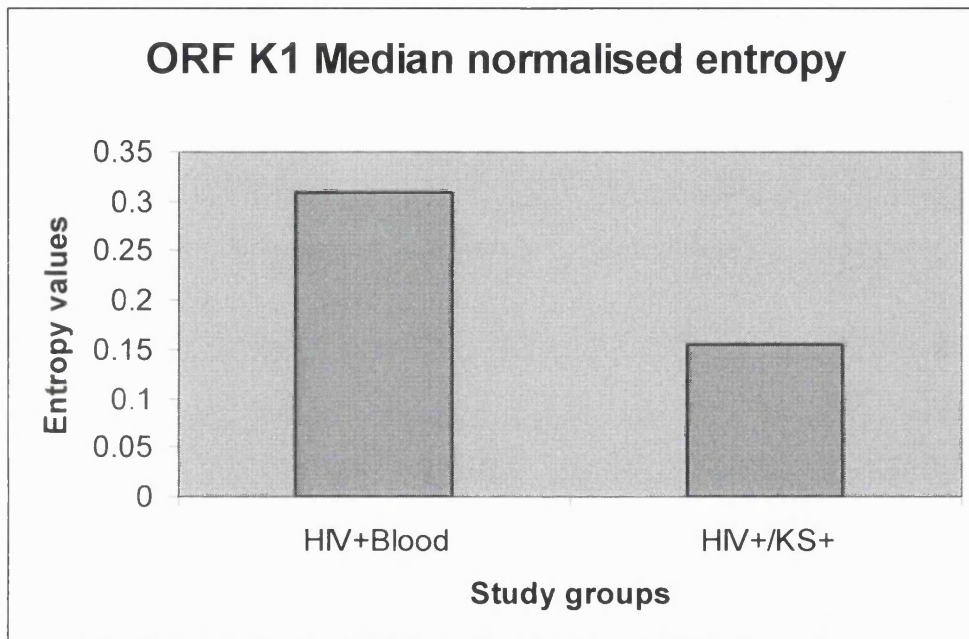
Figure 3.33 Median of the mean genetic distance in the ORF K1 fragment between groups. This is a graphic representation comparing median values of MGDs obtained from the two groups from which ORF K1 DNA could be amplified. The Y axis is numbered with MGD values. It is seen that the blood of the HIV positive group has the higher median MGD value compared to the group comprising KS lesional tissues.

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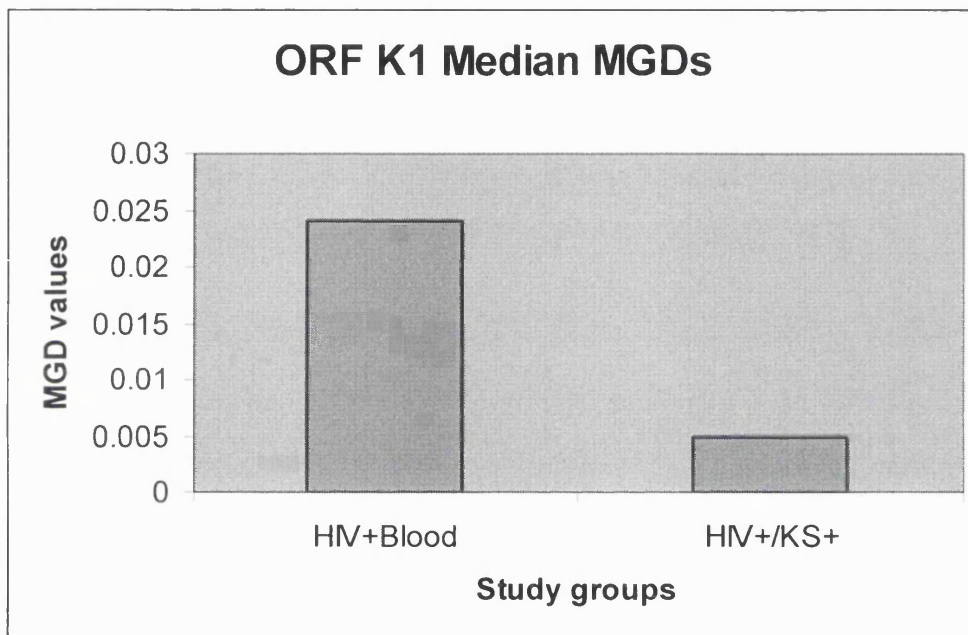
3.1.6 Estimation of *Taq* polymerase misincorporation rates

To determine the extent by which *Taq* polymerase misincorporation could be responsible for the variability in the clinical specimens, the same protocol of SSCP and sequence analysis was applied to clones derived from BCBL cell lines. The results are shown in Figures 3.34 to 3.36. Only in one cell line was a variant that differed from the predominant isolated. Figure 3.36 shows that in the K1 region of BC-2 cell line there was a difference of one nucleotide compared to the predominant, providing the lone example of *Taq* polymerase misincorporation. Therefore, while PCR-induced misincorporation did occur, it did so only very infrequently.

Artefactual misincorporation was therefore unlikely to account for the variation in DNA sequence observed in ORF 26 and ORF K1 clones derived from blood and KS lesions.

Figure 3.34 SSCP analysis of HHV-8 ORF 26 DNA from BCP-1 cells. This SSCP picture shows that all 20 clones amplified from HHV-8 ORF 26 BCP-1 cells are identical in their DNA sequence. Sequencing was not performed as the clones appear to be homogenous.
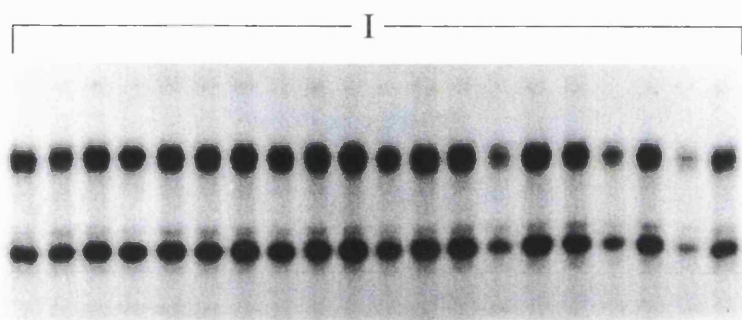
Figure 3.35 SSCP analysis of HHV-8 ORF K1 DNA from BC-1 cells. This SSCP picture shows that all clones but one amplified from HHV-8 ORF K1 BC-1 cells are identical in sequence. There is a slight shift in the banding pattern for the clone marked with an asterix. Sequencing was performed in the predominant type and compared with the sequence of that clone: no mutation was detected.


```
C T T A C A C G T T G A C C T G T C T G T C T A A T G C A T C C T T G C C A A T A T C C T G G T A T T G C A A C A A T A  Majority
              10                  20                  30                  40                  50                  60
  1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  BC1K1-I
  1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  BC1K1-*

C T C G G C T T T T G C G A C T G A C G G A G A G A A G A G T C A T T C T T G A C A C C A T T G C C T G C A A T T T T A  Majority
              70                  80                  90                 100                 110                 120
 61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  BC1K1-I
 61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  BC1K1-*

C T T G T G T G G A A C A A T C T G G G C A T C G A C A G A G C A T T T G G A T T A C A T G G C G T G C A C A A C C T G  Majority
             130                 140                 150                 160                 170                 180
121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  BC1K1-I
121  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  BC1K1-*

T C T T A C A A A C C T T G T G T G C A C A G C C A T C A A A C A C A  Majority
             190                 200                 210
181  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  BC1K1-I
181  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  BC1K1-*
```

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Figure 3.36 SSCP analysis of HHV-8 ORF K1 DNA from BC-2 cells. This SSCP picture shows that all clones but one amplified from HHV-8 ORF K1 BC-2 cells are identical in sequence. There is a shift in the banding pattern of the clone marked as II. The predominant sequence was compared with the sequence of that clone and a base substitution was detected at position 110.

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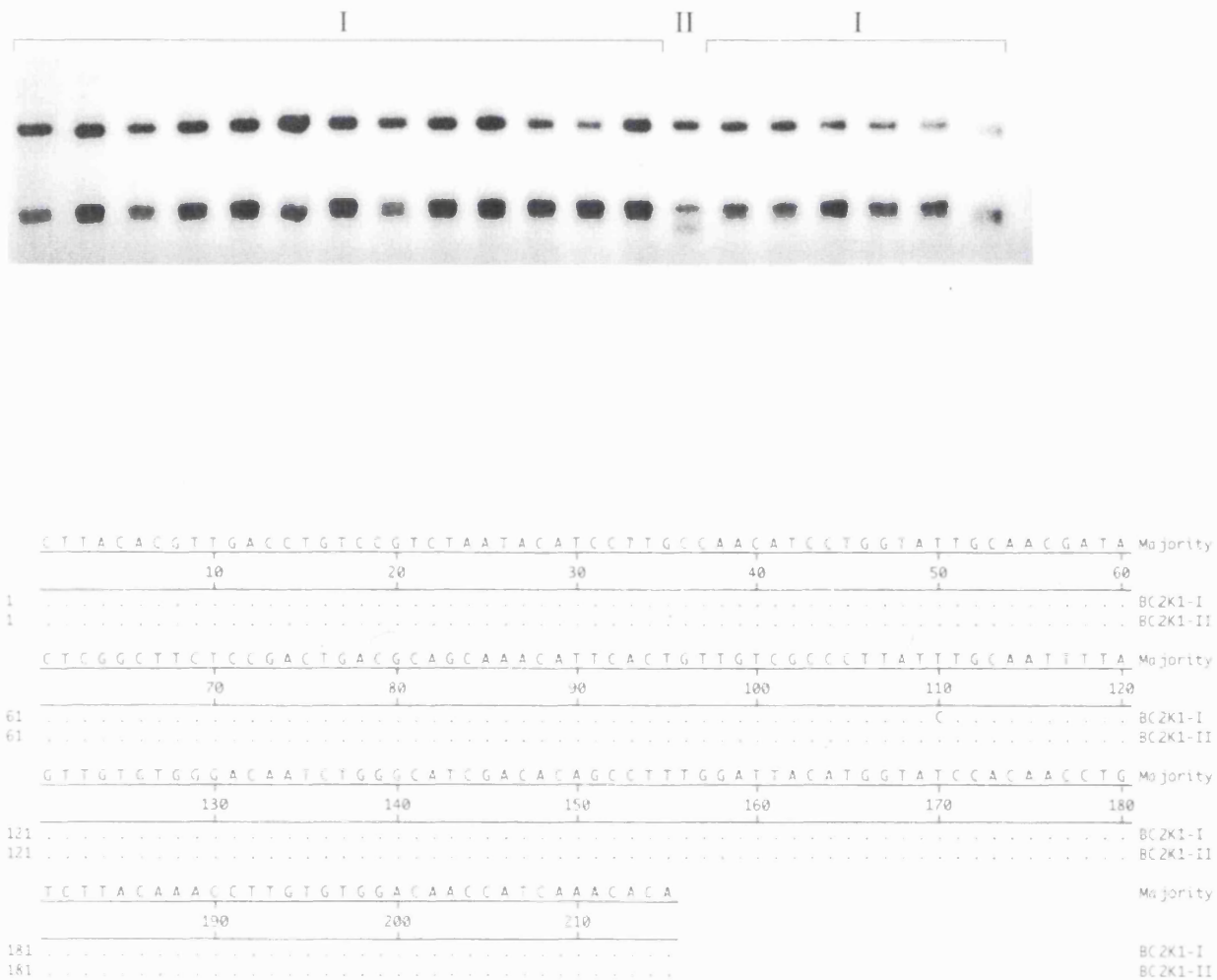
3.1.7 Statistical analysis of ORF 26 and ORF K1 sequence differences

3.1.7.1 ORF 26 sequence variability

Median values were used in order to make comparisons between groups. Compared with blood of HIV-infected patients, whose median normalised entropy value was 0.260, the values in the health care volunteers group (0.099), iatrogenically immunosuppressed (0.066) and KS lesional tissue (0.066), all three considered as a whole, was significantly less (P=0.002). The variability within the latter three groups was not statistically significant (P=0.845). Median values were also used to compare MGDs. The MGD value of the HIV-positive blood group was significantly more compared to the values of the other three groups considered as a whole (P=0.017).

3.1.7.2 ORF K1 sequence variability

No statistical tests were performed to compare ORF K1 variability due to the small number of samples that could be amplified, particularly from the HIV-positive group. Despite this, some observations could be made from these experiments. Compared with the blood of HIV-positive patients, the median normalised entropy value of KS lesional tissues was lower (0.3057 vs 0.1560). Compared with the HIV-positive patients, the median MGD value of KS lesions was also lower (0.024 vs 0.005).

Chapter 4 – Results: Effect of HIV-1 protease inhibitors on the frequency of HHV-8 DNA detection in blood of HIV-infected patients

4.1 Results

4.1.1 Summary of changes in CD4 + cell count, HIV load and HHV-8 DNA status over time

The data was obtained from 1, 2 or 3 samples from 132 HIV patients giving 176 samples in total. One hundred and five samples were collected prior to the introduction of PrI. After PrI therapy was started, 33 were collected twice and 4 three times. Once the 132 sample sets were linked, they were analysed according to the variables in question: CD4+ T cell count, HIV plasma load or proportion positive for HHV-8 DNA. Figures 4.1 to 4.3 summarises how the three variables changed over time. Each patient is represented by a line joining 1, 2 or 3 points according to the number of samples per patient.

4.1.2 Change in PrI treatment status over time

The effect of introducing PrI can only be assessed by comparisons of samples from the same patient before and after the introduction of the PrI treatment. This means that only 33 patients for whom there were samples both before and after the introduction of PrI treatment could be used in this analysis. 17 were already on RTI treatment and stayed on it while 16 who were initially receiving no treatment started RTI at the same time as they started PrI. These two groups were distinguished in the analysis to assess whether the RTI treatment influenced the effect of PrI.

Figure 4.1 Summary of the influence of PrI treatment upon CD4+ T cell counts. This is a graphic representation of the changes in CD4+ T cells in cells/microlitre (Y axis). Patients had one, two or three readings, represented by the test numbers on the X axis.

CD4 counts as they change over time
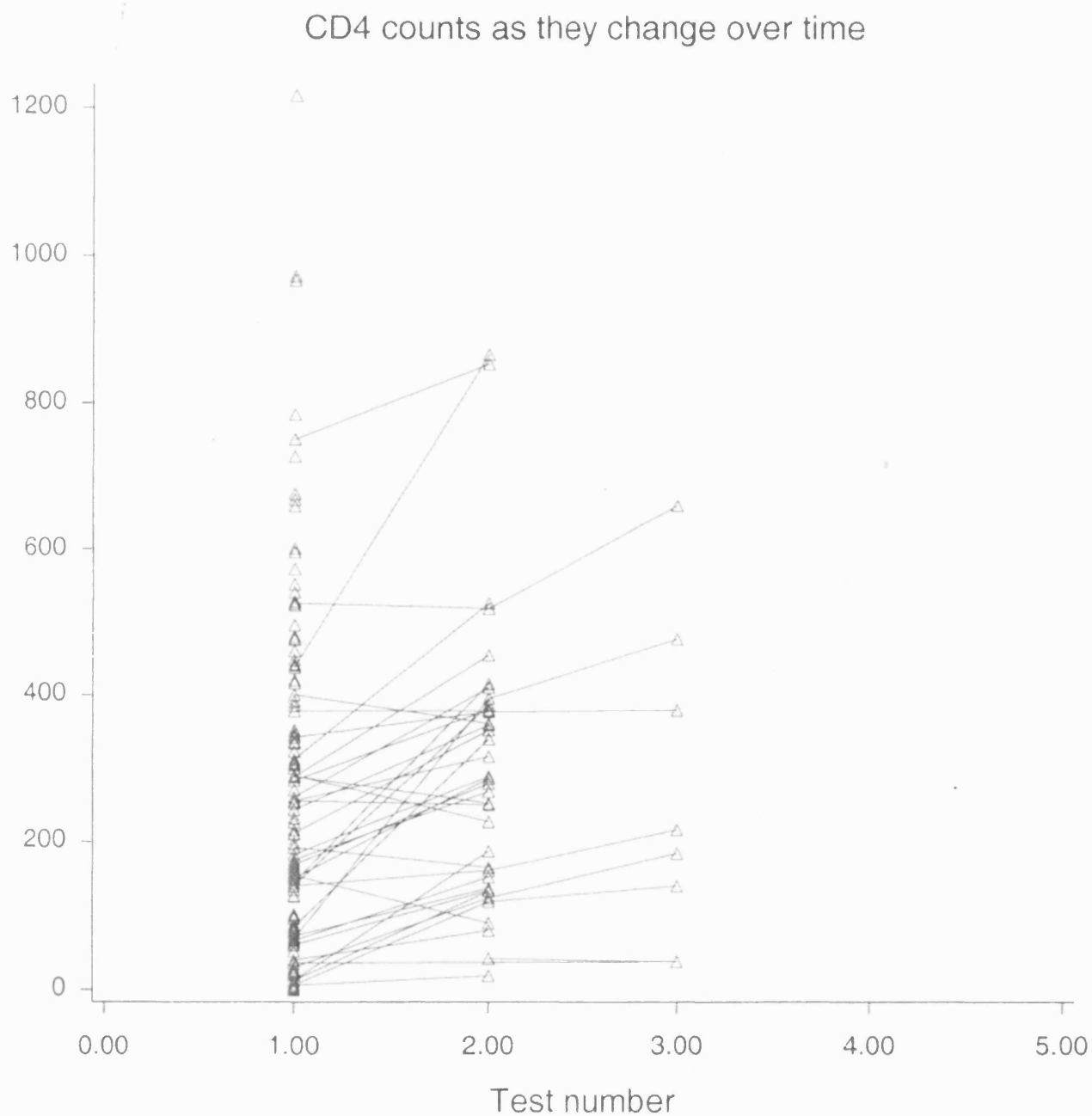
Figure 4.2 Summary of the influence of PrI treatment upon HIV load. This is a graphic representation of the changes in log of copies/microlitre (Y axis). Patients had one, two or three readings, represented by test numbers on the X axis.

Log(HIV loads) as they change over time
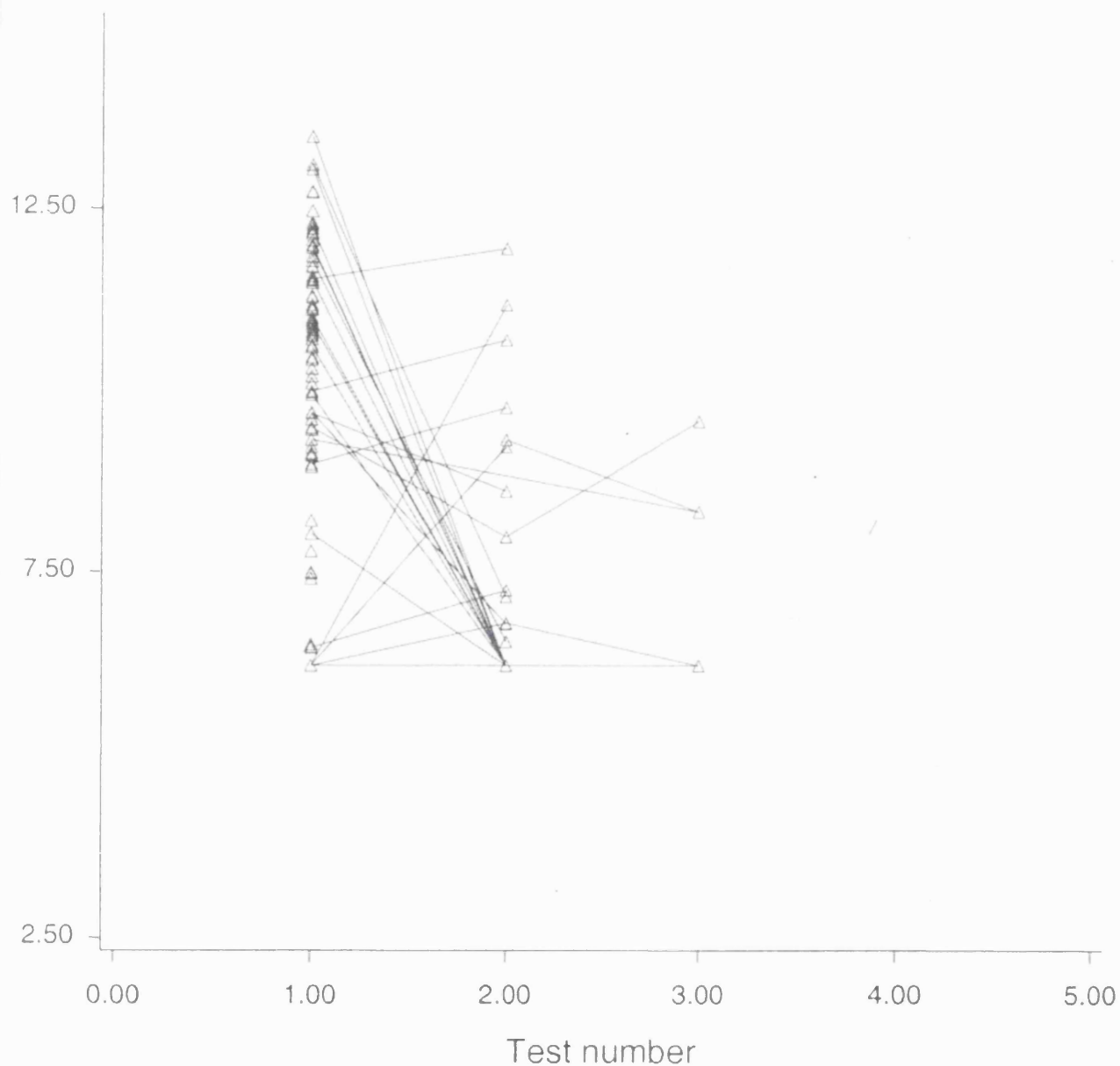
Figure 4.3 Summary of the influence of PrI treatment upon HHV-8 DNA detection. This is a graphic representation of the changes in HHV-8 DNA positivity. Y axis is scaled from 1 (negative for HHV-8 DNA) to 2 (positive for HHV-8 DNA). Patients had one, two or three readings, here represented by test numbers on the X axis.

HHV8 status as it changes over time
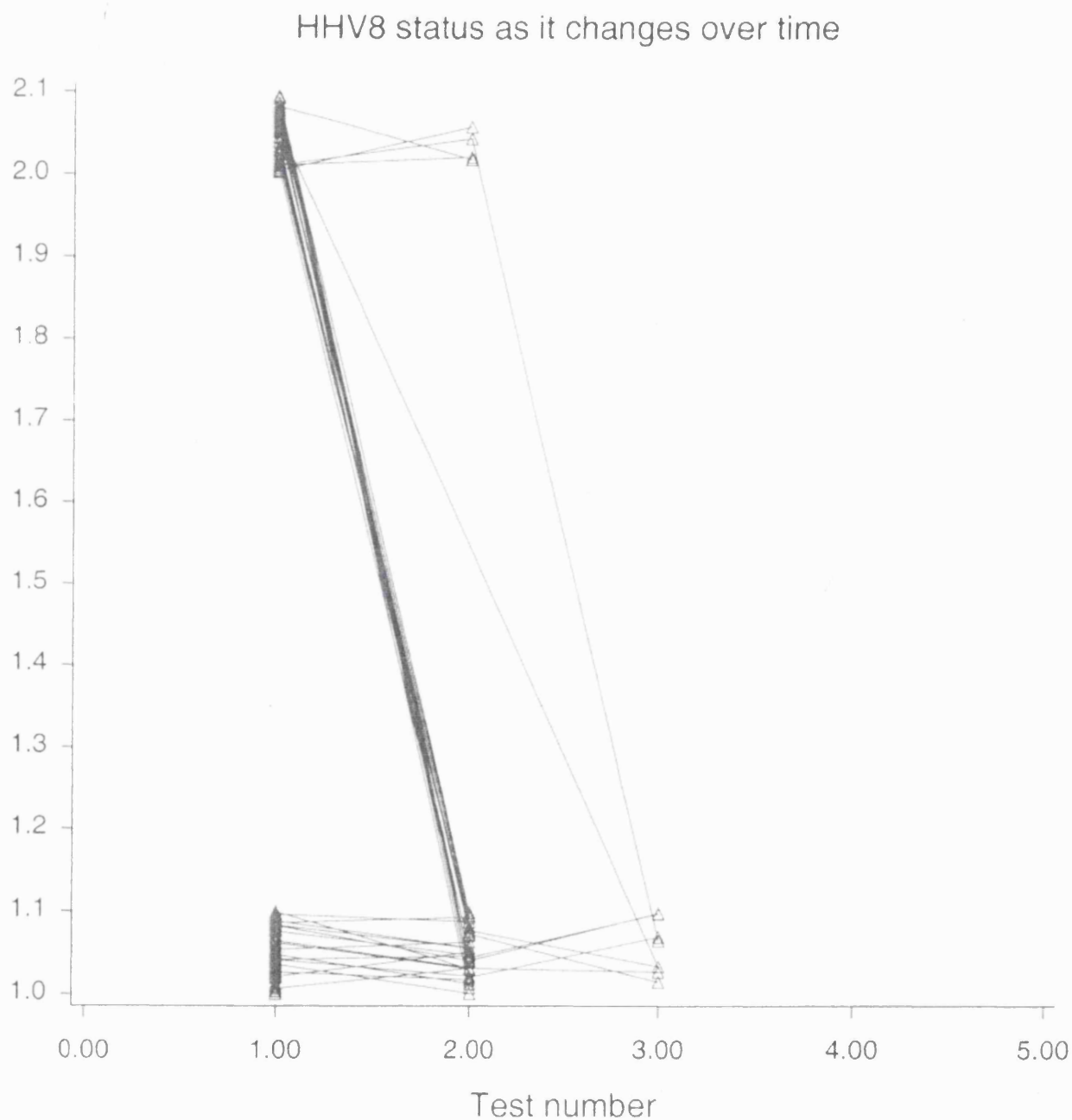
4.1.3 Effect of PrI treatment on CD4+ T cell count

In order to evaluate how CD4+ T cell count changed over time, 'before' and 'after' measures were taken from each of the 33 patients from whom longitudinal data could be derived. The mean CD4+ T cell count before and after the introduction of PrI by RTI treatment status is indicated in Table 4.1.

A change in status from no treatment to treatment using both RTI and PrI led to a mean increase in CD4+ T cell count of 114.2 cells/μL (95% CIs 30.0 to 198.4). If patients were already receiving RTI, then introducing PrI led to a mean increase of 89.2 cells/μL (95% CIs 41.6 to 136.9). Both increases were significantly greater than zero, suggesting that the use of these drugs were responsible for the increased CD4+ T cell count found here. Figure 4.4 graphically shows how mean CD4+ T cell count changed before and after introducing PrI. Figure 4.5 graphically shows how the difference in CD4+ T cell count changed before and after PrI treatment.

To obtain the CIs and perform significance tests it was necessary to assume that the changes from sample 1 to sample 2 have a normal or at least a reasonably symmetrical distribution. The plot in Figure 4.6 suggests that for CD4+ T cell changes this assumption is reasonable.

The differences in CD4+ T cell count before and after PrI treatment were analyzed using Student's t-test. The results are shown in Table 4.2. So both of these increases in CD4+ T cell count are significantly greater than zero.

Further statistical tests were performed in order to evaluate if the CD4+ T cell count increases were significantly different if the patient was already receiving RTI. The t-test comparing the two increases showed that the p value of differences was 0.5198 (t = 0.65; degree of freedom = 31; 95% CIs = -50.20 to 100.10). There was no evidence that the CD4+ T cell count increases differed (p=0.5198). This suggests that the PrI treatment increased CD4+ T cell count by the same amount whether or not the patient was already receiving RTI, although in the first group the increase could result from either of the two treatments since they were both being introduced at the same time.

Table 4.1 Changes in CD4+ T cell count (in cells/μL) according to PrI and RTI treatment status. This table summarises CD4 counts data according to the treatment regimen in the two groups studied. The mean CD4+ T cell counts, alongside with the standard deviation and the number of patients is shown. The difference of the means (after, less before introduction of PrI) is also shown.

RTI treatment		**PrI Before** No	**PrI After** Yes	Difference (After – Before)
No→Yes	Mean	229.81	344.00	114.19
	S.D.	179.25	231.35	135.20
	N	16	16	16
Yes→Yes	Mean	158.06	247.29	89.24
	S.D.	150.78	154.44	79.45
	N	17	17	17
Total	Mean	192.85	294.18	101.33
	S.D.	166.60	198.55	109.02
	N	33	33	33

Table 4.2 Differences in CD4+ T cell count before and after PrI treatment using Student's t-test. This table summarises the statistical tests used to evaluate CD4+ T cell counts before and after treatment in the two groups studied. The 95% confidence intervals values are also shown.

RTI	t value	p value	95% confidence intervals		
No→Yes	3.378	0.0041	30.0	to	198.4
Yes→Yes	4.631	0.0003	41.6	to	136.9

Figure 4.4 Mean CD4+ T cell count before and after introducing PrI treatment. Two groups of patients are represented here. Patients who started both RTI and PrI at the same time are represented by the dark line connected by squares. The group that had been on RTI and then put on PrI is represented by the line connected by diamonds.
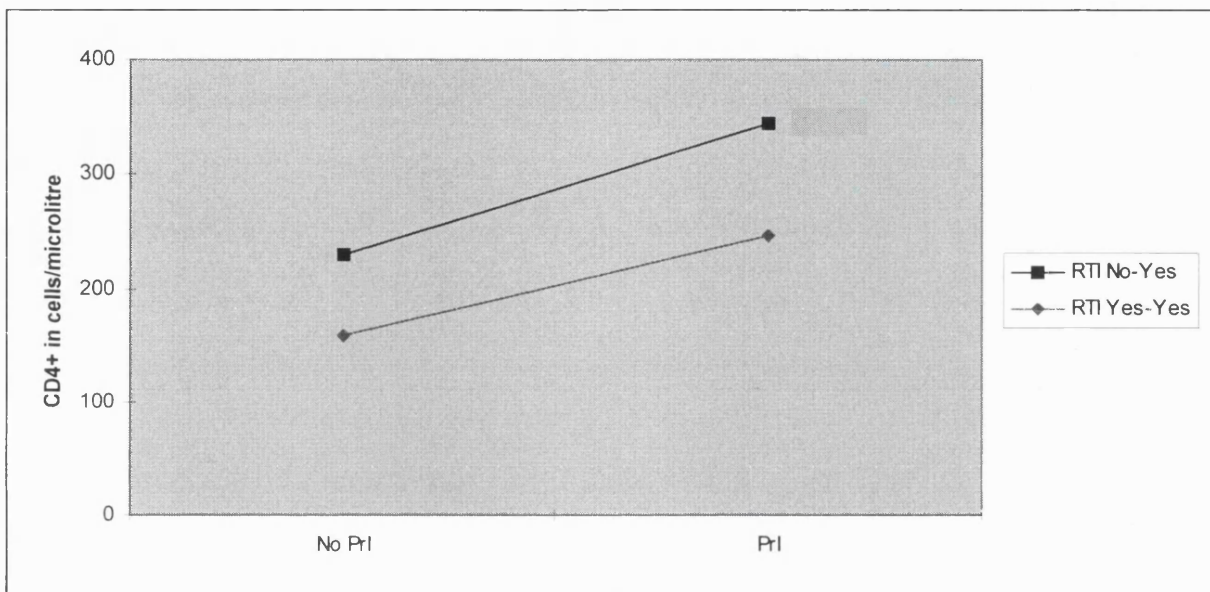
Figure 4.5 Differences in CD4+ T cell count according to PrI and RTI treatment. This figure also shows how CD4+ T cell counts in cells/microlitre changed after treatment, but it focuses on the 95% CIs and the median of before and after measures. The line on the left (1), represents patients who started both RTI and PrI at the same time and, on the right (2), patients who had been on RTI later followed by PrI.

Mean changes and 95%CIs CD4+ T cell associated with starting of PrI treatment

* CD4+ in cells/microlitre   **RTI sequence**
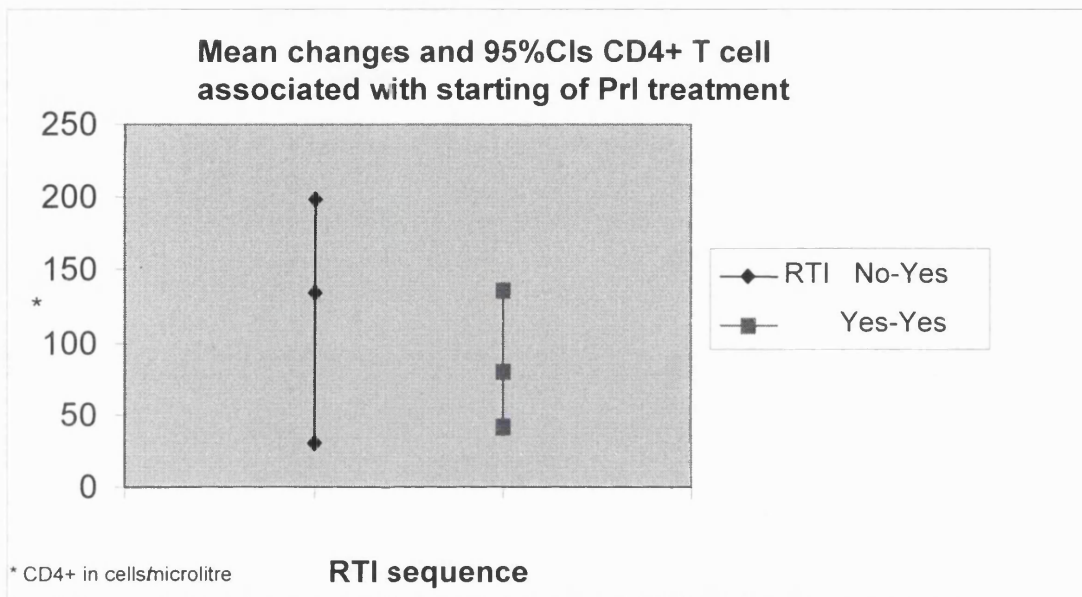
Figure 4.6 Histogram showing distribution of CD4+ T cell counts. The histogram overlaps closely the hypothetical bell shaped curve, demonstrating that the data has a reasonably normal distribution.

Distributions of the Changes in CD4 counts about the mean
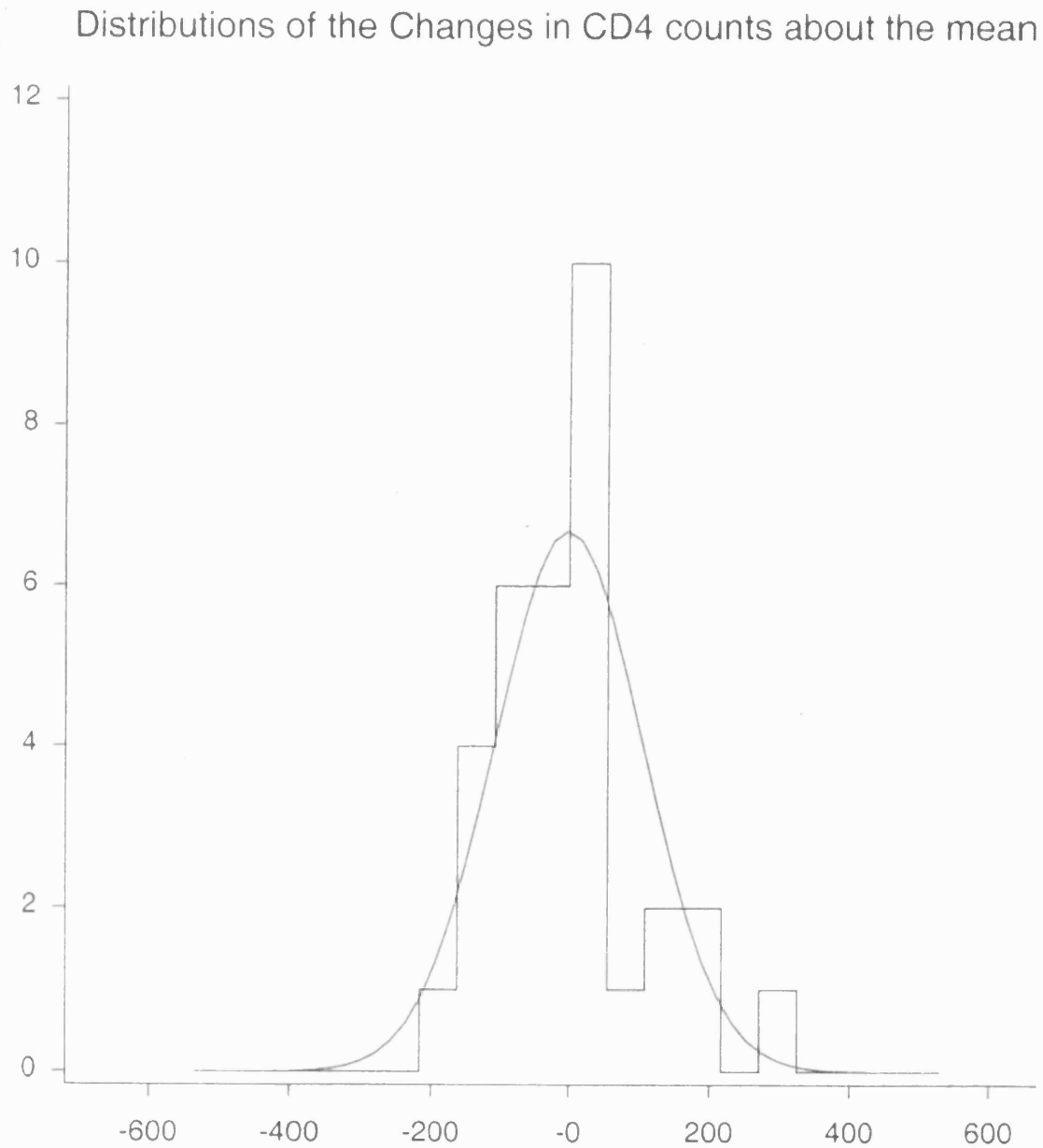
4.1.4 Effect of PrI treatment on HIV-1 plasma viral load

The same procedure (before and after treatment) was applied in order to evaluate changes in the HIV RNA plasma viral load. Each of the 33 patients who provided longitudinal data had their HIV plasma viral load measured before and after treatment. The mean HIV-1 RNA plasma viral load before and after the introduction of PrI by RTI treatment status is given in Table 4.3 Figure 4.7 graphically shows how HIV-1 plasma viral load changed according to PrI treatment status. Figure 4.8 graphically shows how the differences in load changed before and after PrI treatment.

A change in status from no treatment to both RTI and PrI led to a mean decrease in HIV plasma viral load of 105271.3 copies/ml (95% CIs -233192 to 22650). If patients were already receiving RTI, then introducing PrI led to a mean decrease of 54211.65 copies/ml (95% CIs -128773 to 20350).

Again, there was a need to assume that the changes in HIV-1 plasma viral load have a reasonably symmetrical distribution. The plot in Figure 4.9 shows that, in fact, it was not very symmetrical. Nonetheless it is unlikely that adjusting this for example by working on the logarithmic scale or some equivalent manoeuvre will make a substantial difference (A. Swan, personal communication).

The differences in HIV plasma viral load before and after PrI treatment were analysed using Student's t-test. The results are shown in Table 4.4.

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Neither of these differences reached the 5% level of significance, although for the first group the level of significance approached 5%. As it was done with CD4+ T cell count, whether there was a significant difference if the patient was already receiving RTI was also tested by the t-test. There is no evidence that the PrI effect differed according to the RTI treatment status (p = 0.3912; t = -0.87; degree of freedom = 31; 95% CIs = -166134 to 64015).

Table 4.3 Change in HIV-1 plasma viral load (in copies/ml) according to PrI and RTI treatment status. This table summarises HIV load data according to the treatment regimen in the two groups studied. The mean HIV load, alongside with the standard deviation and the number of patients is shown. The difference of the means (after-before introduction of PrI) is also shown.

RTI treatment		**PrI Before** No	**PrI After** Yes	Difference (After – Before)
No→Yes	Mean	110449.88	5178.63	-105271.25
	S.D.	202176.82	17765.88	205505.72
	N	16	16	16
Yes→Yes	Mean	68106.88	13895.24	-54211.65
	S.D.	119178.29	38088.91	124319.00
	N	17	17	17
Total	Mean	88636.82	9669.00	-78967.82
	S.D.	163474.53	29881.48	167915.63
	N	33	33	33

Table 4.4 Differences in HIV load before and after PrI treatment using Student's t-test. This table summarises the statistical tests used to evaluate HIV load before and after treatment in the two groups studied. The 95% confidence intervals values are also shown.

RTI	t value	p value	95% confidence intervals		
No→Yes	-2.049	0.0584	-233192	to	22650
Yes→Yes	-1.798	0.0911	-128773	to	20350

Figure 4.7 HIV-1 plasma viral load before and after introducing PrI treatment. The two groups of patients are represented here. Patients who started both RTI and PrI at the same time are represented by the dark line connected by squares. The group that had been on RTI and then put on PrI is represented by the line connected by diamonds.
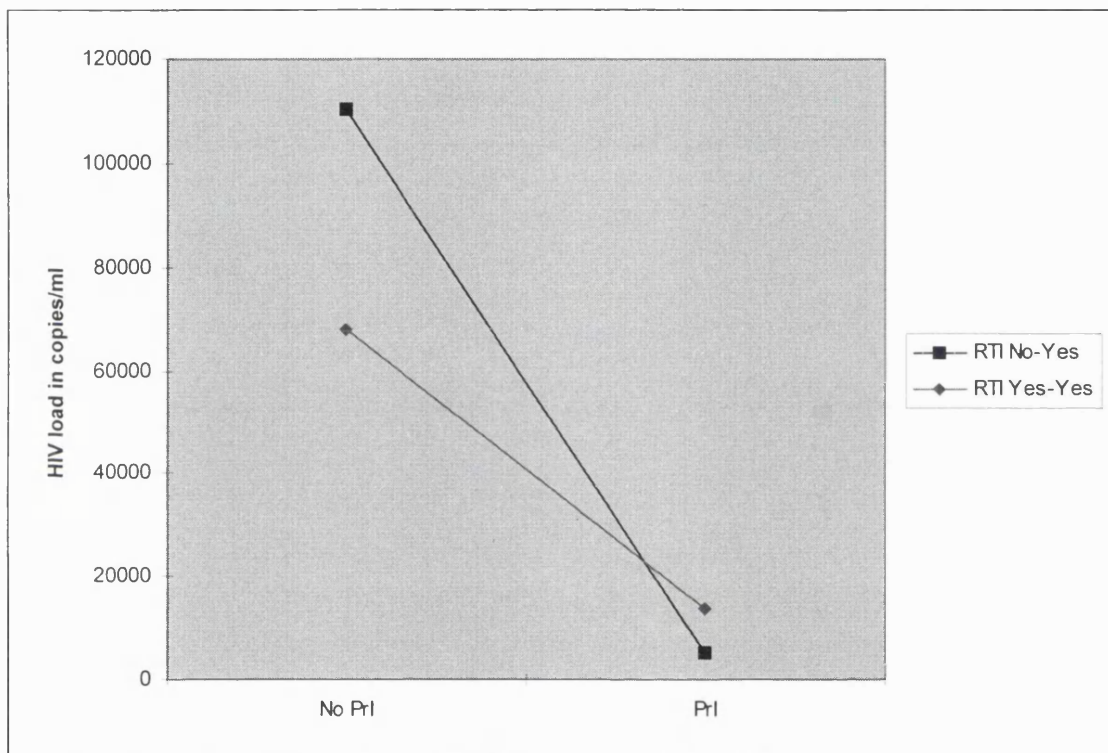
Figure 4.8 Differences in HIV load according to PrI and RTI treatment status. This figure also shows how HIV load in copies/ml changed after treatment, but it focuses on the 95% CIs and the median of before and after measures. The line on the left, represents patients who started both RTI and PrI at the same time and on the right, patients who had been on RTI then put on PrI.

Mean changes and 95% CIs HIV load associated with starting PrI treatment
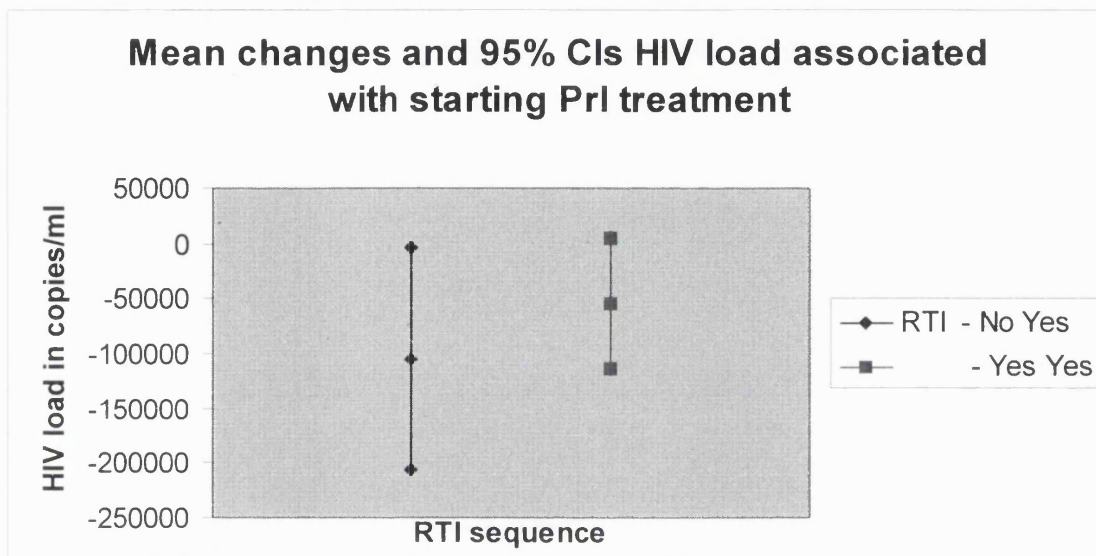
Figure 4.9 Histogram showing distribution of HIV-1 plasma viral load. This histogram does not overlap the hypothetical bell shaped curve, demonstrating that the data is not normally distributed. Despite this a parametric test was used (see text, section 4.1.4, for comments).

Distributions of the Changes in HIV load about the mean
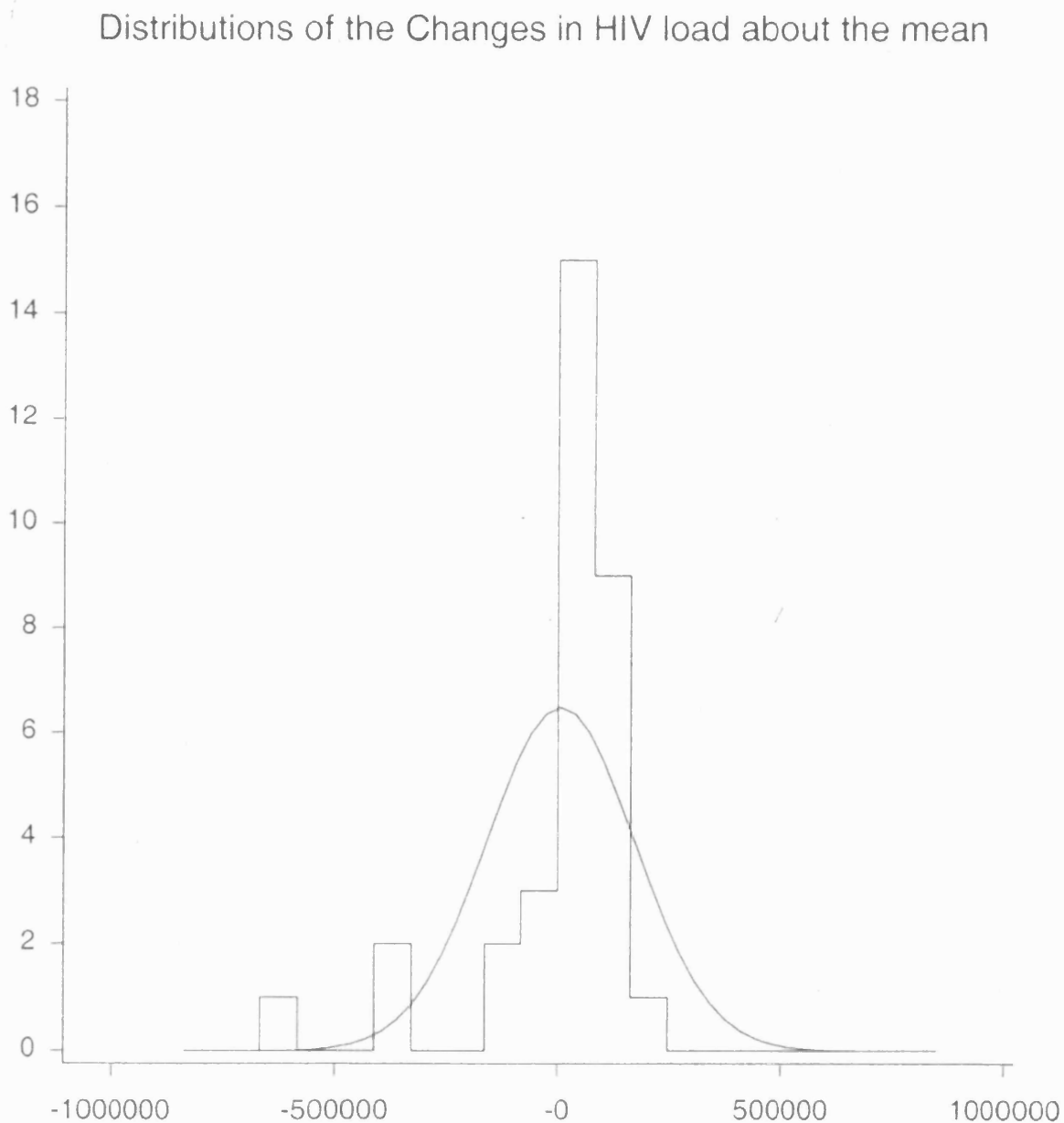
4.1.5 Effect of PrI on HHV-8 DNA status

Considering the whole sample, 42 of 132 patients (35%) were HHV-8 DNA positive before the introduction of PrI. However, as described earlier, only those who could be longitudinally tested were included in this section of the study. Thus, the number of samples that lost their HHV-8 DNA positivity categorised according to PrI and RTI status is shown in Table 4.5. The proportion of patients who was HHV-8 DNA positive before and after PrI treatment is shown in Table 4.6. Figure 4.10 graphically shows how the proportion of patients who were HHV-8 DNA positive changed according to PrI treatment status.

It appears that when both treatments were introduced there was a marked decrease in the proportion HHV-8 DNA positivity. There was also a clear, but lesser decrease associated with the introduction of PrI when RTI was in use throughout. The significance of the decreases was assessed using McNemar's test which compares the probability of being positive after PrI is introduced with the probability of being positive before PrI treatment, using the numbers changing from negative to positive and the numbers changing from positive to negative to obtain an odds ratio for the two probabilities.

Table 4.7 summarises how HHV-8 positivity status differed according to PrI treatment status. The odds ratio for being HHV-8 positive after the introduction of PrI (given RTI also introduced) was 0.00 (95% CIs 0.00 to 0.51). The decrease was significant (p = 0.0077; χ^2 = 7.1). The odds ratio for

being HHV-8 positive after the introduction of PrI (given RTI used throughout) was 0.00 (95% CIs 0.00 to 0.85). The decrease was also significant (p = 0.0412; χ^2 = 4.2). Hence, both decreases were significant implying that the introduction of PrI reduced the prevalence of HHV-8 detection. A formal test could be performed to assess whether this reduction differed with RTI treatment status, but it is clear that the difference, although it may represent a small real effect of RTI on HHV-8 status, was too small to be significant with numbers this small (16 and 17 in the two groups).

Table 4.5 HHV-8 DNA status before and after RTI and PrI treatment. This table summarises the number of patients studied according to HHV-8 DNA status and treatment given.

		PrI	
	HHV-8 DNA status	Before N	After Y
RTI treatment			
No→Yes	-ve	6	15
	+ve	10	1
	Total	16	16
Yes→Yes	-ve	10	16
	+ve	7	1
	Total	17	17
Total		33	33

Table 4.6 Proportion of HHV-8 DNA positivity before and after PrI treatment. This table shows the proportion in % of HHV-8 DNA positivity before and after treatment divided according to the groups studied. The 95% confidence intervals are also shown.

Before PrI	+ve	Total	Proportion +ve	95% CIs		
RTI No→Yes	10	16	62.5	35.4	to	84.8
RTI Yes→Yes	7	17	41.2	18.4	to	67.1
After PrI						
RTI No→Yes	1	16	6.3	0.2	to	30.2
RTI Yes→Yes	1	17	5.9	0.1	to	28.7

Figure 4.10 Proportion of HHV-8 DNA positivity before and after PrI treatment. This figure graphically shows how the proportion of HHV-8 DNA positivity in terms of percentage (Y axis) changed before and after treatment. The two groups of patients are represented here. Patients who started both RTI and PrI at the same time are represented by the dark line connected by squares. The group that had been on RTI and then PrI was introduced is represented by the line connected by diamond shaped line. There is a reduction in HHV-8 DNA positivity in both groups.

Proportion of HHV-8 DNA positivity associated with starting of PrI treatment

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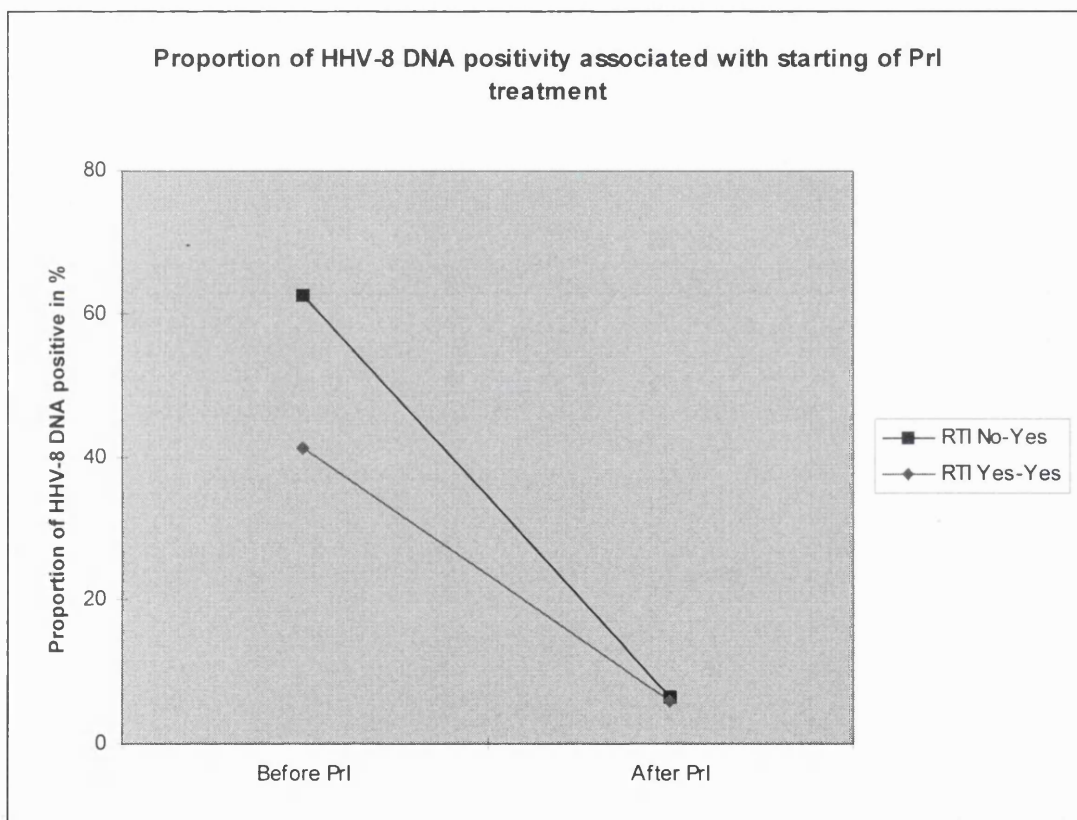
Table 4.7 Changes in HHV-8 DNA positivity over time. This table summarises the results of HHV-8 DNA detection and how it changed over time.

		After PrI		
Before PrI		HHV-8 status		Total
RTI treatment HHV-8 status		-ve	+ve	
No→Yes	-ve	6	0	6
	+ve	9	1	10
	Total	15	1	16
Yes→Yes	-ve	10	0	10
	+ve	6	1	7
	Total	16	1	17
Total		31	2	33

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4.1.6 Changes in HHV-8 IgG status

Fifteen HIV positive male patients, from whom more than one plasma samples could be collected, were enrolled in this study. The results of the indirect immunofluorescence assay are summarised in Table 4.8.

In view of the small number of specimens studied, fluorescence intensity of 1+ and lower was considered as negative, and intensities of 2+ or more were considered positive. Numbers of IFA positives and negatives grouped in this way are summarised in Table 4.9. Only three individuals showed a drop in the HHV-8 IgG antibody levels. All the others remained unchanged after the introduction of PrI. The odds ratio for changing HHV-8 IgG status after the introduction of PrI was 0.00 (95% CIs 0.00 to 2.42). The difference was not significant (p = 0.2482; χ^2 = 1.3).

Table 4.8 Number of samples divided according to intensity of HHV-8 IgG immunofluorescence. This table summarises the data collected for the 15 patients from whom plasma samples were available. It shows how the intensity of IgG antibodies changed over time according to PrI treatment.

IFA before PrI	IFA score after PrI				
	-	1+	2+	3+	Total
-	3	0	0	0	3
1+	0	4	0	0	4
2+	0	0	2	0	2
3+	1	1	1	3	6
Total	4	5	3	3	15

Table 4.9 Qualitative indirect immunofluorescence assay results in relation to PrI therapy status. This table shows the number of changes from being HHV-8 IgG positive to negative or vice-versa.

IFA		After PrI		
		+ve	-ve	Total
Before	+ve	9	3	12
PrI	-ve	0	3	3
Total		9	6	15

Chapter 5 – Discussion

5.1 Discussion - Intra host genetic variability

KS is thought to be a benign proliferation rather than a true malignant neoplasm. This is because of its multifocality, lack of features of typical tumour spindle cells (such as aneuploidy and nuclear atypia), lack of permissive growth in cell culture, and spontaneous regression upon withdrawal of immunosuppressive agents in iatrogenically induced disease. In some cases multiple KS lesions can arise from an independent cellular progenitors in the same patient (Gill *et al*, 1998). These findings suggest that KS lesions may originate from infection by different HHV-8 strains at distinct body sites. Alternatively, if HHV-8 does play a role in the pathogenesis of KS, different HHV-8 strains already found in progenitor KS cells may become preferentially amplified following clonal proliferation of these cells.

The results of the first section of this work demonstrate that a single host, whether co-infected or not with HIV-1, may harbour more than one HHV-8 variant. Peripheral blood of HIV positive patients was more frequently infected with multiple strains of HHV-8 in comparison with blood of heath care volunteers and BMT patients, or oral KS lesional tissue of HIV-infected persons.

A number of factors may underly the presence of multiple HHV-8 variants. Severe immunosuppression, associated with high HIV-1 viral load, as supposedly found in the HIV positive blood group, may lead to HHV-8 reactivation and a higher rate of replication. HIV-1 *tat* is implicated in the

transactivation of HHV-8 from peripheral blood mononuclear cells of HIV-infected patients and in *tat*-treated BCBL-1 cells (Harrington *et al*, 1997). This could then be responsible for the variability found in this group.

Another possible explanation is that the differences observed are due to the differences in the number of infected cells in KS lesional tissue and cell lines compared with blood of HIV infected individuals. A low amount of starting material, as may be found in peripheral blood specimens, could result in a large degree of variability seen in this group, possibly due to PCR artefact. This is, however, unlikely, as the number of cells infected in the post-transplant patients and health care workers were also very low and yet did not show high variability when compared to the leukocytes of the HIV positive group.

PCR artefacts, e.g., *Taq* polymerase misincorporation, are unlikely to be responsible for the degree of variability shown in this study, as demonstrated by the findings relating to BCBL cell lines. PCR-induced misincorporation occurred only infrequently, suggesting that the findings of variability, particularly in the HIV positive blood group are genuine.

It is possible that co-infection with more than one HHV-8 strain is due to immunosuppression alone, although this is unlikely as the transplant patients group did not manifest a high degree of variability, but of course it may be that the degree or type of immunosuppression associated with the BMT patients is not related with reactivation of HHV-8.

It may be possible that exposure to a large number of sexual partners and to anal intercourse account for the high frequency of multiple HHV-8 carriage. This would be consistent with what has been reported for CMV, multiple CMV infections having been reported to occur at different time points (Verbraak *et al*, 1998). However, such a scenario does not appear to be likely, as the variants found in this study were derived from a tight phylogenetic cluster. A phylogenetic analysis of all the ORF 26 sequences amplified in this study (Figure 4.35) shows that there is indeed a clustering of minor variant sequences.

Further work amplifying more variable regions of the HHV-8 genome, i.e. ORF K1, is necessary to clarify whether the variability found in this study follows a quasispecies-like distribution or a mixed infection pattern, in accordance to what has been described above for CMV.

HHV-8 is thought to infect primarily B cells, although it has also been found to a lesser frequency in CD8+ T cells (Sirianni *et al*, 1997). In KS tissues, HHV-8 is located in endothelial and spindle cells (Boshoff *et al*, 1995a). Simultaneous application of immunohistochemical staining and *in situ* hybridisation have identified cells that permit HHV-8 replication to be of monocytic origin (Blasig *et al*, 1997). The identification of HHV-8 in CD4+, CD8+ and monocytic cells allow the possibility for different cell populations to be infected with distinct strains of HHV-8. An inter-compartmental intra-host diversity could therefore be responsible for the variability found in this study. However, further work is required to substantiate this hypothesis of infection

of different leukocyte subsets. Such work can include selective fractionation of leukocytes subsets from peripheral blood or fresh lesional tissues and the determination of HHV-8 subgenomic sequences amplified from these fractionated cells to confirm genetic heterogeneity.

5.2 Discussion – Effect of HIV-1 protease inhibitors on HHV-8 replication

The results of this section of the study show a correlation between the use of HIV-1 protease inhibitors and the frequency of HHV-8 DNA detection by PCR from peripheral blood of HIV-infected male patients. The decrease in detection rate may be due to a direct effect of the protease inhibitors on HHV-8 replication. However, as discussed previously, in an earlier study ritonavir showed no *in vitro* effect on HHV-8 replication (Kedes and Ganem, 1997). In addition, HHV-8 protease does not share homology at the nucleotide or protein level with HIV-1 protease (Unal *et al*, 1997), nor have the same structure compared to HIV-1 protease as studied by x-ray crystallography (Navia *et al*, 1989; Wlodawer *et al*, 1989). A direct effect on HHV-8 replication *in vivo* would therefore seem unlikely.

An alternative explanation, linking the reduced frequency of HHV-8 DNA detection with recovery of the immune system, may be more plausible. CD4+ T cell counts has been observed by others to be raised after combination therapy (Autran *et al*, 1997). Such an improvement is confirmed by the results of the present study, where changing from no treatment to treatment by both RTI and PrI led to a mean increase in CD4+ T cell count of 114.2 cells/μL, and if patients were already on RTI, PrI treatment, led to a

mean increase of 89.2 cell/μL. Such findings are consistent with the observation that KS regresses after cessation of immunosuppressive therapy in transplant patients (Montagnino *et al*, 1994).

HIV-1 RNA plasma viral load has been reported by others to decrease considerably after the addition of protease inhibitors in combination therapy (Li *et al*, 1998; Notermans *et al*, 1998). These findings were confirmed in this study, when it was observed that the majority of patients achieved dramatic reduction in HIV RNA plasma viral load below the cut off limit (< 500 copies/ml) after PrI therapy. This reduction may contribute to the disappearance of HHV-8 DNA in peripheral blood. In this scenario, suppression of HIV-1 *tat* expression would play a role in inhibiting HHV-8 replication. HIV-1 *tat* is implicated in the transactivation of HHV-8 from peripheral blood mononuclear cells of HIV-infected patients and in *tat*-treated BCBL-1 cells (Harrington *et al*, 1997). It is possible that *tat* secreted by HIV-1 infected cells can be taken up by HHV-8 latently infected cells, subsequently upregulating HHV-8 replication.

In this study, the effect of PrI on the proportion of HHV-8 DNA positivity was greater in RTI-naive patients (p=0.0077) when compared with those already on RTI (p=0.0412). These findings may be explained by patients already on potent antiretroviral therapy but not on PrI having already gained recovery of the immune system, which then substantially reduced the HIV-1 plasma viral load. The overall effect would still be the suppression of HIV-1 *tat* expression, thus limiting the level of HHV-8 replication.

It is also clear from the present study that HHV-8 IgG antibody levels may not necessarily change after the introduction of protease inhibitors in the combination therapy of HIV infected patients. Antigen-derived proteins may still be able to induce the production of antibodies even from patients who had undetectable HHV-8 DNA in their peripheral blood. Certainly, HHV-8 can establish latent infection, like other herpesviruses, and by using an IFA assay which detects both lytic and latent antigens, a direct correlation between antibody and DNA detection may not be observed.

The results of this study show the value of RTI and PrI in suppressing HHV-8 replication in HIV-infected patients, and explain why KS is now rarely seen in patients on HAART. However, it is not known for what length of time HAART can effectively suppress HIV-1 viral replication (Kempf *et al*, 1998). There is still a possibility that HIV and HHV-8 replication may rebound. Furthermore, the best means to maintain patient compliance (in view of the fact that medication must be taken several times a day) is still unclear. Side-effects of HAART, which can be severe, and unpredictable interactions with a number of other medications, adversely affect patient compliance. In addition, the high cost of the treatment can restrict access to the HAART treatment regimen in several developing countries. For these reasons, it would still be important to engage in the development of drugs that specifically target HHV-8 replication. Such drugs would be useful in the treatment of the other forms of KS that are not AIDS-related, in particular the aggressive form of endemic (African) KS.

Chapter 6 - Conclusions and suggestions for areas of further studies

6.1 Conclusions

The results of the first part of this study (Chapter 3) show that a given host whether coinfected by HIV or not may be infected by more than one HHV-8 strain. However, blood of HIV-coinfected hosts tends to carry more HHV-8 variants. Whether infection by multiple HHV-8 strains represents a quasispecies-like distribution or reflects multiple episodes of infection, as has been observed for CMV infection, remains unclear. It is more unlikely that multiple infections explain the variation, as sequences of the minor variants appear as clusters of genetically close variants. Thus quasispecies formation is a more likely explanation, and reflects better what happens when progression in HIV infection allows activation of latent HHV-8.

This part of this study also shows that HHV-8 strain variability in KS lesions is restricted. This phenomenon may reflect clonal proliferation of a particular spindle/endothelial HHV-8 infected cell.

The results of the second part of this study (Chapter 4) show that the use of HIV-1 protease inhibitors is associated with a decrease in the frequency of HHV-8 DNA detection in peripheral blood of HIV-infected patients. Whether this is a direct or indirect effect remains unclear. It is also found that changing from no treatment to both RTI and PrI is associated with an increase in CD4+ cell counts and a decrease in HIV load. By contrast, HHV-8 IgG antibody levels do not change over time after the introduction of PrI.

6.2 Suggestions for areas of further studies

It is now important to determine whether the genetic variability of HHV-8 observed is due to the presence of quasispecies or the presence of multiple strains. This can only be done by further optimising PCR for the K1 region. Once PCR related sensitivity problems are overcome, more data can become available to answer this question.

Another area of interest would be to evaluate the clinical and virological impact of drugs that specifically target HHV-8 replication. It is unlikely that HIV-1 protease inhibitors will be completely effective in the management of KS (both HIV-associated and non-HIV-associated). Hence, a new class of compounds requires to be developed.

It is essential that more reliable studies on the global epidemiology of HHV-8 in the general population become available. At present, data regarding the seroprevalence of HHV-8 are contradictory. Studies using more sensitive and specific assays will clarify this issue, helping to better understand the role of HHV-8 in the pathogenesis of KS and other diseases.

Lastly, the global occurrence of HHV-8 inter-host variability and its clinical implications need to be evaluated. There may be geographical and disease associations particular to specific strains of HHV-8.

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APPENDIX 1 - METHODS FOR AUTOMATED SEQUENCING

Computer analysis of sequencing data

After each sequencing run, launch the programme "Analysis" (Applied Biosystems) to display the gel image. Grey tracker lanes indicate where each sample is and the sample files are generated from these positions. Each sample file can be viewed in one of four ways: (1) file information, which is details about the run; (2) raw data which are the data prior to analysis by the "Analysis" programme; (3) analysed data; and (4) sequence data, i.e., data found in the standard IUPAC 5 letter code (A,G,C,T,N).

Base calling of reanalysing sequence data

This enables the raw data to be reanalysed, and is often necessary because the computer will have assigned first and last bases incorrectly which may result in the contig assembly programme being unable to recognise overlapping sequences.

Launch the programme "Analysis", pull down the File menu and select Open. When the dialogue box appears it is possible to select the sample file requiring reanalysis. The window menu is used to select Raw Data, Analysed Data and Controller. The custom tool on the controller is used to locate the beginning of the data, normally located after the dye terminator peaks at the start of the raw data (these are produced from the unincorporated dye terminators). Record the X-axis number for the first base, found in the lower left hand corner of the Raw Data window. Similarly record the number of the last

base. As small PCR products are used, discrete raw data are produced and it is easy to locate the end point. Pull the Analysis menu down, select Call Bases and enter the start and end co-ordinates into the appropriate boxes. This process produces a new sample file that is written over the existing file. However, the raw data remains unaltered. Using the tools on the controller it is possible to look at the reanalysed Analysed Data. Check the chromatogram to ensure that the correct bases have been called, and where ambiguous data are. The base in question can be changed to an "N" in hope that it can be resolved from the sequencing of the other strand. The sequence data are presented in two formats: the sample file and ASCII text files.

Using SeqEd version 1.03

This programme allows contigs to be assembled from the ABI sequence data. Open SeqEd (Applied Biosystems) and pull the File menu down to select New Layout. To import the ABI sequences, pull the Sequences menu down and choose Import Sequence. The sequences of interest are then imported. Any anti-sense sequences can be made to read in the sense direction by highlighting the file name on the left hand side of the layout, then pulling down the Sequences menu and selecting reverse complement sequence.

The sequences can now be aligned by highlighting the sequences of interest, pulling down the Align menu, then choosing overlap, comparative or multiple. Once the alignment is complete, the sequences can be compared for mismatches; this is done by using the Sequences menu, selecting create shadows and then compare two sequences; starred areas indicate

mismatches. These areas are highlighted and viewed once again using the Sequences menu and then Display Chromatograms. Inspect the chromatograms closely. Those bases that are indeterminate are labelled as "N" for unknown. Repeat this process until all starred sites have been examined. A unanimity sequence is created by highlighting the names of all the sequences used, using the Sequences menu pick create shadow and then unanimity sequence. Freeze this unanimity sequence by using freeze shadow from the Sequences menu. This can now be exported in the form of a text file by using export sequence, and also from the Sequences menu. The unanimity sequence can also be used to determine the amino acid sequence. By selecting create shadow and translate codons to amino acids from the sequence menu it is possible to get the DNA sequence translated into "universal" code in all three reading frames with either three letter or single letter amino acid code. This layout can now be saved for future reference.

Using EDITSeq of Lasergene Navigator

This programme enables sequence data to be converted into a format easily recognisable to the *Lasergene Navigator* suite of programmes.

Open the *Lasergene Navigator* and select the Sequence Editing and Analysis option. Enter sequence data in one of two ways, by importing text files created by SeqEd or by manually typing in the sequence. If the data is to be manually entered, the computer can be used to proof read the data vocally. This is done by using the Digitizer menu and choosing Macintosh Voice. To import data, pull the File menu down and select Open, the data

has to be in the DNAstar format. If, however, the data is in another format use the File menu to select Import. This can then be translated into an amino acids by highlighting the DNA sequence and selecting Translate DNA from the Goodies menu. Export these sequences as documents containing sequence data with comments. Save these files for use in other programmes in the *Lasergene Navigator* suite.

Using MegAlign of Lasergene Navigator

This programme enables the degree of similarity between different sequences to be analysed by comparing nucleotides and/or amino acids. .

To use this programme select Multiple Sequence Alignment from the *Lasergene Navigator.* Using the File menu and selecting New enables sequences to be entered into the worktable for alignment. Alternatively a pre-existing alignment can be opened and altered by choosing Open from the File menu. To enter sequences, pull the File menu down and select Enter Sequences. When the dialogue box appears, enter in sequences and/or other alignment files by highlighting their names and selecting Add. Once the desired sequences have been entered, select Done. Add both DNA and amino acid sequences at the same time with the DNA sequences being automatically being translated into amino acids from base one. If only a selected part of the sequence is required, it is possible to select this part from within MegAlign; this is known as subranging, alternatively alter the EditSeq file before entry into the programme. To set the subrange, open the Options

menu followed by <u>Set Sequence Limits</u> and <u>By Coordinates</u>. A dialogue box will appear; enter positions of the first and last base required.

Alignment of multiple sequences was performed either <u>By Clustal Method</u> or <u>By Jotun Hein Method</u> from the <u>Align</u> menu. Clustal is generally preferred. However, Jotun Hein is used when the sequences are derived from a common source. The alignment can be viewed in four different formats: the <u>Alignment Report</u>, <u>Sequence Distances</u>, <u>Residue Substitution</u> and <u>Phylogenetic Tree</u>.

Appendix 2 - Informed consent forms

Informed consent for patients in England

Title of Research: Intra host genetic variability of HHV-8

Name of Investigator: Professor Stephen Porter / Dr. Jair C. Leao

I

....
(Full name)

of

....
(Address)

hereby fully and freely consent to participate in the above research project.

I agree that my general practitioner may be notified of my participation in the trial and that he may release information on my past history. I have informed an investigator of any drug I am presently taking.

I understand and acknowledge that the investigation is designed to promote medical knowledge.

I understand that I may withdraw my consent at any stage in the investigation. I acknowledge the purpose of the investigation and accept any risks involved from the drugs or other procedures. The nature and purpose of such procedures has been detailed to me in an information sheets and has been explained to me by: .

Dr/Mr/Mrs

Signed   Date

DECLARATION BY THE INVESTIGATOR

I confirm that I have provided an information sheet and explained the nature and effect of the procedures to the volunteer and that his/her consent has been given freely and voluntarily.

Signed

Name

Informed consent for the patients in Brazil

Universidade Federal de Pemambuco
Hospital das Clinicas
Comissao de etica

No. do Protocolo de estudo:

"Consentimento Informado"

Titulo do Projeto: Variabilidade genetica do HHV-8

Objetivo: Identificar diferentes Linhagens e estudar rotas de transmissclo do HHV-8 e a partir dai estabelecer um metodo de agrupamento de acordo com o sequenciamento genotipico viral.

Metodologia: Vinte pacientes HIV positivo, do Servico de Doencas Infecciosas e Parasitarias, deste hospital, serao avaliados, atraves de exame de sangue de rotina, de onde se requisitara 10 ml, afim de se tentar DNA viral, bem como esfregacos de mucosa bucal e de lingua, utilizando-se curetas cirurgicas, um procedimento simples e nao invasivo e ainda a coleta de saliva, utilizando-se se necessario uma substancia estimulante do fluxo salivar, suco de limao por exemplo e finalmente coleta de saliva da glandula Parotida, atraves do copo de Lashley, mais uma vez um procedimento indolor ao paciente. Complementando o estudo, sera aplicado um questionario, de carater confidencial.

Riscos: Este e um estudo seguro, que nao apresenta riscos ao paciente que se dispunha a colaborar. 0 sangue sera coletado obedecendo a rotina do servico, onde na maioria das vezes, e procedido sem maiores problemas ao paciente, entretanto estes exames podem levar a um dolorimento leve ou pequeno hematoma (arroxeamento) no local onde houve a puncao. Com relacao ao esfregaco de lingua e de mucosa bucal, este e tambem um procedimento seguro, pois a curetagem (raspagem) e feita de forma superficial e finalmente a coleta de saliva, nao oferece nenhum risco ao paciente, sendo no entanto um procedimento que pode levar algum tempo, principalmente naqueles que apresentam um fluxo salivar diminuido.

Beneficios: Os beneficios deste estudo sao de carater subjetivo e a longo prazo. Lembre-se de que voce estara contribuindo para a obtencao de informacoes de natureza clinica e laboratorial, que poderao favorecer outras pessoas com problemas semelhantes ao seu, alem de ajudar na tentativa de ser elucidada algumas questoes ainda nao resolvidas pela comunidade cientifica, com relacao a este novo virus.

Altemativas: A sua participacao neste estudo clinico laboratorial, e voluntaria. Voce podera decidir nao colaborar neste protocolo de pesquisa, e

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assim como a qualquer momento podera interromper a sua participacao sem qualquer prejuizo para o seu tratamento no Servico. Da mesma maneira se o medico ou outros responsaveis pelo estudo poderao decidir pela interrupcao no estudo baseado em criterios tecnicos. Os dados de seu arquivo serao mantidos confidencialmente. Os resultados poderao ser utilizados para apresentacao, sob codigo, isto e, sem divulgar seu nome ou outra forma que possa identifica-lo(a).

Desta forma, eu _____

R.G. no._____ Expedido por_____

Abaixo assinado, atesto que li e entendi o conteudo deste consentimento informado e aceito de livre expontanea vontade, participar deste estudo, e que esclareci todas as minhas duvidas com, Dr. Jair C. Leao e Dra. Silvia Lemos Hinrichsen, responsaveis pelo estudo.

Paciente _____

Testemunha _____

Investigador _____

Autorizo ainda, que o Dr. Jair Carneiro Leao, faca fotografias com finalidade cientifica, desde que nao apareca meu rosto, ou qualquer outra forma que eu possa ser identificado(a).

Paciente _____

Investigador _____

APPENDIX 3 - PAPERS AND PRESENTATIONS RELATED TO THE THESIS

Papers

Triantos D, **Leao JC**, Porter SR, Scully CM, Teo CG. Tissue distribution of Epstein-Barr virus in hosts coinfected by HIV. (1998) *AIDS* **12 (16)**: 2146-6.

Triantos D, Boulter AW, **Leao JC**, Di Alberti L, Porter SR, Scully CM, Birnbaum W, Johnson NW, Teo CG. Diversity of naturally occurring Epstein-Barr virus revealed by nucleotide sequence polymorphism in hypervariable domains in the BamHI K and N subgenomic regions. (1998) *Journal of General Virology* **79**: 2809-17.

Leao JC, Hinrichsen SL, Freitas BL, Porter SR. Herpesvirus humano-8 e o sarcoma de Kaposi. (1999) *Revista da Associacao Medica Brasileira* **Jan-Mar 99**: 55-62.

Leao JC, Bez C, Hinrichsen SL, Porter SR, Scully CM, Teo CG. Relative restriction of HHV-8 subgenomic sequence heterogeneity in KS tissues and leucocytes of health care workers and iatrogenically immunosuppresed BMT patients compared to peripheral blood of HIV-infected patients. (Manuscript in preparation).

Leao JC, Kumar N, Mclean K, Swan A, Porter SR, Scully CM, Teo CG. Effect of HIV protease inhibitors on the frequency of HHV-8 detection from leucocytes of HIV patients. (Manuscript submitted to AIDS).

Oral and poster presentations

Leao JC, Di Alberti L, Kumar N, Porter SR, Scully CM, Teo CG. Relative restriction of HHV-8 subgenomic sequence heterogeneity in KS tissues compared to peripheral blood of HIV-infected patients. **Kaposi's sarcoma associated herpesvirus and related agents - University of California, Santa Cruz. July 25-28, 1998.**

Di Alberti L, **Leao JC**, Porter SR, Scully CM, Teo CG. Mutational analysis of HHV-8 ORF 26 reveals at least eight genetic groups of the virus worldwide. **British Society for Dental Research. April, 1998.**

Leao JC. Human herpesvirus 8 in dentistry. Lecture for MSc students in Oral Medicine, **Eastman Dental Institute for Oral Health Care Sciences. November, 1998.**

Bez C, **Leao JC**, Porter SR, Scully CM. Lack of association between human herpesvirus 8 and recurrent aphthous stomatitis. **British Society for Dental Research. April, 1999.**