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Multiple Infection by Human Herpesvirus-8 and Cytomegalovirus

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

Doctor of Philosophy
University of London

Mohammed Beyari
BDS MSc MFD RCSI (Ireland)

Oral Medicine

Eastman Dental Institute for Oral Health Care Sciences

University College London

February 2005
Abstract

To substantiate the hypothesis that multiple infection by human herpesvirus 8 (HHV-8) and by cytomegalovirus (CMV) commonly occurs, intraperson nucleotide variation in DNA amplified from hypervariable subgenomic domains of HHV-8 and CMV was evaluated in a group of people living in Malawi, where both viruses circulate hyperendemically.

Mouth rinses, throat gargles, palatal exfoliates and blood were sampled from 89 people (age range: 4 m to 45 y). In 24 people (27%), HHV-8 DNA could be amplified in >1 sample; 9 (38%) were seropositive for human immunodeficiency virus-1 and 7 (29%) exhibited Kaposi’s sarcoma. Sequence variation was sought in DNA segments derived from: the domain in open reading frame (ORF) 73 that encodes latent nuclear antigen; the Bam330 segment of ORF 26; and the V1 region of ORF K1. Restriction fragment-length polymorphism analysis, nucleotide sequencing, PCR cloning and denaturing gel gradient electrophoresis were applied to study the sequence diversity of these segments. Significant intraperson/inter- and intra-sample sequence polymorphisms could be found in 15 people (62.5%). Variation in urine-derived V1 sequences could, in addition, be evaluated: in 5 people, the sequences were monotypic, and in 2, urinary and oral sequences were genotypically different.

Variation in hypervariable domains in the gO and gN regions of CMV was studied in urine and saliva samples of 77 people. In 41 individuals (53%), DNA could be amplified from at least one domain, and, in 14 (18%), from both domains. In 4 individuals (5%), urinary and oral sequences were genotypically different. The
extent of inter- and intra-person variation in the 2 CMV domains studied was significantly less than in K1/V1 of HHV-8.

Multiple infection by HHV-8 and by CMV is common. It would be important to determine if such infection reflects coinfection occurring during initial transmission or superinfection, as the latter implies that vaccination might be ineffective to prevent and control the spread of the viruses.
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Declaration

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

Mohammed Beyari
Oral Medicine
Eastman Dental Institute for Oral Health Care Sciences
University College London
University of London
256, Gray’s Inn Road
London WC1X 8LD

February 2005
Acknowledgments

First of all, I would like to thank God who has given me the strength and health to complete my thesis.

I am extremely grateful to my supervisors, Dr Chong Gee Teo and Professor Stephen Porter for their helpful guidance and support without whom the completion of this work would not have been possible.

I am grateful to Umm-Alqura University for their generous funding of my studies.

I am also grateful to the following:

1- Dr Tim Hodgson who travelled to Malawi to collect the samples studied in this thesis
2- Dr Rachelle Cook for her technical assistance and kindness
3- Mr Florian Bobet for teaching me many of the techniques
4- My friends and colleagues at the Health Protection Agency and Eastman Dental Institute for their help and friendship.

Special thanks go to my parents, for their encouragement and love to my wife, for her warm support.
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>Bax</td>
<td>Bax protein, 21 kDa homologous partner of Bcl-2</td>
</tr>
<tr>
<td>BCBL</td>
<td>body cavity based lymphoma</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B cell leukaemia / lymphoma-2 genes</td>
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<tr>
<td>b-FGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BMT</td>
<td>bone marrow transplant</td>
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<tr>
<td>bp</td>
<td>base pair</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CC</td>
<td>Subfamily of chemokines which have 2 adjacent cysteine residues</td>
</tr>
<tr>
<td>CCR</td>
<td>CC chemokine receptor</td>
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<tr>
<td>CD</td>
<td>Castleman's disease</td>
</tr>
<tr>
<td>CXC</td>
<td>subfamily of chemokines which have 2 cysteine residues separated by single amino acid</td>
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<tr>
<td>cdk</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CPE</td>
<td>cytopathogenic effect</td>
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<tr>
<td>DGGE</td>
<td>denaturing gradient gel electrophoresis</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>deoxynuclotide triphosphate</td>
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<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EDTA</td>
<td>disodium ethylenediaminetetra-acetate</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FLICE</td>
<td>Fas-associated death domain-like interleukin-1 beta-converting enzyme</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>FLIP</td>
<td>FLICE inhibitory protein</td>
</tr>
<tr>
<td>GC</td>
<td>guanine-cytosine</td>
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<td>GPCR</td>
<td>g protein coupled receptor</td>
</tr>
<tr>
<td>GVHD</td>
<td>graft versus host disease</td>
</tr>
<tr>
<td>HAART</td>
<td>highly active antiretroviral therapy</td>
</tr>
<tr>
<td>HBV</td>
<td>hepatitis B virus</td>
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<tr>
<td>HHV-6</td>
<td>human herpesvirus 6</td>
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<td>HHV-7</td>
<td>human herpesvirus 7</td>
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<tr>
<td>HHV8</td>
<td>human herpesvirus 8</td>
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<td>HIV</td>
<td>human immunodeficiency virus</td>
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<td>HSV</td>
<td>herpes simplex virus</td>
</tr>
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<td>HVS</td>
<td>herpesvirus saimiri</td>
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<tr>
<td>IDUs</td>
<td>injection drug users</td>
</tr>
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<td>IFA</td>
<td>immunofluorescence assay</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
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<td>IL-6</td>
<td>interleukin 6</td>
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<td>IL-8</td>
<td>interleukin 8</td>
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<tr>
<td>IRD</td>
<td>internal repeat domain</td>
</tr>
<tr>
<td>IRES</td>
<td>internal ribosome entry site</td>
</tr>
<tr>
<td>IRF</td>
<td>interferon regulatory factor</td>
</tr>
<tr>
<td>KS</td>
<td>Kaposi’s sarcoma</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposi’s sarcoma-associated herpesvirus</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>LNA</td>
<td>latency associated nuclear antigen</td>
</tr>
<tr>
<td>LUR</td>
<td>long unique region</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MCD</td>
<td>multicentric Castleman's disease</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage inhibitory protein</td>
</tr>
<tr>
<td>MPC</td>
<td>magnetic particle concentrator</td>
</tr>
<tr>
<td>NF</td>
<td>nuclear factor</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>ORF</td>
<td>opening reading frame</td>
</tr>
<tr>
<td>Onco M</td>
<td>oncostatin M protein</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PEL</td>
<td>primary effusion lymphoma</td>
</tr>
<tr>
<td>PKR</td>
<td>protein kinase</td>
</tr>
<tr>
<td>pRB</td>
<td>retinoblastoma tumour suppressor</td>
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<tr>
<td>PHYLIP</td>
<td>phylogeny inference package</td>
</tr>
<tr>
<td>RDA</td>
<td>representational difference analysis</td>
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<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>Rta</td>
<td>replication and transcription activator</td>
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<tr>
<td>RV</td>
<td>rhadinovirus</td>
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<td>TAE</td>
<td>tris acetate EDTA</td>
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<td>Taq</td>
<td>Thermus aquaticus</td>
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<tr>
<td>Tat</td>
<td>transactivator protein</td>
</tr>
<tr>
<td>TBE</td>
<td>TRIS borate EDTA</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
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</tr>
<tr>
<td>TCID</td>
<td>tissue culture infectious dose</td>
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<td>TGFβ</td>
<td>transforming growth factor-beta</td>
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<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol 13 acetate</td>
</tr>
<tr>
<td>TR</td>
<td>terminal repeat</td>
</tr>
<tr>
<td>UL</td>
<td>unique long</td>
</tr>
<tr>
<td>US</td>
<td>unique short</td>
</tr>
<tr>
<td>UPGMA</td>
<td>unweighted pair group method with arithmetic means</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>VZV</td>
<td>Varicella-zoster virus</td>
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</table>
Chapter 1

Introductory Considerations
1.1 *Herpesviridae*

*Herpesviridae* is the name assignment to a family of enveloped, double-stranded DNA viruses with relatively large complex genomes. 'Herpes' means 'to creep' and reflects how herpesviruses infections obtrusively spread from infected cells to adjacent healthy cells. The viruses replicate in the nucleus of a wide range of vertebrate hosts. Such viruses have been isolated from humans, horses, cattle, mice, pigs, chickens, turtles, lizards, fish, and even in some invertebrates, such as oysters (Roizmann *et al.*, 1992).

1.1.1 Virion structure

All herpesvirus virions have four structural elements:

- **Core**, which consists of a single linear molecule of dsDNA.
- **Capsid**, which surrounds the core, which is icosahedral, consists of 162 capsomeres, and is 100 nm in diameter.
- **Tegument**, which is situated between the capsid and envelope, and is amorphous, and consists of viral enzymes, some of which are needed to subvert the host cell's chemical processes to direct them towards virion production, some to defend against the host cell's immediate responses, and others for which the function is not yet understood.
- **Envelope**, the outer layer of the virion, which is composed of altered host membrane and comprises a dozen unique viral glycoproteins (Roizmann *et al.*, 1992).
1.1.2 Genome characteristics

Herpesvirus genomes range in length from 120 to 230 kbp and contain 60 to 120 genes. Because replication takes place inside the nucleus, herpesviruses can exploit both the host's transcription machinery and its DNA repair enzymes to support a large genome with complex arrays of genes. Herpesvirus genes may be characterized as either essential or dispensable for growth in cell culture. Essential genes regulate transcription and are needed to construct the virion. Dispensable genes for the most part function to enhance the cellular environment for virus production, to defend the virus from attack by host immune system and to promote cell-to-cell spread. Large numbers of dispensable genes are required for a productive \textit{in vivo} infection: it is only in the restricted environment of laboratory cell cultures that they are dispensable. All herpesvirus genomes contain lengthy terminal repeats, both direct and inverted (Roizmann \textit{et al.}, 1992; Roizmann and Pellett, 2001).

1.1.3 Biological properties

Four biological properties characterize members of the \textit{Herpesviridae} family.

1. Herpesviruses express a large number of enzymes involved in metabolism of nucleic acid (e.g. thymidine kinase), DNA synthesis (e.g. DNA helicase/primase) and processing of proteins (e.g. protein kinase).

2. Synthesis of viral genomes and assembly of capsids occurs in the nucleus.

3. Productive viral infection is accompanied by inevitable cell destruction.

4. Herpesviruses are able to establish and maintain a latent state in their host and reactivate following cellular stress. Latency involves stable maintenance of the viral genome in the nucleus with limited expression of a small subset of viral genes.
1.1.4 Strategies for success

The success of herpesvirus infections depends upon several strategies. The first is the fast, efficient way, which involves the virion invading the host cell, turning off host protein synthesis and releasing viral DNA into the nucleus, thereby permitting replication and virion production to start immediately. Another strategy is to thwart attacks from the host. Tactics include inhibiting splicing of mRNA, blocking presentation of antigenic peptides on the cell surface and blocking apoptosis (cell death) induced by viral gene expression. A third strategy is to hide their bare, circularized genome in the nucleus of lymphocytes, neurons and other host cells, and then to revert to productive infection months, even years later. These latent herpesvirus infections are often benign, but reactivation from that state can be devastating in newborns and in immuno-suppressed individuals.

1.1.5 Herpesviridae subfamilies

The members of the family Herpesviridae have been classified by the Herpesvirus Study Group of the International Committee on the Taxonomy of viruses (ICTV) into three subfamilies (i.e., the alphaherpesvirinae, the betaherpesvirinae, and the gammaherpesvirinae) based on biologic properties. The same study group classified a small number of herpesviruses into genera based on DNA sequence homology and similarities in genome sequence arrangement (Figure 1.1).

_α-herpesvirinae_: The members of this subfamily are classified on the basis of variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, and capacity to establish latent infections primarily but not exclusively in sensory ganglia. This subfamily contains
Figure 1.1 Classification of Herpesviridae (Reproduced from Moore et al., 1996b).
the herpes simplex viruses (HSV) 1 and 2, and varicella-zoster virus (VZV).

**β-herpesvirinae**: A characteristic of the members of this subfamily is the restricted host range. The reproductive cycle is long and the infection progresses slowly in culture. Infected cells frequently enlarge (cytomegalia). The virus can be maintained in latent form in secretory glands, lymphoreticular cells, kidney and other tissues. This family contains the genera cytomegalovirus (CMV), human herpes virus-6 (HHV-6) and human herpes virus-7 (HHV-7).

**γ-herpesvirinae**: *In vitro*, all members of this subfamily replicate in lymphoblastoid cells and some cause lytic infections in some types of epithelioid and fibroblastic cells. Viruses in this group are usually specifically tropic for either T or B lymphocytes. Latent virus is frequently demonstrated in lymphoid tissue. This subfamily contains Epstein-Barr virus (EBV) and Kaposi’s sarcoma-associated herpes virus (KSHV), also known as human herpes virus-8 (HHV-8).

### 1.1.6 Human herpes viruses

The rest of this chapter reviews key features of herpesviruses that are shed in the mouth, which particular emphasis on HHV-8 and CMV.

#### 1.1.6.1 Herpes simplex virus

1.1.6.1.1 Pathogenesis and epidemiology

HSV infects the skin, mucous membrane and neurones of the dorsal root ganglia, in which it maintains lifelong latent infection. The virus can reactivate and spread centrifugally down neuronal axons in spinal or trigeminal nerves. It may be shed
asymptomatically in saliva (for HSV-1 predominantly) or genital secretions (for HSV-2 predominantly), and potentially cause destruction of the skin, mucosa and, occasionally, the major organs (Dwyer and Cunningham, 2002).

Primary HSV-1 infection occurs when a susceptible person, usually a child, comes into close contact with a person with primary or recurrent infection. Primary infection in children is often asymptomatic, but can cause stomatitis severe enough to require hospitalisation. Symptoms are more common in adolescents and adults. Most HSV-1 seroconversions occur in the first five years of life, and by adulthood, 80% of individuals possess HSV antibodies. However, as the HSV-1 seroprevalence in children is falling in many developed countries, the risk of primary HSV-1 infection in adults is increasing. HSV-1 may also cause genital herpes; the increasing prevalence of this infection may be due to increasing practice of orogenital contact.

HSV-2 infection is usually acquired sexually from early adulthood, often in people with pre-existing HSV-1 infection. Accordingly, clinical infection with HSV-2 may be considered an initial rather than a primary infection (the latter occurs when an adult acquires HSV-2 in the absence of HSV-1 antibodies). HSV-2 usually causes genital herpes, but is also a rare cause of herpes labialis. The major influence on HSV-2 acquisition is the number of lifetime sexual partners, with women generally infected at an earlier age than men. Most transmissions occur via asymptomatic viral shedding in genital secretions (Johnson et al., 1989). Both HSV-1 and HSV-2 infections recur often, with genital infections more likely to recur if caused by HSV-2 than HSV-1.
1.1.6.1.2 Clinical features

1.1.6.1.2.1 Orofacial infection

In primary HSV infection of the oropharynx, the most common manifestation is gingivostomatitis. Shallow ulcers form at the buccal mucosa and under the tongue, and may also occur at the hard palate (this feature differentiates HSV infection from herpangina caused by coxsackie virus). These ulcers may be accompanied by fever and submandibular lymphadenopathy. Autoinoculation from the primary infection may transmit infection elsewhere on the body (e.g., herpetic whitlow) (Stanberry et al., 2000).

Recurrent orolabial infections ("cold sores") may be triggered by stimuli such as fever, stress, cold, menstruation and ultraviolet radiation. Lesions usually occur on the vermilion border of the lips but may develop elsewhere on the face, including inside the nose. There is often a prodromal tingling or itching at the site of recurrence. Asymptomatic oral shedding of HSV is common and can transmit the virus. Lesions may be widespread in people with eczema and severe in those who are immunocompromised (Stanberry et al., 2000).

1.1.6.1.2.2 Genital infection

Symptomatic primary genital infection is moderately severe (more so in women) and lasts up to three weeks. Common signs include fever, dysuria, widespread ulceration, inguinal lymphadenopathy, malaise and pain. It may be accompanied by radiculomyelitis, with urinary retention and neuralgia, and secondary bacterial infection. Perianal infections and proctitis may occur, especially in homosexual men. Genital infection often leads to significant emotional and psychosexual disturbance.
About 20% of HSV-2-seropositive patients experience overt recurrences of genital herpes; lesions are more limited in area and severity than primary infection. About 60% of HSV-2 seropositive patients have lesions but may not recognise their herpetic nature, especially if they are small or atypical. The remaining 20% have true asymptomatic viral shedding. Genital HSV-2 recurrences usually last longer than oral HSV-1 recurrences and are more frequent in the six to 12 m after initial infection (Johnson et al., 1989; Koutsky et al., 1992).

1.1.6.1.2.3 Neonatal infection

This varies in prevalence around the world, from 1 in 2500 live births in the United States to 1 in 13 000 births in Australia, reflecting the wide variation in HSV-2 seropositivity in different regions and socioeconomic groups (Mindel et al., 2000). Most neonatal HSV infection occurs peripartum through an infected birth canal, although rarely it may occur in the postpartum period from direct contact or even from congenital infection. Although most cases (about 70%) are due to HSV-2, more cases of HSV-1-associated neonatal disease are being observed in the course of the current rise in HSV-1 genital infections. Primary maternal genital herpes in the last trimester, particularly around the time of labour, leads to infection in about a third of babies. Neonatal HSV may also follow symptomatic or asymptomatic recurrences; most babies with neonatal herpes are born to mothers with asymptomatic viral shedding. The clinical picture ranges from disease localised to the skin, eyes and mouth to encephalitis and disseminated disease. Neonatal herpes may recur, necessitating long-term antiviral therapy (Brown et al., 1997).
1.1.6.1.2.4 Other infections

HSV-1 keratitis may present with conjunctivitis or a unilateral dendritic (branching) ulcer. If recurrences are frequent and severe, they may involve deeper corneal layers, leading to interstitial or disciform keratitis and possible loss of vision. Adult herpes encephalitis is a severe focal encephalitis caused by direct viral invasion of the brain (usually by HSV-1), typically in the frontotemporal and parietal areas. It occurs in all age groups but is uncommon — about 1 in 10^6 population per year. Immunocompromised people are at risk of severe HSV infection, including progressive and extensive oral and genital herpes and disseminated herpes (Siegal et al., 1981; Whitley et al., 1989).

1.1.6.2 Varicella–zoster virus

1.1.6.2.1 Pathogenesis and epidemiology

VZV is acquired through the respiratory route. From the upper respiratory tract, it disseminates to lymph nodes and then via lymphocytes back to the skin, resulting in the rash of chickenpox. Most people in developed countries are infected with VZV in childhood, with 90% seropositive by adulthood. Chickenpox is usually a mild disease in healthy children, but more severe in adults. It is often itchy and heralded by posterior cervical lymphadenopathy and fever, after an incubation period of two weeks. The rash is centripetal, being concentrated on the body rather than the limbs, and the lesions evolve through papular, vesicular and crusting stages (Dwyer and Cunningham, 2002).

After primary infection, VZV becomes latent in dorsal root or cranial nerve ganglia. In 0.3% to 0.5% of the population, the virus reactivates causing herpes zoster
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(Shingles). The dermatomes most affected are C-3, T-5, L-1 and L-2 (Greenberg, 1996). Prodromal neurological symptoms of herpes zoster relate to pain rather than the typical paraesthesiae observed in recurrent herpes simplex. The rash of zoster is often intensely pruritic and spreads throughout the course of affected nerve, evolving through papular, vesicular and crusting stages and usually presenting unilaterally. It lasts two to four weeks. The most troubling symptom is pain, which may be self-limited or persist beyond the rash for up to a year ("postherpetic neuralgia"). Another relatively common complication is zoster ophthalmicus (2%–4%), which follows involvement of the first division (ophthalmic) of the trigeminal nerve. Infection ranges from keratitis to the more severe iritis. About 1% of immunocompromised people with herpes zoster develop severe complications involving the eye, brain or liver (Dworkin et al., 1997; Cohen et al., 1999). The oral lesions of primary infection are of minor significance compared to cutaneous manifestations. Oral lesions of zoster are pathognomonic and characterized by a prodrome, followed by a unilateral vesicular eruption that soon ulcerates. Though oral complications are rare, postherpetic neuralgia, tooth exfoliation and mandibular necrosis have been reported (Manz et al., 1986).

1.1.6.3 Epstein-Barr virus

1.1.6.3.1 Pathogenesis and epidemiology

The discovery of EBV was reported in 1964 by Epstein and co-workers (Epstein et al., 1964). More than 90% of adults worldwide are infected with EBV and carry the virus as a life-long persistent infection, with latent infection of B lymphocytes. The virus is spread by infected saliva and occasionally by blood transfusion. However, there are some reports of EBV detection in genital secretions, suggesting the
possibility of sexual transmission. Transmission from a transplanted organ can also occur with subsequent infection of a previously seronegative recipient (Macsween and Crawford, 2003).

It is now known that there are two types of EBV (1 and 2, or A and B) circulating in the community, which show variation in DNA sequence of the latent genes. They show no specific disease association. Type 1 is more prevalent in the West, whereas types 1 and 2 are equally prevalent in Africa.

Primary infection in childhood is often subclinical or mild, whereas in adolescents or young adults primary EBV infection causes infectious mononucleosis (IM), also known as kissing disease because of its association with saliva exchange. IM is typified by fever, pharyngitis, lymphadenopathy and splenomegaly. Rashes, including macular erythema, petechiae and urticaria may occur, and concurrent administration of ampicillin results in a rash (Cohen, 2000).

EBV has been implicated in a wide variety of other diseases such as Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, non-Hodgkin's lymphoma and T-cell lymphomas (Cohen, 2000; Macsween and Crawford, 2003). An important oral lesion caused by EBV is hairy leukoplakia, which occurs in HIV-infected individuals as well as in some immunocompromised patients. It is present as a white corrugated lesion involving the lateral borders of the tongue, although involvement of other part of oral mucosa has also been described. It is characterised by hyperkeratosis, acanthosis, koilocytosis and lack of an inflammatory response. EBV
DNA is abundantly present in the upper, keratinised epithelial cells of the lesions. (Triantos et al., 1997).

1.1.6.4 Cytomegalovirus

This will be discussed at length in a separate section, Section 1.3.

1.1.6.5 Human herpes virus-6

The discovery of HHV-6 was first reported in 1986 (Salahuddin et al., 1986). It is tropic mainly for CD4+ T cells; however, replication can also occur in CD8+ cells, macrophages, and epithelial cells. CD46 has recently been identified to be a cellular receptor for HHV-6 (De Araujo et al., 2002). The virus is ubiquitous in the population, with greater than 90% seropositivity in adults, and has a worldwide distribution. Infection with HHV-6, transmitted by saliva, usually occurs during the first 2 years of life (Stoeckle, 2000). Immunocompromised patients, especially those who have undergone solid transplant, are at greater risk of HHV-6 disease (Emery, 2001). Two distinct variants of HHV-6 have been characterized, designated A and B. The virus is the cause of exanthema subitum which is a common self-limiting febrile disease of infants. It may also lead to mononucleosis, pneumonia, meningitis and encephalitis. There is also evidence suggesting that HHV-6 is a cofactor in HIV infection in its speeding of the progress of immunosuppression (De Araujo et al., 2002).

1.1.6.6 Human herpes virus-7

The isolation of HHV-7 was first reported in 1990 (Frenkel et al., 1990) from CD4+ cells of healthy adults. DNA analyses have shown that HHV-7 is related closely to
HHV-6. HHV-7 shares with HHV-6 similar cell tropisms, disease associations and antigenic epitopes. Infectious HHV-7 has been isolated from the saliva of as many as 90% of healthy individuals in different parts of the world, and saliva is also the most likely vehicle of transmission. Primary infection usually occurs during childhood but later than that caused by HHV-6, usually in children of 2 y, with prevalence greater than 90% by the ages of 6 to 10 y. There is no clear association between HHV-7 and a specific clinical disorder, although it has been associated with febrile illness in children, exanthema subitum and pityriasis rosea (De Araujo et al., 2002).

1.1.6.7. Human herpes virus-8

The virus will be discussed in a separate section below.

1.2 Human herpes virus 8

Kaposi’s sarcoma (KS) is a vascular tumour that was brought to the attention of the medical community over a century ago in 1872 by the report of purple-coloured nodular skin lesions in five elderly men by Moritz Kaposi. Four epidemiological forms of KS based on clinical and epidemiological differences are now recognised: classic KS, endemic-HIV-negative KS found in Africa, iatrogenic KS, and HIV-associated or epidemic KS. In 1994 Chang and co-workers reported the identification of fragments of herpesvirus-like DNA in AIDS-KS biopsy samples. HHV-8 is now accepted as the etiological agent for all the various forms of KS (Chang et al., 1994).

1.2.1 Epidemiological forms of KS

Figure 1.2 illustrates clinical aspects of the 4 forms of KS. Classic KS was the form described by Kaposi. It occurs predominantly in elderly patients of Mediterranean,
Figure 1.2 Different forms of KS. Upper left: classic KS; brownish, livid, confluent plaques and papules on the forefoot and toes. Lower left: immunosuppression KS; multiple livid papules and nodules on the dorsum of the foot; note the swollen ankle. Upper right: AIDS-KS; red-purple macules and papules on the medial aspect of the sole and foot. Lower right: endemic African KS; multiple confluent nodules and plaques on the thigh with prominent oedema of the lower thigh and ankle; some tumours are ulcerated (Reproduced from Hengge et al., 2002a)
East European, Arabic or Jewish ancestry (Franceschi and Geddes, 1995; Antman and Chang, 2000). The incidence peaks after the 6th decade of life (DiGiovanna and Safai, 1981). It affects the extremities, is generally indolent and is more common in men than women (sex ratio estimated to be from 3:1 to 10:1) (Franceschi and Geddes, 1995). Classic KS runs a chronic course and rarely metastasises. Patients survive an average of 10-15 y before dying of unrelated causes. Complications include lymphoedema, hyperkeratosis and other neoplasms such as non-Hodgkin's lymphoma and cutaneous malignant melanoma (Iscovich et al., 1999).

KS was already present in equatorial Africa for many decades preceding the HIV epidemic (Oettle, 1962): a 1971 report, e.g., showed that KS accounted for 3% to 9% of all reported cancers in Uganda (Taylor et al., 1971). A study published in 1993 showed that KS in HIV-negative and HIV-positive patients accounted for approximately 50% of all tumours diagnosed in men in some countries in central Africa (Wabinga et al., 1993). African or endemic KS is usually more aggressive than the classic form of KS (D'Oliveira and Torres, 1972). It presents as benign nodular cutaneous disease predominantly in young adults (mean age 35 y) or as a florid mucocutaneous oral visceral disease. In young children, it can be aggressive, with localised cutaneous disease progressing to invade adjacent soft tissues and bones or rapidly disseminating to lymph nodes and visceral organs, usually in the absence of cutaneous lesions. In the absence of HIV infection an underlying immunodeficiency is generally not found (Kestens et al., 1985).

The third form of KS has been described in iatrogenically immunosuppressed organ transplant recipients and in wide spectrum of patients receiving chronic
immunosuppressive therapy (Qunibi et al., 1988; Farge, 1993; Szende et al., 1997). It occurs more frequently in some ethnic groups in whom endemic or classic KS occurs (Qunibi et al., 1988; Franceschi and Serraino, 1995). This variant of KS may also be aggressive, involving lymph nodes, the mucosa and the viscera but remission can occur spontaneously after discontinuation of immunosuppressive therapy (Brooks, 1986).

AIDS-KS or the epidemic is the fourth variant of KS. It was observed about 20 y ago in western homosexual men with AIDS, and was the most common neoplasm occurring in patients with AIDS before highly active anti-retroviral therapy (HAART) became available (Beral et al., 1990; Rabkin et al., 1995). The overall risk of KS in AIDS patients is estimated to be more than 20 000 times greater than that of the general population and 300 times that of other immunosuppressed patients (Beral et al., 1990). Striking differences in risks of acquiring AIDS-KS were observed between different HIV transmission groups, with the risk in homosexual men being more than men with haemophilia and the risk in women acquiring HIV from bisexual men being higher than heterosexual intravenous drug users (Haverkos and Drotman, 1985; Beral et al., 1990). This variant is typically lymphoadenopathic and tends to involve the viscera and mucosa as well as the skin (Figure 1.3). It is commonly multifocal and symmetrical at presentation. Before HAART became available oral KS was among the first clinical manifestations of AIDS (Silverman S Jr et al., 1986). The KS lesion may appear as a red-purple macule, an ulcer, or as a nodule or mass. Intraoral KS occurs on the heavily keratinized mucosa, the palate being the site of predilection in more than 90% of reported cases; other affected sites include the gingiva, tongue and the buccal mucosa (Silverman et al., 1986).
Figure 1.3 Manifestations of AIDS-KS. Upper left: multiple livid, irregular papules and plaques. Note the prominent periorbital and perinasal oedema. Upper right: disseminated purple-black nodules and tumours on the chest in Christmas tree-like distribution along the lines of Blaschko. Lower left: mucous membrane involvement with reddish tumours and livid plaques on the upper gum. Lower right: violaceous well demarcated plaque with prominent borders on the tip of the glans penis (Reproduced from Hengge et al., 2002a).
1.2.2 Histogenesis of KS

The 4 clinico-epidemiological forms of KS have indistinguishable histological features. KS is a complex multifocal vascular lesion at the early stage, “the patch stage”; the histological picture is that of endothelial-lined spaces surrounding normal blood vessels; there is often a variable degree of inflammatory infiltrate. The “plaque” stage is characterised by an increased growth of spindle-shaped vascular processes in the dermis, along with the formation of slit-like vascular spaces filled with erythrocytes. In the late, “nodular” stage, the lesion is comprised primarily of spindle shaped cells arranged in large sheets, some of which are undergoing mitosis, with slit-like vascular spaces and haemosiderin pigmentation (Cockerell, 1991). Figure 1.4 shows a schematic diagram of the histogenesis of KS and its surrounded microenvironment.

The spindle cells are thought to be the tumour cells that form the bulk of established KS but their histogenesis is unknown. The majority express lymphatic endothelial cell markers, including CD31 and CD34 markers. However, some express markers for smooth muscle cells, macrophages and dendritic cells (Nickoloff and Griffiths, 1989; Sturzl et al., 1992) suggesting that spindle cells represent a heterogeneous population of cells, or cells that arise from a pluripotent mesenchymal precursor cell. All KS spindle cells express vascular endothelial growth factor receptor VEGFR-3, which is usually expressed only by lymphatic endothelium and by neoangiogenic vessels, but not by mature vascular endothelial cells, indicating that KS spindle cells may be of the endothelial lineage that can differentiate into lymphatic cells (Dupin et al., 1999).
Figure 1.4 Schematic of KS pathogenesis. Some HHV8–infected endothelial cells together with HIV–infected monocytes and lymphocytes (marked with a "+"+) will eventually lead to development of atypical endothelial cells that may be transformed when exposed to a particular microenvironment resulting in a polyclonal cell population with spindle-shaped morphology. These spindle cells bearing a number of growth factor receptors will contribute to the formation of slit-like vessels with a discontinuous endothelial lining. During the course of the disease a few (or a single) predominant clones may grow out leading to an oligo or mono-clonal cell population in advanced KS stages (Reproduced from Hengge et al., 2002b)
KS, like most tumours, produces and responds to cytokines. Spindle cells or infiltrating CD8+ lymphocytes and macrophages express high levels of interleukin 6 (IL-6), basic fibroblast growth factor (bFGF), gamma interferon, and a variety of other cytokines (Ensoli et al., 1989; Miles et al., 1990; Sirianni et al., 1998; Fiorelli et al., 1998; Dupin et al., 1999).

AIDS-KS cell lines have been shown to secrete large amounts of biologically active IL-6, which can promote spindle cell proliferation (Miles et al., 1990; Dupin et al., 1999). Gamma-interferon can promote the formation of KS spindle cells with an angiogenic phenotype and transform endothelial cells to resemble spindle cells.

bFGF is an angiogenic factor, highly expressed in early lesions that promotes spindle cell growth (Fiorelli et al., 1998). Gamma-interferon can induce KS-like tumour formation in nude mice and these tumours in turn produce bFGF (Ensoli et al., 1989).

HIV-1-associated KS exhibits a more aggressive clinical course than the other types of KS. Therefore, it has been proposed that HIV-1 gene products may positively influence KS development (Ensoli et al., 1994). The tat gene in HIV-1 codes for an early trans-activator protein (Tat) essential for HIV-1 viral replication (Fisher et al., 1986). It is released into the extracellular fluid during acute HIV infection, can promote spindle cell growth in vitro (Ensoli et al., 1993) and induces an angiogenic phenotype in nude mice (Vogel et al., 1988). Normal endothelial cells display a spindle cell-like morphology when exposed to Tat in the presence of other cytokines (e.g. IL-1, tumor necrosis factor, and gamma interferon) in culture. Indeed, it has
been proposed that these cytokines, whose production is up-regulated in persons with HIV-1 infection, work synergistically with Tat in KS development (Barillari et al., 1992). Recent studies have indicated that Tat may influence the specific tissue distribution of KS (nose, oral and genital mucosa) observed in HIV-associated KS (Prakash et al., 2000).

1.2.3 The discovery of HHV-8

A viral aetiology for KS was suspected long before the onset of the AIDS epidemic (Oettle, 1962). During the 1970s herpesvirus-like particles were found by electron microscopy in tumour cells which were thought to be CMV (Giraldo et al., 1972). Subsequent studies identified, in lesional tissues, the DNA of CMV, HHV-6, human papilloma viruses (HPVs) and other viral or bacterial pathogens, all put forward as candidate aetiologic agents. However, a minority of KS lesions was found to carry DNA of the implicated viruses (Huang et al., 1992; Kempf et al., 1995; Monini et al., 1996b).

Although no etiologic agent was identifiable, epidemiologic studies in the late 1980s suggested that a sexually transmitted agent was the cause of KS and was being transmitted independently of HIV. The suspected agent was presumed to be more efficiently transmitted through homosexual than heterosexual contact, resulting in a much higher incidence rate among gay male AIDS patients than other AIDS risk groups. The agent was also likely to be poorly transmissible through blood products since KS prevalence rates among HIV-infected transfusion recipients and haemophiliacs were relatively low (Beral et al., 1990; Beral, 1991; Beral et al., 1992).
Given that there may be a separate KS agent, it was reasoned that the genomic difference between proposed infected (KS) and uninfected (skin) tissues from an individual patient with AIDS-KS should be that of the infectious agent. A subtractive hybridization technique, representational difference analysis (RDA), based on polymerase chain reaction (PCR) (Lisitsyn et al., 1993) enrichment of unique DNA fragments was employed by Chang and colleagues (Chang et al., 1994). With the use of genomic DNA from an AIDS-KS lesion that was compared to unaffected DNA from the patient, two unique DNA fragments were isolated. These two DNA fragments (KS330Bam and KS631Bam), which showed high homology to known herpesvirus sequences, were used as Southern hybridization probes and for the generation of PCR primers. Both segments were homologous to two members of the \( \gamma \)-herpesvirus sub-family. KS330Bam was 51% homologous to opening reading frame (ORF) 26 of *Herpesvirus saimiri* (HSV) and 39% homologous to ORF BDLF1 of EBV. KS631Bam was homologous to a tegument protein coded by ORF 75 of HSV and ORF BNRF1 of EBV. The unique sequences were found in 90% of KS lesions from AIDS patients. Subsequent studies showed evidence that the vast majority of KS lesions from patients with AIDS and from other groups at risk of KS carry DNA of this novel virus (Ambroziak et al., 1995; Boshoff et al., 1995; Schalling et al., 1995; Chang and Moore, 1996).

### 1.2.4 Establishing the causal role of HHV-8 in KS

Four observations linked HHV-8 causally to the aetiopathogenesis of KS:

i) HHV-8 DNA is present, by PCR in all epidemiological forms of KS, in all fresh biopsies tested and in the vast majority of paraffin-embedded material (Ambroziak et al., 1995; Boshoff et al., 1995; Schalling et al., 1995; Moore and Chang, 1995;
Chang and Moore, 1996). The virus is found in HIV-positive and HIV-negative patients with KS (Ambroziak et al., 1995). To strengthen the molecular epidemiological association between HHV-8 and KS further, it was demonstrated by PCR in situ hybridization, RNA in situ hybridization and immunohistochemistry that HHV-8 is present in spindle cells in nearly all KS lesions (Boshoff et al., 1995; Li et al., 1996; Staskus et al., 1997; Rainbow et al., 1997; Davis et al., 1997; Sturzl et al., 1997; Dupin et al., 1999; Kellam et al., 1999).

ii) HHV-8 sequence may be detected by PCR in the peripheral blood of HIV-positive individuals before the onset of KS lesions (Whitby et al., 1995; Lefrere et al., 1996; Moore et al., 1996c), indicating that those at risk of KS have a higher viral load than those not at risk.

iii) Seroprevalence studies indicated that in general populations at risk of developing KS, there was a higher prevalence of HHV-8 infection (to be discussed in more detail later). The incidence of developing KS correlated with the prevalence of HHV-8 infection in a given population.

iv) Like most other human herpes viruses, HHV-8 may be in a latent stage of infection early in the course of the clinical disease (Reitz et al., 1999), infecting a small proportion of spindle cells, but, as KS advances, HHV-8 can be detected in nearly all tumour spindle cells (Dupin et al., 1999; Sturzl et al., 1999).
1.2.5 Other diseases associated with HHV-8

1.2.5.1 Primary effusion lymphoma

Primary effusion lymphomas (PELs), also known as body cavity-based lymphomas, are a type of high grade non-Hodgkin's lymphoma that develop nearly exclusively in the pericardial, peritoneal and pleural cavities as lymphomatous effusion, usually in the absence of any identifiable tumour mass. Most cases occur in HIV-infected individuals (Cesarman and Knowles, 1997), but a small number of patients with PEL who are HIV-seronegative have been identified (Klepfish et al., 2001). Cesarman and co-workers reported that HHV-8 was specifically associated with PEL, and they noted that PEL contains high viral copies when grown in vivo, rendering them useful for the characterisation of individual strains of the virus (Cesarman et al., 1995).

Most tumour cells are immunoblast-like cells, but some large anaplastic lymphocytes multilobulated nuclei may also be present (Cannon and Cesarman, 2000). Phenotyping may be difficult because the cells characteristically lack the lineage-restricted markers of both T and B lymphocytes. Clonal immunoglobulin gene rearrangement found in all cases studied has firmly established PEL as a B-cell neoplasm (Cesarman and Knowles, 1999). Co-infection of PEL cells by both EBV and HHV-8 has been observed, but association with HHV-8 alone has also been reported. Notably, most of PEL in HIV-seropositive patients are EBV-positive. (Said et al., 1996; Cesarman et al., 1996). PEL lacks rearrangements of the c-myc gene, which in the presence of EBV may play a role in development of Burkitt's lymphoma. Thus it is possible that HHV-8 acts in conjunction with EBV to induce full transformation (Miller, 1990; Cesarman et al., 1995). Thus far, no effective therapy has been established for HIV-associated PEL. Consequently, the prognosis is
poor, and the reported median survival is about 18 weeks. Of interest are reports of clinical improvement after HAART (Oksenhendler et al., 1998).

1.2.5.2 Multicentric Castleman's disease
Castleman’s disease (angiofollicular lymphoid hyperplasia or giant lymph node hyperplasia) is a rare, nonmalignant, usually polyclonal form of lymphadenopathy (Frizzera, 1992). Castleman’s disease is classified histologically into the hyaline-vascular type and the plasma cell type. Clinically, it may be classified into two forms: (i) localized Castleman’s disease, which is usually of hyaline-vascular histologic type, is located in the mediastinum in 70% of affected patients, and can usually be cured by surgical excision; or (ii) a systemic variant, multicentric Castleman disease (MCD), usually of plasma cell type (Frizzera, 1992). MCD adopts an aggressive, often fatal clinical course and usually presents with multifocal lymphadenopathy and a variety of systemic symptoms, such as fever, rash, cytopenia, and hypergammaglobulinemia. This disease is common among AIDS patients: HHV-8 DNA sequences could be found in all HIV-related cases of MCD in contrast to less than half in HIV-seronegative cases. (Soulier et al., 1995). Moreover, HHV-8 viral load in the peripheral blood of MCD patients tends to correlate with the increased severity of symptoms, worse prognosis (Grandadam et al., 1997) and exacerbations (Oksenhendler et al., 2000). The clinical course of HHV-8-positive MCD tends to be worse, particularly among HIV-seropositive individuals, and is not always improved by therapy with HAART (Zietz et al., 1999; Dupin et al., 2000). HHV-8 DNA has been localised in plasmablasts of B cell lineage in the mantle zone of the B-cell follicles (Kellam et al., 1999; Katano et al., 2000). The role of HHV-8 pathogenesis in MCD is not clear. However, viral IL-6 (vIL-6) is highly expressed in
a subset of plasmablasts (Dupin et al., 1999) and may drive HHV-8-infected naïve B cells to become plasmablasts and to develop various lymphoproliferative lesions. In addition, patients with MCD may develop other HHV-8-associated diseases: PEL (Ascoli et al., 2001), KS (Soulier et al., 1995), or both (Codish et al., 2000).

1.2.5.3 Other disorders

HHV-8 infection has been proposed as an aetiological agent in a number of diseases including multiple myeloma (Rettig et al., 1997), some reactive lymphadenopathies (Luppi et al., 1996), pemphigus vulgaris (Memar et al., 1997), a variety of skin carcinomas and lymphomas, and sarcoidosis (Di Alberti et al., 1997). Despite these associations, no conclusive evidence has been found to strongly link any of these diseases to infection with HHV-8.

1.2.6 HHV-8 taxonomy

Shortly following the discovery of HHV-8, the entire HHV-8 genome of 165 kilobases was reported (Russo et al., 1996; Moore et al., 1996b). The virus was assigned the trivial name, Kaposi’s sarcoma-associated herpesvirus and the proper name, human herpesvirus-8, now used interchangeably in the literature. The γ-herpesviruses are known to infect lymphocytes (Roizmann et al., 1992) and are often implicated in the development of lymphomas (Neipel et al., 1998). The γ-herpesvirus subfamily is further divided into two genera, rhadinovirus (γ-2) and lymphocryptovirus (γ-1). EBV is a γ-1 herpesvirus and is the prototypic member of this group.
When HHV-8 was discovered, it was thought to be very closely to herpes virus saimiri (HVS) of New World monkeys, the prototype $\gamma$-2 related herpes viruses (rhadinovirus) that infects squirrel monkeys (Albrecht et al., 1992). However, it has recently become apparent that rhadinoviruses are more closely related to HHV-8 of Old World monkeys, including several macaque species and African green monkeys (Rose et al., 1997; Greensill et al., 2000). Extensive studies have been done to find evidence of herpesvirus infection in primates and as a result, many species of Old World primates are being revealed to be infected with $\gamma$-2 herpesviruses. Old World primate rhadinoviruses can be divided into two distinct lineages: RV1 and RV2. HHV-8 belongs to the RV1 lineage, and is closely associated phylogenetically to primate rhadinoviruses. Other viruses belonging to RV1 are retroperitoneal fibromatosis herpesvirus (RFHVMm) from rhesus macaque (Macaca mulatto), RFHVMn from pig-tailed macaque (Macaca nemestrina) (Rose et al., 1997); ChRV1 from the African green monkey (Chlorocebus aethio) (Greensill et al., 2000), pan rhadino-herpesviruses (PanRHV1a and b) from the chimpanzee (Pan troglodytes) and GorRHV1 from the gorilla (Gorilla gorilla) (Lacoste et al., 2001).

RV2 viruses include rhesus rhadinovirus (RRV) from the rhesus macaque (Macaca mulatta) (Searles et al., 1999), MneRV2 from the pig-tailed macaque (Macaca nemestrina) (Schulz, 2000), ChRV2 from the African green monkey (Chlorocebus aethiops) (Greensill et al., 2000), and MndRHV2 from the mandrill/drill (Mandrillus sphinx Mandrillus leucophaeus) (Lacoste et al., 2000). The presence of RV1- and RV2-type rhadinoviruses in many Old World primates points to possibly yet undiscovered rhadinoviruses of the RV2 lineage capable of infecting humans (Lacoste et al., 2001).
Chapter 1 Introductory Considerations

1.2.6.1 The HHV-8 genome

Complete genome sequences of two HHV-8 isolates have now been determined, one from a PEL cell line (Russo et al., 1996) and the other from a KS specimen (Neipel et al., 1997), both revealing the characteristic co-linear genomic organization of the *rhadinoviruses* (Figure 1.5). HHV-8 genome is 140.5 kb in length and is of low GC content (53.5%). It comprises a segment called L-DNA or long unique region (LUR) that is flanked by numerous 801-base pair terminal repeat (TR) regions of high GC content (84.5%) called H-DNA. The L DNA region of HHV-8 genome has at least 81 ORFs and five internal repeat regions (Russo et al., 1996; Neipel et al., 1997). HHV-8 ORFs are named according to their homology with HVS ORFs. Unique regions are numbered consecutively and given K as a prefix (i.e. K1 to K15) with newly discovered unique ORFs designated by the addition of a decimal point (i.e. K4.1) (Russo et al., 1996; Schulz, 1998).

As with other γ-herpesviruses, essential ORFs that code for viral replication and assembly proteins are arranged in blocks and are referred to as the conserved genes. There are approximately 67 such genes conserved among members of herpesvirus subfamilies. In HHV-8, these include: ORF 25, coding for the major structural protein; ORF 9, for DNA polymerase; and ORF 17, for a proteinase and assembly protein (Moore et al., 1996b). Non-conserved gene blocks in the HHV-8 genome lie between the conserved blocks and contain some ORFs that are found in other *rhadinoviruses* including homologues to mammalian proteins. A characteristic of HHV-8 and other γ-herpesvirus is the similarity of a large number of the ORFs to known cellular genes, suggesting that some of these genes may have been pirated from the host chromosomes during the process of virus/host co-evolution.
Figure 1.5 The HHV-8 genome. The genome consists of a long unique region (140.5 kb) encoding for over 80 open reading frames (ORFs), surrounded by terminal repeat regions (TRs) consisting of 801 base pair direct repeat units with a high G+C content. The ORFs are named after the corresponding genes in herpesvirus saimiri, and genes without significant homology are given the designation 'K'. Three large regions (I, II and III) contain genes conserved among the Rhadinoviruses, whereas the regions between them contain unique genes. Many of these unique genes encode homologues for host cellular proteins (shown in red with dotted outlines; genes without known homologues are shown in yellow with dotted outlines). Genes that are potentially important in the pathogenesis of KS are labelled.

Abbreviations: CCP, complement control protein; v-cyc, viral D-type cyclin; vFLIP, viral FLICE inhibitory protein; vGPCR, viral G-protein-coupled receptor; vIL-6, viral interleukin 6; viRF, viral interferon regulatory factor; kb, kilobase; LANA, latency-associated nuclear antigen; vMIP, viral macrophage inflammatory protein (Reproduced from Neipel et al. 1999).
Some of these genes are involved in down-modulating the immune response, evading cellular systems important for targeting infected cells, nucleotide biosynthesis and for cell growth and differentiation. They include the vBcl-2, vIL-8R, and vMIP-IK, vIL-6, and the D type viral cyclin, whose functions are usually similar to that of their cellular homologues (Russo et al., 1996; Neipel et al., 1998).

1.2.6.2. HHV-8 genotyping

The HHV-8 genome is well conserved, with most coding regions in isolates obtained from PEL cell lines or in sub-genomes characterised from KS biopsies showing only 0.1% nucleotide variation between the sequences at central genomic region (Russo et al., 1996; Neipel et al., 1997). Initial studies conducted on the genetic variability and polymorphism of HHV-8 focused on two small gene fragments of the ORF 26 (minor capsid gene) and ORF 75 (tegument region) from KS specimens. They showed the existence of three groups (A, B and C), with a very low overall nucleotide variability up to 2%, but less than 0.1% within each group (Zong et al., 1997; Kasolo et al., 1998; Fouchard et al., 2000). Type A was predominant in areas associated with classical KS, whereas the B and C strains were more prevalent in Africa. All the three strains were represented among American AIDS patients but the A type predominated (Zong et al., 1997; Luppi et al., 1997).

The left end of the genome where ORF K1 is located has a hot spot of genetic variability. ORF K1 encodes a transmembrane protein with structural similarity to the immunoglobulin receptor family of proteins and is expressed in the early lytic phase after induction by TPA in PEL cell lines (Lagunoff and Ganem, 1997; Lee et al., 1998). The transforming potential of the ORF K1 protein in vivo was reported by
Lee et al. (1998). While it has no homologues in other herpesviruses, there is an equivalent position in the HVS genome from which transforming proteins are encoded (Lee et al., 1998).

The K1 encodes 289 amino acids. The predicted protein comprises a single peptide in the N-terminal region (amino acids 1-19), a predicted hydrophobic transmembrane domain in the C-terminal region, a small intracellular domain (amino acids 227-276), and a highly variable extracellular domain in the central region (amino acids 20-226). In the central region, the position between amino acids 51-92 (variable region 1 or VR1) and between amino acids 191-231 (VR2) are the most heterogenous. Certain segments within and connecting these two blocks are conserved presumably to maintain the tertiary structure of the protein (Meng et al., 1999).

Meng et al. (1999) reported a K1 sequence-based system for HHV-8 strain differentiation and genotyping and have shown that HHV-8 falls into at least four distinguishable genotypes (I-IV). Genotype I may be further divided into four subtypes, A-F. Among 63 distinct ORF K1 proteins, Zong et al. (1999) also identified four main genotypes, A-D, which differed from each other by between 15% and 30% of their amino acids; they further observed 13 clades or subtypes based on amino acid differences of 5% or greater (i.e. A1...A5, C1 etc). A more recent comprehensive study of 139 samples, from which at least VR1 sequence data were available, has yielded 22 defined ORF K1 subtypes (Zong et al., 2002).

The B subtype is almost exclusively associated with patients in or from sub-Saharan Africa and the D subtype has been found only in patients of Pacific Island heritage,
whereas the A and C subtypes are distributed throughout the USA, Europe, Asia and the Middle East (Zong et al., 1999; Zong et al., 2002). Subtype E is hyperendemic in Amerindians in Brazil (Biggar et al., 2000) and Ecuador (Whitby et al., 2004). In addition, a novel subtype has been identified only in South Africa, classified as subtype N (Alagiozoglou et al., 2000). B subtype sequences are divergent in ORF K1 but show little phylogenetic clustering, thus allowing only three further subgroups to be distinguished (B1, B2 and B3) based on 4 amino acid changes in the VR2 regions (Zong et al., 2002) and a deletion in the B3 variant (Treurnicht et al., 2002). AIDS KS patients in the US are normally infected with subtypes A1, A4 and C3. The predominance of these few subtypes indicate reactivation of endogenous HHV-8 variants associated with the AIDS epidemic in USA and may have been introduced by immigrants from endemic Europe, the Middle East, and Africa, as well as horizontal transmission events occurring locally (Zong et al., 1999; Zong et al., 2002).

The wide geographical and ethnic distribution of ORF K1 subtypes suggests that the major subgroups originated in paleolithic times through isolation and founder effects, associated with the expansionary migrations of anatomically modern humans first into sub-Saharan Africa starting 100,000 years ago (B subgroup), then into South Asia, Australia and the Pacific beginning 60,000 years ago (D subgroup), and, finally, as two major branches into Europe and North Asia (both via the Middle East) about 35,000 years ago (A and C subgroups). These patterns also imply that (outside of the AIDS epidemic) the virus is primarily transmitted familially (e.g., parent to child and between siblings) and that recombination events associated with multiple infection are relatively rare (Zong et al., 1999; Hayward, 1999).
At least two major variants of HHV-8 can be distinguished using the K15 gene between the ORF75 and the terminal repeat, situated at the right-end of the genome. The two variants, named P (predominant or prototype) and M (minor) alleles, show only 30% amino acid sequence identity (Poole et al., 1999). Each allele comprises eight exons specifying a protein with 12 membrane-spanning domains and a C-terminal cytoplasmic domain. This structure resembles latent membrane protein 2 (LMP2) of EBV, except that LMP2 possesses an N-terminal, rather than a C-terminal, cytoplasmic domain (Sample et al., 1989). The K15 proteins possibly play a role in signal transduction (Hayward, 1999). The P allele is the more frequent among HHV-8 genomes characterized, and has been found in association with all five K1 subtypes (Poole et al., 1999). The rarer M allele has thus far been found in association with the A, B and C subtypes (Poole et al., 1999).

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

1.2.6.3 Gene expression

As with the other herpesviruses, HHV-8 exhibits both latent (nonproductive) and lytic (productive) replication, both of which are characterized by virtually distinct gene expression programs. HHV-8 establishes latency in the host (Gao et al., 1996a) and is under stringent latent replication control in PEL cell lines (Russo et al., 1996). While a minority of cells in culture may express lytic cycle proteins at any given time, chemical induction with tetradecanyl phorbol acetate (TPA) is needed for the full expression of lytic gene products in PEL cells in vitro. Three broad categories (classes) of gene expression have been described (Sarid et al., 1998), based on their expression outcomes after TPA induction: class I (which are constitutively expressed regardless of TPA treatment under standard growth condition); class II (which are expressed without TPA but are induced to higher transcription levels by TPA treatment and typically represents regulatory and viral DNA replicative genes, as well as the majority of the viral homologues of cellular genes); and class III (which are transcribed only following TPA treatment and encoded most of the viral structural and replication genes). Table 1.1 show latent, immediate early, early and late genes in HHV-8

1.2.6.3.1 Latent infection

HHV-8 becomes latent in the majority of cells it infects. During this stage of the virus life cycle the viral genome resides in the nucleus as closed circular episomal
### Table 1.1 Latent, immediate early, early and late genes of HHV-8.

<table>
<thead>
<tr>
<th>Latent genes</th>
<th>K10.5, K11.5, K12, K13, ORF72, ORF73</th>
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<tr>
<td>Immediate early genes</td>
<td>K3, ORF70, K4, K4.1, K4.2, ORF45, ORF50</td>
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<tr>
<td>Early genes</td>
<td>ORF6, ORF7, ORF8, ORF9, ORF10, ORF11, ORF16, ORF18 ORF21, K5, K6, ORF31, ORF34, ORF35, ORF36, ORF37 ORF38, ORF40, ORF41, ORF54, ORF56, ORF57, K9, K10 ORF58, ORF59, ORF60, ORF61, ORF62</td>
</tr>
<tr>
<td>Late genes</td>
<td>ORF4, K7, ORF17, ORF19, ORF20, ORF23, ORF24, ORF25 ORF26, ORF28, ORF32, ORF33, ORF39, ORF43, ORF44, K8 K8.1, ORF53, ORF64, ORF65, ORF66, ORF68, ORF69, ORF75</td>
</tr>
</tbody>
</table>
DNA expressing a small subset of viral genes. Potentially, this minimises the number of viral epitopes that are presented by infected cells to cytotoxic T lymphocytes (CTLs). It seems likely that the genes expressed during latency play a major role in the pathogenesis of HHV-8 (Renne et al., 1996a). These genes can be represented as transcripts of class I genes. The right hand cluster of three latent adjacent genes in genome are co-transcribed on two polycistronic mRNAs: latent transcript-1 (LT1) (transcribed by ORF71/ORF72/ORF73), and LT2 (by ORF 71/ORF72) (Dittmer et al., 1998; Talbot et al., 1999).

The major latent loci are as follows:

(i) ORF 71

ORF71 produces vFLIP, a homologue to cellular FLICE (FADD [Fas-associated death domain]-like interleukin-1 beta-converting enzyme) inhibitory proteins (FLIP), also called caspase-8. vFLIPs found in other herpesviruses are known to inhibit fas-mediated apoptosis by preventing the recruitment of caspase-8 (FLICE) to the death-induced signalling complex (Thome et al., 1997). vFLIP in HHV-8 has a similar function and can prevent apoptosis in latently infected cells (Djerbi et al., 1999). ORF71 utilises internal ribosome entry site (IRES)–mediated initiation to produce a functional protein and that ensures the protection of infected cell from CTL-induced cell killing throughout the cell cycle (Bieleski and Talbot, 2001).

(ii) ORF 72

The ORF 72-encoded vCyclin is the viral homolog to cellular cyclin, and is most closely related to cyclin D (Li et al., 1997). Cellular cyclin D binds with cyclin-dependent kinases (CDK) 4 and 6 (Radkov et al., 2000), and this complex...
phosphorylates retinoblastoma protein (pRb), releasing the transcription factor E2F. E2F, in turn, activates the transcription of S-phase genes, including cyclin E, which is needed for G1–S phase transition. vCyclin, like cellular cyclins, is also able to phosphorylate pRb in vitro in complexes with CDKs. The vCyclin-CDK complexes are insensitive to CDK inhibitors (p16INK4a, p21CIP1 and p27KIP1). Thus, exogenous expression of vCyclin from the infecting viral genome prevents CDK inhibitor-imposed G1 arrest, stimulates entry into S-phase, and substitutes for cyclinA/CDK2 to initiate DNA replication (Swanton et al., 1997).

(iii) ORF 73
Latent nuclear antigen-1 (LANA-1) is expressed by ORF 73 and is detected in the majority of KS lesions as well as in cell lines derived from PELs. LANA-1 plays a role in HHV-8 maintenance by tethering the viral episome through the terminal repeat to host chromosome ensuring persistence in the daughter cells after successful rounds of cell division (Ballestas et al., 1999; Cotter and Robertson, 1999). This interaction creates the characteristic pattern of intra-nuclear dots observed in immunofluorescence assays. In normal uninfected cells, G1/S progression is negatively regulated by binding of pRb to E2F transcription factor. In infected cells, LANA-1 competes with E2F for binding of pRb thus freeing E2F to activate the transcription of genes involved in cell cycle progression. LANA-1 also binds p53 and blocks p53-mediated apoptosis (Friborg et al., 1999).

(iv) ORFs K10.5, K11.5 and K12
K10.5, K11.5 and K12 are considered as latent genes but can be induced by TPA during lytic replication of the virus, and are therefore classified as class II transcripts.
(Sarid et al., 1998; Jenner et al., 2001). K10.5 expresses LANA-2 protein, which inhibits p53-induced transcription and apoptosis. LANA-2 is expressed latently only in cells and cell lines (i.e. PEL, PEL cell lines and MCD) of haematopoietic origin but not in KS tumours (Rivas et al., 2001).

The ORF K11.1 gene encoding vIRF-2 is latently expressed in PEL cell lines (Burysek et al., 1999). While IRFs have the ability to bind nucleotides, vIRF-2 can bind oligonucleotides corresponding to nuclear factor kappa B (NF-kappaB) (Burysek and Pitha, 2001). NF-kappa B is a transcriptional regulatory factor that in turn up regulates the expression of a wide variety of inflammatory cytokines and adhesion molecules (Pati et al., 2001). vIRF-2 is constitutively expressed in the nucleus of PEL cells and inhibits the effects of cellular IFN through its interaction with a double stranded RNA activated protein kinase (PKR) (Burysek and Pitha, 2001). PKR is normally induced by IFN and mediates the anti-viral and anti-proliferative effects of IFN (Hovanessian, 1989; Sen and Ransohoff, 1993). By down-regulating the antiviral response caused by PKR, vIRF-2 may be important for maintenance of viral latency (Burysek and Pitha, 2001). Kaposin A encoded by ORF K12 has been shown to induce tumorigenic transformation and is expressed in the PEL cell lines BCBL-1 and KS-1 (Muralidhar et al., 2000).

1.2.6.3.2 Lytic infection

The majority of the HHV-8 genome remains silent during latent infection. The programme of gene expression during lytic replication was revealed by DNA array expression profiling of PEL cell lines without (Jenner et al., 2001) and with stimulation by TPA (Paulose-Murphy et al., 2001). The lytic genes mainly express
class II and class III transcripts (Sarid et al., 1998). The first group of genes expressed after the induction of lytic replication are typically regulators of gene expression, including the immediate early transactivators. Homologues of cellular genes involved in regulation or signal transduction are mostly expressed after the viral transactivators followed by the expression of sets of genes, involved in replication of the viral DNA including the DNA polymerase and its processivity factor. The structural genes and those involved in virus expression and maturation are expressed later, generally after 24 h post-induction (Jenner et al., 2001).

Class II transcripts major lytic genes that are transactivators and homologues of cellular genes are as follows:

(i) ORFs 50, K8 and 57

The first groups of genes expressed after the induction of lytic replication are typically regulators of gene expression including the immediate early transactivators ORF 50 (Rta or Lyta), K8 (Zta or Kb-ZIP) and ORF 57 (post-transcriptional regulator of gene expression) (Jenner et al., 2001). The ORF 50/Rta promoter region is heavily methylated. Demethylation of the promoter by TPA induces lytic cycle replication; demethylation of the Rta promoter region is essential for expression of Rta (Chen et al., 2001). Studies have identified an ORF50 similar to EBV immediate early transactivator, which functions as an immediate early transactivator of HHV-8 lytic replication. It was demonstrated that its protein is capable of stimulating HHV-8 lytic genes through transcription cascades resulting in replication of the virus in vitro similar to the Zta and Rta proteins of EBV (Renne et al., 1996b).
(ii) vMIPs

HHV-8 encodes three secreted chemokines (chemoattractant cytokines) that have unique properties. Viral macrophage inflammatory proteins (vMIP-I, vMIP-II and vMIP-III) are encoded by ORFs K4, K4.1 and K6, respectively, located at the left end of LUR (Moore et al., 1996a). The three chemokines have been shown to induce angiogenesis, suggesting their roles in the pathogenesis of KS. vMIP-I is a specific agonist of CCR8 (Dairaghi et al., 1999; Endres et al., 1999), vMIP-II of CCR3 (Boshoff et al., 1997) and vMIP-III of CCR4 (Stine et al., 2000). All the three major receptors, CCR3, CCR4 and CCR8, are chemoattractant receptors for Th-2 lymphocytes and their recruitment, and inhibit the Th-1 immune response thus potentially protecting HHV-8-infected cells (Stine et al., 2000). vMIP-II acts as an antagonist for co-receptors CCR3, CCR5 and CXCR3 which allow HIV-1 to infect host cell, and can block its entry (Feng et al., 1996; Deng et al., 1996; Boshoff et al., 1997; Kledal et al., 1997).

(iii) vBcl-2

The vBcl-2 is encoded by ORF 16, and shares about 60% homology with human Bcl-2 family members such as Bcl-2 and Bax (Sarid et al., 1997). Bcl-2 is a potent cell death-suppresser that operates by inhibiting apoptosis (Reed, 1994). When over-expressed in cell culture, vBcl-2 prevents apoptosis and prolongs cell life (Cheng et al., 1997). vBcl-2 different from Bcl-2 because it does not dimerise with other member like Bax and is not cleavable by caspases (Bellows et al., 2000). It interacts with and inhibits the proapoptotic function of cellular Bcl-2 family member. All these prolong cell survival to allow for production of viral progeny (Inohara et al., 1998).
(iv) vGPCR

ORF 74 of the HHV-8 genome encodes viral G-protein-coupled receptor (vGPCR) and is most related to the human interleukin 8 (IL-8) receptors (Arvanitakis et al., 1997). vGPCR expressed in NIH3T3 cells has been shown to induce tumour formation when injected into nude mice. Before the discovery of HHV-8, AIDS-associated KS was already known to express elevated levels of vascular endothelial growth factor (VEGF) that lead to angiogenesis, and it was found that vGPCR-transfected cells secrete elevated level of VEGF (Bais et al., 1998). HHV-8 GPCR activates NF-kappa B which in turn up-regulates the expression of a wide variety of inflammatory cytokines and adhesion molecules (Pati et al., 2001).

(v) Viral interferon regulatory factors (vIRFs)

In addition to the latently expressed vIRFs discussed above, HHV-8 encodes two other homologues of cellular interferon regulatory factors (IRFs) lytically: vIRF-1 and vIRF-3. Interferons (IFN) are a group of cytokines with wide ranging effects including defence against viral infection and inhibition of cell growth. IRFs regulate the transcription of IFNs thereby modifying the effects brought about by IFN activity (Reis et al., 1992; Taniguchi et al., 1995).

The ORF K9-encoded vIRF-1 is homologous to cellular IRFs. It inhibits cellular responses to IFNs (Gao et al., 1997; Zimring et al., 1998), and can down-regulate the transcriptional activation induced by IFNs α, β, and γ (Gao et al., 1997; Li et al., 1998). This activity suppresses the host’s IFN-mediated innate immune response to viruses. vIRF-1 also acts as an oncogenic protein by transforming rodent fibroblasts in vitro and causing tumour formation in nude mice (Gao et al., 1997; Li et al.,
1998). It has been demonstrated that vIRF-1 can bind to p53 (Nakamura et al., 2001), a tumour suppressor gene involved in many types of human cancers (Vogelstein et al., 2000). By suppressing phosphorylation and acetylation of p53, vIRF-1 inhibits its activity and prevents apoptosis in the infected cell (Nakamura et al., 2001).

The other lytic viral IRF, vIRF-3, has homology to the cellular IRF-4 and the latent vIRF-2 and also blocks IFN signalling by functioning as a dominant negative inhibitor of IRF-3 and IRF-7. Overexpression of vIRF-3 in mouse L929 cells inhibits virus mediated synthesis of biologically active interferons (Lubyova and Pitha, 2000).

(vi) Viral interleukin 6 (vIL-6)

vIL-6, encoded by ORF K2, is approximately 24% homologous to human IL 6 (hIL-6) (Moore et al., 1996c). vIL-6 is readily secreted by HHV-8-infected cells in PELs during latency and MCD and, to a lesser extent, in KS lesions (Moore et al., 1996c; Parravicini et al., 2000). Similar to hIL-6, vIL-6 activates signal transduction pathways through its interaction with the gp130 receptor, but unlike hIL-6, does not need to interact with the IL6 co-receptor (IL-6R) to activate signalling. However, interaction with both gp130 and IL-6R may increase signalling efficiency (Wan et al., 1999). Both hIL-6 and vIL-6 are able to inhibit apoptosis and induce B-cell proliferation (Moore et al., 1996a). Mice inoculated with vIL-6-transfected NIH3T3 cells develop more highly vascularised tumours than control mice and the tumours also express higher levels of VEGF (Aoki et al., 1999). Although most cells in PEL or MCD do not secrete vIL-6, this cytokine has a paracrine effect on the surrounding
tissue and could be important in HHV-8-related disease progression. For instance, in cases of HHV-8 infected MCD, vIL-6 appears to cause a proliferation of uninfected B cells that comprise the majority of the tumour mass (Parravicini et al., 2000).

Lytic genes encode as class III transcripts (Sarid et al., 1998). They include structural protein genes, such as the gene that encodes major capsid protein (ORF 25), a gene encoding small capsomer-interacting protein (SCIP) (ORF 65), DNA polymerase (ORF9) and thymidine kinase (TK) (ORF 21). Their functions are probably to replace or enhance cellular DNA biosynthesis to aid in virion production (Russo et al., 1996; Neipel et al., 1997).

1.2.7 HHV-8 transmission

1.2.7.1 Sexual transmission

Transmission of HHV-8 is primarily sexual among homosexual men. Thus, in this population, AIDS KS can primarily be considered to be a sexually transmitted disease (Vieira et al., 1997; Martin et al., 1998; Melbye et al., 1998; Dukers et al., 2000). However, the specific mechanisms of transmission remain controversial. Some of the early studies on the relationship between KS and sexual practices report an association between oral-anal sex and AIDS KS (1993; Grulich and Kaldor, 1996; Vieira et al., 1997). Cross-sectional studies have demonstrated an association between the presence of HHV-8 antibodies and anal-genital sex, oral-anal sex, and deep kissing with an HIV-1-positive partner (Melbye et al., 1998; Grulich et al., 1999; Pauk et al., 2000). One study associated specific sexual techniques to seroconversion (Dukers et al., 2000), implicating recent oral-genital sex as a strong risk factor. Few studies have prospectively examined the risk factors for
recent HHV-8 infection, and all have found evidence of sexual transmission among homosexual men, e.g., recent sexual contact with an HIV-infected partner (Martin et al., 1998; Casper et al., 2002).

On the assumption that HHV-8 can be sexually transmitted, the virus would need to be shed at high titres in semen. Indeed, HHV-8 DNA has been detected in prostate tissue biopsies of HIV-seropositive men without KS (Staskus et al., 1997; Diamond et al., 1998) indicating that HHV-8 can be shed from the prostate into the semen. Moreover, HHV-8 DNA detection was reported in semen samples of healthy donors (Monini et al., 1996a). However, later studies have rarely reported positive samples in the semen of patients with KS, and almost never in healthy semen donors (Boshoff and Weiss, 2001). Longitudinal studies of viral shedding have shown that HHV-8 is not substantially shed in semen or rectal tissue, but is persistently found in the oral cavities of some individuals (Pauk et al., 2000; Corey et al., 2002). Infectious HHV-8 have now been recognised to be detected in high titres in saliva of HHV-8-seropositive individuals (Vieira et al., 1997; Koelle et al., 1997; Blackbourn et al., 1998; Pauk et al., 2000). Since oral-genital sex is a common practice among homosexual men, as is deep kissing, saliva rather than semen is now considered as significant vehicle of HHV-8 transmission. The risk of HHV-8 transmission through receptive ano-genital or insertive oro-anal sex may be even lower, because little or no virus has been detected in semen or faeces (Dukers et al., 2000).

Among heterosexuals, correlations with sexual transmission have not been consistently maintained (Smith et al., 1999; Wawer et al., 2001). Heterosexual transmission is not likely to be a major route and seems to require frequent sexual
exposure to occur. HHV-8 prevalence is elevated mainly in heterosexuals who have a high number of sexual partners (Bestetti et al., 1998; Tedeschi et al., 2000; de Sanjose et al., 2002).

1.2.7.2 Parenteral transmission

Many studies have provided some evidence that HHV-8 can be transmitted by blood or blood products (Blackbourn et al., 1997; Regamey et al., 1998; Engels et al., 1999; Cannon et al., 2001). Some studies, which were performed in intermediate or high endemic populations, have shown that the prevalence is somewhat higher among injecting drug users (IDUs) compared with the general population, or an association between HHV-8 infection and injecting behaviour (Cannon et al., 2001; Sosa et al., 2001; Parisi et al., 2002). In countries or populations that are highly endemic for HHV-8, transmission by blood is not believed to be important, but could play a role.

On the other hand, low-prevalence areas generally show similar prevalence of HHV-8 between IDUs and the general population. Although syringe sharing among IDUs is a common means of transmitting HIV-1, hepatitis B virus and hepatitis C virus, it does not result in a similar spread of HHV-8 (Renwick et al., 2002). In a study of persons who received blood products from HIV-1- and HHV-8-coinfected donors, 10 of 14 recipients seroconverted for HIV-1, whereas none seroconverted for HHV-8 (Operskalski et al., 1997). Nonetheless, transmission through blood products cannot be excluded.
1.2.7.3 Vertical transmission

Whether HHV-8 infection may be transmitted from mother to child is still unclear, with studies showing conflicting results. A study in South Africa suggests a rate of vertical transmission as high as 30% (Bourboulia et al., 1998). Another study showed that transmission appears to increase with increasing maternal antibody titres (Sitas et al., 1999). However, most studies have shown that HHV-8 prevalence in children increases with age (Bourboulia et al., 1998; Lyall et al., 1999; Serraino et al., 2001), and that most children born to HHV-8-seropositive mothers possess passively transmitted antibodies and then serorevert by 24 m of age (Calabro et al., 2000). On the other hand, a study in Zambia demonstrated no association between the HHV-8 infection rates among infants born to HHV-8-seropositive mothers (Brayfield et al., 2003). This study showed detection of viral DNA in peripheral blood mononuclear cells (PBMCs) at the time of delivery for 2 of 89 infants born to HHV-8-seropositive mothers, which suggests that in utero infection occurs but rarely (Brayfield et al., 2003). However, a more recent study in Italy suggests that vertical transmission of HHV-8 is unlikely or, at least, very rare (Sarmati et al., 2004).

1.2.7.4 Non-sexual person-to-person transmission

Non-sexual person-to-person transmission and childhood transmission are rare in the developed countries. Blauvelt et al. (1997) showed that transmission before puberty rarely occurs in the United States. In contrast, findings from countries where HHV-8 infection is more widespread, such as African and Mediterranean countries, suggest that the virus can be transmitted among family members and that transmission is associated with close contact and crowding.
A study of HHV-8 prevalence within families reported equally high rates among individuals with classic KS and their relatives; these rates were considerably higher than those among a sample of individuals from the general population who were matched by sex and age. Clustering of HHV-8 seroprevalence between spouses, children, and siblings was demonstrated in a Sardinian study population (Angeloni et al., 1998).

In Middle Eastern countries, evidence of HHV-8 infection in children also supports non-sexual transmission. In Egyptian children, HHV-8 seroprevalence of antibodies exceeded 50% in children older than 6 y and increased steadily up to 10 y, thereafter stabilising (Andreoni et al., 1999). In Egyptian children HHV-8 primary infection may be associated with a febrile illness and skin rash, and HHV-8 infection has been positively associated with close contact with at least two other children in the community, pointing to saliva as an important route of paediatric infection (Andreoni et al., 2002).

Moreover, for Israeli children, HHV-8 seroprevalence also rises with age. Davidovici et al., (2001) found that HHV-8 infection in children of a seropositive mother was influenced by the antibody titre of the mother, but was unassociated with HHV-8-infected father.

Gessain et al. (1999) found that in Cameroon, HHV-8 infection was also common among children. Seroprevalence steadily increased from 27% at age of 4 y to 39% in the 12-14 y group to above 48% in children over 15 y, approaching the level of HHV-8 infection in adults. Correlation with hepatitis B infection suggests that HHV-
8 is transmitted horizontally in conditions of close contact and crowding (Mayama et al., 1998). Plancoulaine et al. (2000) studied a population of villagers of African origin in French Guiana and showed that the HHV-8 seroprevalence was 15% by age of 15 y and rising sharply to about 30% after 40 y; they provided evidence to implicate horizontal familial transmission between children and their mother but not their fathers, and between children and their siblings. A more recent similar study conducted in rural Tanzania also found associations between the HHV-8 serostatus of spouses and between the serostatus of a father and that of his children (Mbulaiteye et al., 2003). In a study of KS patients and their first-degree relatives in Malawi, both identical and non-identical HHV-8 sub-genomic sequences were observed between family members, suggesting transmission of HHV-8 along both intra- and extra-familial transmission routes (Cook et al., 2002b).

A recent study in sub-Saharan Africa demonstrated the absence of HHV-8 DNA in the breast milk of seropositive mothers, suggesting that contact with breast milk is not a likely source of horizontal transmission of virus to infants (Brayfield et al., 2004). However, a study by Dedicoat and co-workers in South Africa showed presence of HHV-8 DNA in 12 out of 43 breast milk samples (Dedicoat et al., 2004).

The high prevalence of infection before adolescence in endemic areas and the higher frequency of the detection of HHV-8 in saliva compared with semen (Pauk et al., 2000) provide indirect evidence of non-sexual transmission. It is thus hypothesized that viral spread through salivary contact might be involved in childhood transmission in endemic countries, although this is not definitely shown (Cattani et al., 1999). However, a recent study in South Africa reported that HHV-8 DNA was
detectable in 145 of 978 maternal saliva samples (mean virus load: 488,450 copies/mL), suggesting saliva is also a significant vehicle of mother-to-child transmission (Dedicoat et al., 2004). Other than close contact and the use of shared items (spoons, plates, etc) in endemic area like Africa, behavioural practices associated with saliva exchange in sub-Saharan Africa may play a role in transmission of HHV-8. The use of saliva in healing medical practices, religious initiation or ritual practices, and feeding practices have been implicated (Wojcicki, 2003).

The source of salivary HHV-8 is unclear. Corey et al. (2002) compared HHV-8 DNA content in whole saliva versus parotid duct secretions and found HHV-8 to be absent or markedly lower in parotid fluid than whole saliva; minor salivary gland biopsies performed in 4 HHV-8 seropositive persons who shed HHV-8 in their saliva showed no evidence of HHV-8 in the biopsy tissues using in situ hybridization. A model of reactivation and shedding similar to EBV has been suggested in which HHV-8 shed into the mouth originates from activation in latently infected B cells infecting the epithelial cells that line the lymph nodes (Sixbey et al., 1984; Ivarsson et al., 1999). In palatine tonsils of two HIV infected HHV-8 seropositive persons, the lytic gene products of HHV-8 in B cells within germinal centre and the epithelium lining of tonsil could be detected, suggesting that lytic replication of HHV-8 in B cells within germinal centre may transfer HHV-8 to epithelium surface either by its migration to the surface epithelium or by contiguous infection (Corey et al., 2002).
1.2.7.5 Transmission to organ transplant recipients

Cases of seroconversion to HHV-8 have been observed after heart transplantation (Emond et al., 2002) and kidney transplantation (Luppi et al., 2000; Luppi et al., 2002). These indicate that HHV-8 may be transmitted from the donor organ during transplantation. Few organ donation recipients develop KS in areas of low seroprevalence, but a higher incidence may be found in areas of higher KS incidence. In most cases, transplant patients are infected with HHV-8 prior to transplantation (Parravicini et al., 1997) and KS may develop as a result of viral reactivation due to drug-induced immunosuppression.

1.2.8 HHV-8 epidemiology

1.2.8.1 Methods used to determine HHV-8 prevalence

Some prevalence studies have relied on detection of HHV-8 DNA using PCR methods. However, only half of KS patients have detectable levels of HHV-8 DNA in PBMCs, indicating that viremia may not be frequent (Whitby et al., 1995; Moore et al., 1996c). For most immunocompetent patients who are infected with HHV-8, clinical signs of KS are not present and DNA is even more difficult to detect in PBMCs. Thus, HHV-8 DNA testing is not suitable for estimating prevalence of infection. Nested PCR may increase the sensitivity of the PCR by 100 to 1000 times (Moore et al., 1996c). Increased sensitivity of PCR can, however, create problems with contamination, creating false PCR positive results and rendering sequence data unusable (Moore and Chang, 1998). Thus, when detection of HHV-8 DNA in semen of a large proportion was reported in healthy Italian blood donors (Monini et al., 1996a), the finding could not be corroborated by other groups suggesting that PCR
contamination had indeed occurred in that study (Howard et al., 1997; Pellett et al., 1999).

Serologic assays for HHV-8-specific antibodies are more sensitive than PCR for detecting either current or past infection with the virus. Current assays can detect viral infection in more than 90% of infected patients (Gao et al., 1996b). First-generation assays were developed using HHV-8-infected PEL cell lines as sources of antigen in either an immunofluorescence assay (IFA) or in immunoblotting assay formats. LANA was the first serologic antigen identified by indirect IFA; while it is specific, it is generally less sensitive (about 80% sensitivity) (Gao et al., 1996b). IFA using lytic antigens can yield higher sensitivity rates than LANA assays. However, they suffer from problems associated with cross reactivity to other herpesvirus antibodies, particularly as no HHV-8-negative PEL cell lines are available to act as controls for non-specific reactions (Lennette et al., 1996). Recent assays using recombinant HHV-8 antigen expressed from another unrelated virus (e.g. Semliki Forest virus) allow high levels of antigen to be expressed and appropriate negative controls to be included. This approach has enhanced both the specificity and sensitivity of the IFA assay. Thus, in the assay developed by Inoue et al. (2000) the ORF K8.1 gene product was used as the antigen, and as it has no homologue to other herpesviruses, cross-reactivity does not occur.

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

Evidence suggests that patients show heterogeneous serologic responses to defined HHV-8 antigens; therefore, combinations of antigen assays may achieve the best detection rates (Lang et al., 1999; Spira et al., 2000). Although early studies suggested wide differences in infection rates in populations at risk of KS based on the type of antigen used (Rabkin et al., 1998), properly performed assays in standardized formats have been found to be highly concordant (Schatz et al., 2001).

1.2.8.2 HHV-8 seroprevalence

Seroprevalence studies show that, unlike most human herpesviruses, HHV-8 does not spread universally among all human populations. The lowest HHV-8 prevalence has been reported in northern Europe and the United States, where the prevalence of infection ranges from 0% to 5% among blood donors (Simpson et al., 1996; Gao et al., 1996b). Initial studies among children in these geographical regions indicated that HHV-8 infection is rare or nonexistent, with 0% prevalence among those HIV-seronegative or -seropositive (Simpson et al., 1996; Moore, 2000). A more recent study of 787 serum specimens obtained from children living in two areas where HHV-8 is not endemic, Germany and USA, showed seroprevalences of between 3% to 4% (Martro et al., 2004). In these areas, HHV-8 infection is mostly concentrated among homosexual men (Martin et al., 1998; Stine et al., 2000). HIV-infected homosexual men have the highest seroprevalence of any risk group. A study in the San Francisco area using samples dating to 1984 reported a seroprevalence of 48%
among homosexual or bisexual men who were HIV-positive but did not have KS (Martin et al., 1998). A similar study conducted in Amsterdam between 1984 and 1996 reported a baseline HHV-8 incidence of 30% among homosexual men who were also HIV-seropositive but had not developed KS (Dukers et al., 2000). In Europe and North America, HIV-1-seronegative homosexual men consistently show a lower seroprevalence of HHV-8 infection than HIV-1-seropositive homosexual men, and higher than observed among blood donors (Kedes et al., 1996; Martin et al., 1998; Melbye et al., 1998). In a recent study on HIV-infected patients without KS in Germany, the seroprevalence was up to 52% of 398 patients (Albrecht et al., 2004). Heterosexual men and women with multiple partners in these Western countries show slightly increased seroprevalences when compared to the general population (Melbye et al., 1998; Whitby et al., 1999).

In South East Asia, prevalence of HHV-8 is variable: in Japan, it ranges between 0.2 – 1.4% (Fujii et al., 1999; Satoh et al., 2001). However, Chen et al. (2004) in Northern Thailand showed a relatively high HHV-8 seroprevalence: of 992 persons studied, consisting of 400 married couples in which the husband and the wife were HIV-seropositive (200 couples), and in which the husband was HIV-seropositive but the wife HIV-seronegative (200 couples), and 200 HIV-seronegative men from a sexually transmitted diseases (STD) clinic: the HHV-8 seroprevalence was 24% in the total population, 28% in the HIV-seropositive group and 18 % in the HIV-seronegative group.

HHV-8 seroprevalences in South America have also been documented. The seroprevalence in blood donors from Central and South America is between 1.9% to
6.7%, with the variability thought to be linked to variation in immune status and exposure to HIV patients in the cohorts tested (Perez et al., 2004). In Brazil, varying seroprevalences, 53% among Amerindians (Biggar et al., 2000) and 16% in urban communities (Freitas et al., 2002), have been reported.

Countries surrounding the Mediterranean and the Middle East are regions of high HHV-8 seroprevalences. In Italy, the prevalence varies between less than 10% in the North to more than 20% in the South (Whitby et al., 1998), with the highest rates observed on the islands of Sicily and Sardinia (Vitale et al., 2001). Wide variations have been observed even within smaller areas, such as northern Italy, where the prevalence ranges from less than 5% in Milan to more than 20% at the lower end of the Po Valley (Whitby et al., 1998; Calabro et al., 1998). An HHV-8 prevalence of 4% has been reported among Italian children younger than 11 y (Whitby et al., 2000). The highest prevalence in the Mediterranean area, exceeding 50%, has been reported among children in Alexandria, Egypt (Andreoni et al., 1999). HHV-8 seroprevalence is also higher in Israeli Jews (10%) than in European and North American blood donors (Davidovici et al., 2001).

In Africa, HHV-8 seroprevalences are very high (de The et al., 1999), particularly in sub-Saharan Africa, where endemic KS has been reported. In this area, the HHV-8 seroprevalence among children ranges from 13% in Cameroon to 37% in Uganda (Mayama et al., 1998; Gessain et al., 1999), and from 36% in Ghana to 47% in Zambia (Olsen et al., 1998; Nuvor et al., 2001). The prevalence among adults ranges from 22% in central Africa to 48% in Zambia (Belec et al., 1998; He et al., 1998). Very high rates, between 76% and 87%, have been reported in the Congo and in
Botswana (Engels et al., 2000), and among older adults in Ghana (43%) and Zambia (71%) (Olsen et al., 1998; Nuvor et al., 2001). The HHV-8 prevalence among prepubescent children from Central Africa ranges between 40% to 50% and shows a positive correlation with age (Kasolo et al., 1997; Mayama et al., 1998; Gessain et al., 1999).

1.3 Cytomegalovirus

CMV is ubiquitous. The virus infects most individuals by adulthood and in developed countries. CMV infection is generally asymptomatic and leads to persistence. Primary and persistent infection may lead to severe disease in the absence of an effective immune response, as in the immunologically immature and the immunocompromised. Its impact on mortality and morbidity has become prominent in recent decades due to the rise in organ allografting, immunosuppressive treatment, and HIV infection. CMV is a leading causal agent of birth defects (Griffiths, 2000; Pass, 2001).

1.3.1 Virology

1.3.1.1 Virion structure

CMV shows typical structural features characteristic of the herpesvirus family. The DNA is contained within a capsid of 162 hexagonal capsomeres. The capsid is surrounded by the ill-defined tegument, which is itself surrounded by a loosely applied lipid envelope. The mature virion particle is 150-200 nm in diameter (Mocarski and Courcelle, 2001).
1.3.1.2 Structure of the CMV genome

The CMV genome is the largest of all herpesviruses and has a high G+C content. It contains an arrangement of unique long (UL), unique short (US) and repeat regions (Mocarski and Courcelle, 2001). The AD169 laboratory strain is the only one in which the genome has been completely sequenced. Analysis of its 230-kbp genome has revealed that it encodes 225 ORFs of ~100 or more amino acids (Chee et al., 1990; Novotny et al., 2001). These ORFs are designated sequentially according to their location within the unique and repeated regions. Comparison of the AD169 amino acid sequences with those of other herpesvirus genomes has shown that the protein products of more than 40 ORFs share high similarity to proteins encoded by α- and γ-herpesviruses (Chee et al., 1990; Karlin et al., 1994), and provided further evidence of a common origin of the three subfamilies. Of the herpesvirus-conserved ORFs, ~25% appear to encode functions related to viral DNA metabolism and replication, whereas the remaining 75% are thought to be involved in the maturation and structural organization of virions. The UL ORF-encoding functions are involved in DNA replication and repair, nucleotide metabolism, or virion structure; the ORFs are grouped in seven conserved gene blocks (A–G) also found in other herpesviruses, such as HSV-1 and EBV. The US ORFs and ORFs located within the repeated regions of the CMV genome are less well conserved than in the other herpesviruses (Chee et al., 1990).

1.3.1.3 CMV proteins

The International CMV Workshop of 1993 agreed on a nomenclature for the description of CMV proteins (Landini and Spaete, 1993). The system designates p for protein, gp for glycoprotein, and pp for phosphoprotein, followed by a numerical
Figure 1.6 Genome organization and ORFs of CMV (Towne strain) based on the genomewide shotgun sequencing of the viral sequence. The Towne genome is composed of a UL region and a US region, both flanked by inverted repeat regions (RL and RS). RL and RS are shown in a thicker format than UL and US. Each of the ORFs is color-coded according to the growth properties of their corresponding virus gene-deletion mutants. The vertical dashed lines represent the splicing junctions (reproduced from Dunn et al, 2003).
assignment to the genetic locus. Of the more than 30 viral proteins found in the complete infectious virion, 4 constitute the capsid; encoded by UL46, UL48.5, UL85 and UL86. Assembly protein (AP), encoded by UL80, is associated with the capsid and may play roles in maturation (Mocarski and Courcelle, 2001).

The phospholipid envelope contains 6 virus-encoded glycoproteins: gpUL55 (gB), gpUL73 (gN), gp UL74 (gO), gpUL75 (gH), UL100 (gM), and gpUL115 (gL). These glycoproteins play essential roles in virus entry into host cells, cell-to-cell spread and virion maturation (Britt and Mach, 1996). Mutational analysis has revealed that disruption of gB, gH, gL, and gM ORFs results in failure to produce infectious progeny (Hobom et al., 2000).

The remaining 20–25 structural virion proteins probably are located in the still poorly characterized amorphous layer between the nucleocapsid and the envelope (Baldick and Shenk, 1996). The tegument proteins may be involved in the maturation of progeny virions, or may influence viral and cellular events in the early stages of infection, such as release of viral DNA from disassembling virus particles. Most tegument proteins are phosphorylated and are highly immunogenic. The most abundant are ppUL32 (pp150 or basic phosphoprotein) and ppUL83 (pp65). Owing to the large amount produced, pp65 is frequently the target antigen used in antigenemia assays for the rapid diagnosis of CMV infections. Tegument proteins such as ppUL69 and ppUL82 (pp71) may play important regulatory roles in both viral and cellular gene expression. They are transactivators of viral gene expression, and can dysregulate cell cycle progression. UL69 is required for efficient viral replication, and arrests cells in G1 by an unknown mechanism (Lu and Shenk, 1999).
UL82 is a transcriptional activator of activating transcription factor (ATF)/cyclic AMP-response element binding protein (CREB) or the activator protein-1 (AP-1)-containing promoter. It also increases the infectivity of transfected viral DNA, is required for viral replication at low multiplicities of infection, and accelerates the G₁ to S transition of quiescent cells (Liu and Stinski, 1992; Baldick et al., 1997).

1.3.1.4 CMV protein-coding genes used for genotyping

(i) gB (gpUL55)
Glycoprotein B (gB; gpUL55), a component of the envelope complex gC-I, is the most widely studied polymorphic viral glycoprotein. It is used for the virus to attach to, and penetrate into, the cell. There are specific receptors on the cell surface for which gB constitutes the ligand. It has been shown that antibodies to gB inhibit virus penetration into the cells and block transmission of infectious virus from cell to cell (Rasmussen et al., 1991; Navarro et al., 1993). Four main gB genotypes (gB-1, gB-2, gB-3 and gB-4) have been found (Chou and Dennison, 1991; Fries et al., 1994; Meyer-Konig et al., 1998), although some rare non-prototypic variants have been described (Shepp et al., 1998; Trincado et al., 2000). Data on the functional role of the main gB types in CMV pathogenesis and their relationship with the clinical outcome of CMV disease and virus cell/tissue tropism are conflicting, and, in some cases, contradictory (Fries et al., 1994; Chern et al., 1998; Gilbert et al., 1999).

(ii) UL144
UL144 ORF encodes a homologue of the tumor necrosis factor receptor. Three phylogenetic groups have been identified based on this ORF, with variability as high as 21% in amino acids among variant groups (Lurain et al., 1999).
(iii) gN (UL73)

gpUL73-gN is encoded by the ORF UL73, is a component of the envelope gC-II complex in association with gM and is able to elicit neutralizing antibodies (Mach et al., 2000; Dal Monte et al., 2001; Pignatelli et al., 2002). UL73 shows 4 main genomic variants, denoted gN-1, gN-2, gN-3 and gN-4. The gN-3 genotype can be divided further into two subgroups (gN-3a and gN-3b) while the gN-4 genotype can be divided further into 3 subgroups (gN-4a, gN-4b and gN-4c) (Pignatelli et al., 2001; Pignatelli et al., 2003). These gN genomic variants are stable during in vitro and in vivo virus replication and show no correlation with gB genotypes. Furthermore, the UL73 gN-encoding gene seems more polymorphic than the gB (Pignatelli et al., 2001). The variants gN-1, gN-3 and gN-4 and their subgroups have been detectable in four geographical areas of North America, Europe, China and Australia, although with different frequencies. The rarest genotype, gN-2, has been observed in people residing in Northern America and Europe, but not identified in Chinese or Australians (Pignatelli et al., 2003).

(iv) gO (UL74)

gpUL74-gO is encoded by the ORF UL74, a newly discovered hypervariable locus in CMV (Paterson et al., 2002; Rasmussen et al., 2002). gO region complexes with UL75 (gH) and UL115 (gL) to form the gCIII viral-envelope complex (Rasmussen et al., 2002). gO has a role in infection by facilitating cell fusion and cellular spread (Paterson et al., 2002). The gO genotypic variants can differ by up to 50% of amino acids (Paterson et al., 2002; Rasmussen et al., 2002).
1.3.2 Epidemiology

The CMV is transmitted both vertically and horizontally. Transmission may occur following primary infection, or reactivation. CMV is shed in various body fluids including saliva, semen, urine and cervicovaginal secretion, frequently in the absence of symptoms. In populations living under good socioeconomic conditions, approximately 40% are seropositive by adolescence, this figure increasing thereafter by approximately 1% annually. By contrast, almost all children from low socioeconomic populations are already infected by the onset of puberty (Griffiths, 2000; Pass, 2001). About 10% of infants are infected by the age of 6 m, following transmission from their mothers via the placenta, during delivery, and by breast feeding (Pass, 1985; Stagno et al., 1986).

1.3.3 Routes of transmission

1.3.3.1 Intrauterine

The frequency of congenital acquired CMV infection in developed countries ranges from 0.5 – 2.2% of all live births (Fowler et al., 1992; Trincado and Rawlinson, 2001). Infection results from either primary maternal infection contracted in pregnancy or from reactivation during pregnancy of CMV in a previously infected mother. The risk of primary maternal infection in a non-immune mother in pregnancy is about 1%, and it carries a 40% risk of congenital infection. Foetal infection is more likely to occur, and to be severe when a seronegative mother acquires primary infection in early pregnancy. The risk of symptomatic congenital infection from reactivation of maternal CMV in pregnancy is lower, although not absent, as pre-existing maternal immunity limits spread to the foetus (Fowler et al., 1992; Pass, 2001).
1.3.3.2 Perinatal

Infection during delivery is the outcome of shedding from the vagina or cervix, followed by ingestion of infected secretions by the offspring. Shedding close to the time of delivery has been noted in 2% to 28% of mothers (Reynolds et al., 1973; Stagno et al., 1982). Breast-feeding is another significant route of perinatal transmission. Transmission is a function of its duration and the relatively low virus load in the milk (Dworsky et al., 1983). It has been shown, for example, that infants nursed for <1 m do not become infected, compared to almost 40% of those nursed longer. Moreover, 70% of infants are infected when the virus can be isolated from the milk, whereas only 10% are infected when the mother is seropositive, but was culture-negative for virus in the milk. PCR studies have also demonstrated a strong relationship between the presence of viral DNA in milk and transmission to the infant (Vochem et al., 1998).

1.3.3.3 Postnatal

The absence of symptoms following postnatal infection makes it difficult to determine what the principal transmission routes are. CMV transmission occurs from child to child in day care centres. Among young children, saliva and urine are the most common vehicles. Infection in children is usually asymptomatic, but infectious toddlers may transmit the virus to adult nursery staff or to mothers (Pass et al., 1990). If any of these adults are pregnant, there is a risk of fetal damage, especially if the mother is seronegative.

Sexual transmission may be significant, particularly as high CMV seroprevalences among patients attending clinics for sexually transmitted disease have been observed
(Shen et al., 1994). Heterosexual contact is a major mode of transmission of CMV among some young adults who shed strains of CMV in the cervix secretion and in semen (Handsfield et al., 1985). Primary infection with CMV via blood transfusions was noted in the mid-1960s, although epidemiological studies have demonstrated that this is an uncommon route. Attempts to culture CMV from fresh donor blood have rarely been successful. It is thus assumed that the virus is latent in the blood cells of healthy donors and is reactivated following transfusion when they encounter an allogeneic stimulus. The nature of the leukocytes carrying latent virus is unknown, although attention is being increasingly focused on those of the monocyte/macrophage lineage (Soderberg-Naucler et al., 1997; Soderberg-Naucler et al., 2001).

CMV is a significant post-allograft pathogen. Several studies have shown that seronegative recipients of an organ from a seropositive donor are at much greater risk of acquiring a primary infection (Peterson et al., 1980; Pollard, 1988). The incidence of CMV infection following allogenic bone marrow transplant (BMT) ranges from 32% to 70%, with an average of 50%, regardless of the prior serological status of the recipient and donor (Ruutu et al., 1990; Rubie et al., 1993).

1.3.4 Diagnosis of CMV infection

1.3.4.1 Viral culture

CMV has been traditionally isolated in fibroblast tissue culture. Fibroblasts undergo a focal cytopathogenic effect (CPE) that is easily confirmed by fluorescent antibody stains. The virus can be recovered from a variety of specimens: urine, tissues, respiratory swabs and washes, body fluids and blood. Conventional viral culture is
the gold standard for diagnosing CMV despite shortcomings such as poor predictive value, difficulties in quantitation, and a long turnaround time (1–3 weeks). Falsely negative cultures may occur if the interval between specimen collection and cell culture inoculation is prolonged. Cell cultures show a lower sensitivity when compared with PCR and nucleic acid probe methods (Sandin et al., 1991). Rapid viral culture (using the shell vial method) is a modification of conventional culture that leads to a reduced reporting time to 48 h from 7 to 14 days. In this method, specimens are centrifuged onto fibroblast monolayers, incubated briefly, then stained before the appearance of CPE with a monoclonal antibody to early antigens (Gleaves et al., 1989; Rabella and Drew, 1990).

1.3.4.2 Histopathology
CMV infection of a cell is recognized by the presence of characteristic intranuclear “owl’s eye” inclusions, which have a surrounding halo and marginated chromatin. Cells that bear these inclusions may be found in the kidney, lung, oesophagus, liver and salivary glands. While histopathologic diagnosis is specific, false negatives due to sampling error are common (Mattes et al., 2000).

1.3.4.3 PCR detection of CMV DNA
PCR has provided an invaluable diagnostic tool in virology because of its ability to detect minute amounts of viral nucleic acid in clinical specimen. PCR-based techniques are increasingly being used to detect and quantitate CMV load in blood or plasma. As the virus is seldom detected in plasma (as opposed to leucocytes) in healthy carriers, the presence of CMV DNA in plasma indicates active viral replication (Spector et al., 1998). The use of quantitative PCR techniques allows a
measurement of 'viral load' in plasma: such assays have been advocated for the monitoring of immunosuppressed patients, and a rise in viral load above a predetermined threshold necessitates anti-CMV prophylaxis, even in the absence of a clinical syndrome attributable to CMV (Emery et al., 2000).

1.3.4.4 Antigen detection

The assay consists of direct staining of leukocytes with monoclonal antibodies directed against the lower matrix protein (pp65) (Grefte et al., 1992). pp65 is present in the CMV virion and is detectable in infected peripheral mononuclear cells. The results are expressed as the number of antigen-positive cells relative to the number of the cells used to prepare the slide. This assay is a sensitive method of estimating the systemic CMV load. Its advantages include a short processing time (less than 6 h) and lack of requirement of highly specialized laboratory. However, it needs to be processed immediately, and the results are subject to variation in personal skills (Boeckh and Boivin, 1998).

1.3.4.5 Serology antibody detection

Serological determination of a past or recent CMV infection in immunocompetent individuals rests on detection of virus-specific IgG or IgM antibodies. Occurrence of a primary infection is conventionally deduced from the seroconversion response. A specific IgM response may be serologic evidence of a recent primary infection. IgM testing time is not reliable because false positive reactions may result in the presence of rheumatoid factor or antinuclear antibodies, and true positive reaction may appear in patients who had experienced past CMV infection. Testing the avidity of CMV-specific IgG is another way of CMV serologic diagnosis and has proven to be a
powerful tool for distinguishing primary from past or recurrent CMV infection in pregnant women and solid organ transplant recipients (Blackburn et al., 1991; Lazzarotto et al., 1999). It is defined as the strength with which the IgG attaches to antigen. IgG avidity matures with the length of time following primary infection. Thus, IgG produced within the first 3 to 5 m following primary infection exhibits low avidity, whereas IgG produced several months or years later exhibits high avidity and indicates past or recurrent infection (Prince and Leber, 2002).

1.3.5 Clinical features

1.3.5.1 Congenital infection

Approximately 30% of congenitally infected newborns develop symptomatic disease, including the most severe manifestation of cytomegalic inclusion disease, which includes growth retardation, jaundice, hepatosplenomegaly, thrombocytopenia, retinitis, along with CNS involvement in the form of encephalitis and seizures. 18% develop long-term sequelae such as hearing loss (Gaytant et al., 2002). Children infected during the first trimester of pregnancy are at increased risk of complications, although infections at any stage of pregnancy can produce symptomatic disease (Boppna et al., 1999). Prior maternal CMV antibodies are protective against transmission of the virus, but not against development of symptomatic disease once infection has occurred (Boppna et al., 1999; Gaytant et al., 2002). Infection during pregnancy is often asymptomatic; however, infected pregnant women when questioned closely, often admit to fatigue (Revello and Gerna, 2002).
1.3.5.2 Infection in immunocompetent hosts

Most perinatally infected infants do not develop acute symptoms, although occasionally pneumonitis may occur in first 3 m of life (Brasfield et al., 1987). Postnatal infection in the immunocompetent child or adult is almost always asymptomatic, but may account for 8% of all the cases of mononucleosis (Nesmith and Pass, 1995). Clinical manifestations of mononucleosis due to CMV are very similar to that caused by the more common EBV. Persistent fever, myalgia, headache, cervical lymphadenopathy, splenomegaly and nonspecific constitutional symptoms are common, and may persist for weeks.

1.3.5.3 Infection in the immunocompromised host

CMV is a significant opportunistic pathogen in immunocompromised patients, e.g., solid organ transplant recipients, bone marrow transplant (BMT) recipients and AIDS patients. Primary infection, reactivation of latent virus, and reinfection are all possible and are often clinically silent. The onset of infection is marked by spiking pyrexia, which may resolve in a few days. Its severity is roughly parallel with the level of immunosuppression, and is most prominent in BMT recipients and AIDS patients (Pass, 2001).

1.3.5.4 Disease in solid organ transplant recipients

Clinical signs of CMV infection in transplant recipients may be absent or severe, although severe infection is now less frequent as a result of better prophylaxis. CMV infection may be associated with chronic rejection and renal artery stenosis in renal transplant recipients (Brennan, 2001), with accelerated coronary artery stenosis in heart transplant recipients (Hosenpud, 1999), and with the ‘vanishing bile duct’
syndrome in liver transplant recipients (van den Berg et al., 1996). None of these associations has been definitively established as being caused by CMV.

1.3.5.5 Disease in bone marrow transplant recipients

Pneumonitis is the most serious manifestation of CMV infection after BMT, occurring in 10-15% of allogeneic BMT recipients with a mortality of 80% prior to the advent of antiviral therapy. The clinical picture is that of interstitial pneumonitis in the absence of any other identifiable pathogen, with increasing arterial hypoxaemia, and progression to respiratory failure. It is suggested that graft versus host disease (GVHD) may contribute to the lung injury in CMV pneumonitis in BMT recipients. The relationship between CMV and GVHD is controversial, with proposals that CMV may predispose to GVHD, and vice versa (Broers et al., 2000).

1.3.5.6 Disease in patients with AIDS

The incidence of CMV infection in HIV-seropositive patients is close to 100%, and coinfection with multiple strains is common (Baldanti et al., 1998). Retinitis and gastrointestinal disease are common manifestations of CMV infection in HIV-infected patients, but the commonest is retinitis, which may be seen in up to 25% of patients with AIDS. It is characterized by haemorrhagic retinal necrosis, spreading along retinal vessels; when disease encroaches on the macula sight is threatened (Jacobson, 1997). However, HAART has significantly decreased the incidence of CMV-induced disease (particularly retinitis) (Jabs et al., 2002).
1.4 Hypothesis and aim of the current study

The molecular epidemiology of HHV-8 and CMV was investigated in patients with KS and their relatives in Malawi, a region of high HHV-8 and CMV endemicity. It was hypothesised that hosts living in such a hyper-endemic region are prone to multiple HHV-8 infection and multiple CMV infection. To substantiate this hypothesis, nucleotide and amino acid sequence polymorphisms in hypervariable regions of the HHV-8 and CMV genomes carried in samples obtained from various anatomical compartments were investigated.
Chapter 2

Methods and Materials
2.1 Amplification of HHV-8 sub-genomic DNA

2.1.1 Patients and samples

The study group included patients with presumptive cutaneous, oral and nodal KS \( n = 22 \) attending the Central Hospital of Blantyre, Malawi, Africa, and their first-degree relatives \( n = 67 \). In all cases of nodal KS, the diagnosis was confirmed histologically. Ethical approval for the study was granted in the United Kingdom and locally (Cook et al., 2002b). An identification system described in a previous publication (Cook et al., 2002b) was applied: index cases of KS were assigned the "i" suffix, and their family members assigned numerals; letters denoted family assignments. Venous blood, urine and oral samples were obtained after informed consent was given. The following oral samples were collected: mouth rinse, throat gargle, and palatal exfoliate. Table 2.1 shows the characteristics of the study group. This study group is the same as that reported by Cook et al. (2002b). Table 1-5 in Appendix 2 shows DNA detectability of all samples for different regions of HHV-8 and CMV from all patients in this study.

2.1.2 Preparation of whole blood

Peripheral blood was collected in EDTA-treated vacutainers and stored, if necessary, for not more than two days at 4\(^\circ\) C. Plasma was separated from blood by centrifugation at 1200 rpm for 5 min with 1 ml of supernate being then removed. The remaining blood was resuspended. To separate different leukocyte fractions from the blood, Dynabeads (Dynal A.D., Oslo, Norway) were utilised. Dynabeads are small magnetically charged uniformly shaped beads coated with a specific monoclonal antibody. For this study Dynabeads coated with antibodies (mouse IgG2a
# Table 2.1

<table>
<thead>
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<th>Index case</th>
<th>Vincristine given?</th>
<th>Spouse (age)</th>
<th>Children/siblings (age/sex)</th>
<th>Mother (age)</th>
<th>Father (age)</th>
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<td>B</td>
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<td>B7(40y)</td>
<td>B7(40y)</td>
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<tr>
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<tr>
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<td>Y2(8y/F), Y3(5y/F), Y4(3y/F)</td>
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<td>Z1(5y/F)</td>
<td>Z2(22y)</td>
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</tbody>
</table>

*KSHV K1/V1 DNA amplified from blood (CD45 +); bKSHV K1/V1 DNA amplified from saliva; tKSHV-seropositive; hHIV-1-seropositive; tKSHV ORF 26 DNA amplified from blood (CD45 +); tKSHV ORF 26 DNA amplified from saliva.

Table 2.1 Characteristics of the patients with Kaposi’s sarcoma and their family members (reproduced from Cook et al., 2002b).
monoclonal antibody, clone EO1) against CD45 and (mouse IgG1 monoclonal antibody, clone 9G11) against CD31 were used. To 1 ml of whole blood, 50 μl of Dynabeads was added to give an approximate final concentration of $2 \times 10^7$ beads/ml. The whole blood and Dynabead mixture was incubated at 4°C for 20 min. Dynabeads were collected using a magnetic particle concentrator (MPC) (Dynal), washed 3 times with 1 ml of phosphate buffer saline (PBS) containing 2% foetal calf serum, and eluted into 250 μl of nuclease-free water.

2.1.3 Preparation of oral samples

A sampling order of the oral components had been imposed to minimize cross-contamination between compartments: the throat-gargle sample (collected after 30 s of gargling with 5 ml of PBS, while patient’s head tilted back), the mouth-rinse sample (collected after retaining 5 ml of PBS in the mouth for 2 min, during which gentle lateral flexions of the neck were encouraged), and the palatal-exfoliate sample (eluted in 1 ml of PBS after applying 5 rotations of an exfoliative cytology brush (Oral CDX, Henry Schein Company) to the palatal mucosa posterior to the upper incisors). All oral samples were subjected to low-speed centrifugation, after which the pellets were resuspended in 1 ml of PBS, split into 250-μL aliquots, and stored at -70°C until required.

2.1.4 Preparation of urine samples

Mid-stream urine samples were collected in a 5 ml tube then subjected to low-speed centrifugation, after which the pellets were resuspended in 1 ml of PBS, split into 250-μl aliquots, and stored at -70°C until required.
2.1.5 DNA extraction from blood

Dynabead preparations were extracted using the Geneclean III DNA extraction kit (BIO 101). 6M sodium iodide was added to the sample in the first step to lyse the cells and to inactivate any nuclease activity. A suspension of silica particles (EZ-glassmilk) was introduced and with mechanical agitation, the DNA became bound to the silica particles. The DNA was eluted in 50 μl of nuclease-free water.

2.1.6 DNA extraction from oral samples

200 μl aliquots of the pellet portion of the mouth rinse sample, gargle and brushed palatal samples were extracted using QIAamp kit (QIAGEN). Samples were eluted in 200 μl of nuclease-free water.

2.1.7 DNA extraction from urine

200 μl aliquots of the pellet portion of urine sample were extracted using QIAamp kit (QIAGEN). Samples were eluted in 200 μl of nuclease-free water.

2.1.8 PCR amplification of DNA from ORFs 26 and K1

The primary PCR for HHV-8 ORF 26 (also referred to as "KS330"), was carried out in a 25-μl reaction mixture containing nuclease-free water, PCR buffer (200 mM Tris HCL (PH 8.4), 500mM KCl), 4.0 mM MgCl₂, 10 mM each dNTP, 1 unit Taq DNA polymerase (Invitrogen), 20 pmol each first round primer and 2 μl extracted DNA. The secondary PCR mix was identical except 20 pmol of each inner primer was used and 2 μl of the first round product was added as template.
First-round PCR amplification of the first variable region of HHV-8 ORF K1 (K1/V1) was carried out in a 50-μl reaction mixture containing nuclease-free water, 1X buffer number 8 (Stratagene) containing (3.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.8), 75 mM KCl), 20 pmol each outer primer, 10 mM each dNTP and 1 unit Taq DNA polymerase (Invitrogen). To this 5 μl of extracted DNA was added. The second-round conditions were the same as the first except the addition of 20 pmol of each inner primer and 2 μl of the first round product was added as template.

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

Primer sequences for ORF 26 and ORF K1 are shown in Table 2.2. PCR using outer KS330 primers produce a product 233 bp in length from position 355 to 588 and using the inner primers yield a 211-bp product from position 366 to 577. Nucleotide positions are numbered according to the sequence deposited in GenBank under accession number U75698. PCR using the ORF K1 outer primers produces a 255-bp fragment from position 568 to 823 in the BCBL-1 K1 sequence (GenBank accession number U86667), while PCR using the inner primers produced a 246-bp fragment from position 573 to 819 in the BCBL-1 K1 sequence.
<table>
<thead>
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<th>Region</th>
<th>Position</th>
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<tr>
<td>ORF 26</td>
<td>Outer-sense</td>
<td>KS 1§</td>
<td>5'AGCCGAAAGGATTCCACCAT</td>
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<tr>
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<td>Outer-antisense</td>
<td>KS 2§</td>
<td>5'TCCGTGTTGTCTACGTCCAG</td>
</tr>
<tr>
<td></td>
<td>Inner-sense</td>
<td>Ksinn1†</td>
<td>5'TTCCACCATTGTGCTGAAT</td>
</tr>
<tr>
<td></td>
<td>Inner-antisense</td>
<td>Ksinn2†</td>
<td>5'TACGTCCAGACGATATGTGC</td>
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<td>ORF K1/V1</td>
<td>Outer-sense</td>
<td>K1inn5†</td>
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<td>K1inn6†</td>
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</tr>
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<td>Inner-sense</td>
<td>K1-N‡</td>
<td>5'GAGTGATTCAACGCCTTAC</td>
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</tbody>
</table>

§ (Di Alberti et al, 1997)
† (Zong et al., 2002)
‡ (Cook et al., 2002b)

**Table 2.2** Oligonucleotide primers used for the amplification of HHV-8 ORF 26 and ORF K1 fragments
2.1.9 PCR amplification of ORF 73 DNA

The internal repeat domain (IRD) of HHV-8 ORF 73 was amplified by nested PCR from extracts of a selected samples that were PCR-positive for ORF K1 and/or ORF 26. The IRD of ORF 73 is a highly variable region and the size of the product varies between 1300 bp to 1900 bp in HHV-8 infected cell lines (Zhang et al., 2000). For nested PCR, a primary PCR mixture was made up to 25 µl total volume containing nuclease-free water, 1.5 mM MgCl₂, 10 mM each dNTP, 2X PCRx enhancer solution (Invitrogen), 1.25 units platinum Taq DNA polymerase (Invitrogen), 50 pmol each outer primer, and 2 µl of extracted DNA. The second-round PCR was carried out under the same conditions except for the addition of 2 µl primary product and 50 pmol of each inner primer. The nucleotide sequences for first and second round primers are shown in Table 2.3. Amplification for both rounds of nested PCR was carried out for 35 cycles of 94°C for 30s, 58°C for 30s and 68°C for 2 min, preceded by a 5 min denaturation step and ending with a final extension step of 5 min at 68°C (Zhang et al., 2000; Cook et al., 2002a).

2.1.10 Separation and visualisation of PCR products

PCR products were mixed with 2 µl Orange G loading dye and loaded onto a 2% agarose gel (SB fine gel, Severn Biotech Ltd.) along with 1 µg of 1 kb DNA ladder (Invitrogen) to estimate the size of the PCR product. Electrophoresis took place in a 1X TRIS-borate-EDTA buffer (TBE, Invitrogen) after which the gel was stained in a solution of 5 µg/ml ethidium bromide in TBE buffer. The bands were visualised using short wave UV transillumination.
<table>
<thead>
<tr>
<th>Region</th>
<th>Position</th>
<th>Name</th>
<th>Primer Sequence</th>
</tr>
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<tbody>
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<td>ORF 73 IRD</td>
<td>Outer-sense†</td>
<td>IRD1-F</td>
<td>5’ACGCCAACCACCTACATCT</td>
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<tr>
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<td>Outer-antisense†</td>
<td>IRD1-R</td>
<td>5’TCACTGTCGCTAACACAGG</td>
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<tr>
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<td>Inner-sense*</td>
<td>LNAII F</td>
<td>5’ATGGGGACACGAGATTAGC</td>
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<tr>
<td></td>
<td>Inner-antisense*</td>
<td>LNAII R</td>
<td>5’CGACCCGTGAAAGATATG</td>
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</tbody>
</table>

† (Cook et al., 2002a)
*(Zhang et al., 2000)

**Table 2.3** Oligonucleotide primers used for the amplification of the IRD of HHV-8 ORF 73.
2.1.11 Procedures to minimise contamination

To minimize contamination during PCR, DNA extraction, PCR reagent preparation, thermocycling, and post-PCR procedures were conducted in dedicated rooms. Appropriate negative and positive control specimens were included in each PCR. In addition, DNA extraction and PCR were repeated for those samples showing intra-individual HHV-8 subgenomic sequence variation.

2.2 Amplification of CMV sub-genomic DNA

2.2.1 PCR amplification of ORF UL73 (gN) DNA

The primary PCR for CMV ORF UL73 (gN) was carried out in a 50-µl reaction mixture containing nuclease-free water, PCR buffer (200 mM Tris HCL (PH 8.4), 500 mM KCl), 25.0 mM MgCl₂, 10 mM each dNTP, 1 unit Taq DNA polymerase (Invitrogen), 10 pmol each of first round primers and 5 µl extracted DNA. The secondary PCR mix was identical except that 10 pmol of each inner primer was used and 2 µl of the first round product was added as template. Amplification of UL73 region was carried out under the PCR thermocycling conditions: samples were heated to 96°C for 1 min followed by 35 cycles of 96°C for 1 min, 55°C for 1 min, and 72°C for 1 min followed by a 10 min extension period at 72°C. Primer sequences for ORF UL73 are shown in Table 2.4. The ORF UL73 outer primers produced a 416-bp fragment from position 106668 to 107084 in the AD169 sequence (GenBank accession number BK000394). The inner primers produced a 304 bp fragment from position 106728 to 107031 in the AD169 sequence.
2.2.2 PCR amplification of ORF UL74 (gO) DNA

The first round PCR for CMV ORF UL74 (gO) was carried out in a 50-μl reaction mixture containing nuclease-free water, PCR buffer (200 mM Tris HCL (PH 8.4), 500 mM KCl), 25.0 mM MgCl₂, 10 mM each dNTP, 1 unit Taq DNA polymerase (Invitrogen, Paisley, UK), 10 pmol each of first round primers and 5 μl extracted DNA. The secondary PCR mix was identical except 10 pmol of each inner primer was used and 2 μl of the first round product was added as template. Amplification of UL73 region was carried out under the PCR thermocycling conditions: samples being heated to 95°C for 3 min followed by 35 cycles of 96°C for 1 min, 55°C for 1 min, and 72°C for 1 min followed by a 10 min extension period at 72°C. Primer sequences for ORF UL74 are shown in Table 2.4. Outer ORF UL74 primers generate a product 1400 bp in length from position 107056 to 108456 and the inner primers yielded a 433-bp product from position 107065 to 107503. Nucleotide positions are numbered according to the complete genome sequence of the AD169 strain of CMV deposited in GenBank under accession number BK000394.

2.3 DNA sequencing

2.3.1 Automated sequencing

All samples were sequenced using the Beckman CEQ2000 automated capillary array sequencer. Samples for sequencing were separated by electrophoresis on a 2% agarose gel and bands identified. Bands were then excised and purified using a spin column based purification kit (Amersham) following the manufacturer’s instructions. To estimate how much DNA to add the sequencing reaction, 2 μl of purified product was electrophoresed through an agarose gel and compared with 1 μg of 1 kb ladder (Invitrogen). Purified DNA was added to the PCR sequencing reaction consisting of
# Chapter 2 Methods and Materials

<table>
<thead>
<tr>
<th>Region</th>
<th>Position</th>
<th>Name</th>
<th>Sequence</th>
</tr>
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<td>ORF UL73</td>
<td>Outer-sense</td>
<td>gN-up(^{1})</td>
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<td>Inner-sense</td>
<td>gN-3</td>
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<td>gN-4</td>
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<td>Inner-sense</td>
<td>gO2(^{4})</td>
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<td>Outer/Inner-antisense</td>
<td>UL74-2(^{4})</td>
<td>5' GACATTGCTGGATCCAGACTTTA</td>
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§ (Pignatelli et al., 2003)

‡ (Paterson et al., 2002)

**Table 2.4** Oligonucleotide primers used for the amplification of CMV ORF UL73 and ORF UL74 fragments.
the following components: purified DNA 3 μl, sterile water 3 μl, inner PCR primers (3pmol) 2 μl, and 12 μl Beckman CS sequencing kit mix (Beckman) to a total volume of 20 μl.

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

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

2.3.2 Analysis of sequence data

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

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

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

DNADIST creates a DNA matrix using one of three possible models of substitution; Jukes and Cantor (Jukes and Cantor, 1969), Kimura (Kimura, 1980) and a maximum likelihood model (Felsenstein, 1981). The Kimura “2-parameter” method is used most often and assumes that independent changes occur at all sites with equal probability and allows for differences in transition and transversions rates. The output format of DNADIST is a matrix where genetic distances between sequences
are roughly equivalent to percent nucleotide divergence values created using Megalign. The genetic distance values between species can be used in other programs to estimate branch lengths and draw trees. The Kimura "2-parameter" method was used to create distance matrixes from sequence data in this study.

Trees were drawn using the NEIGHBOR program, which utilised either the neighbour-joining algorithm (Saitou and Nei, 1987) or the UPGMA method of clustering (Sneath and Sokal, 1973). NEIGHBOR creates successive clusters of closely related sequences and minimises branch lengths as the lineages join. No further rearrangements were made to the tree and because NEIGHBOR does not assume an evolutionary clock and the resulting tree is unrooted when viewed in TREEVIEW programme.

2.4 Restriction fragment-length polymorphism analysis of PCR sequence diversity in products

2.4.1 Amplification of ORF 73 DNA for RFLP analysis

The IRD of HHV-8 ORF 73 was amplified by nested PCR as described in Section 2.1.9.

2.4.2 Detection of PCR products

PCR products were separated and visualised as described in Section 2.1.10 except that samples were run on a 1% agarose gel alongside 1 μg of a 1 kb ladder.
2.4.3 Restriction digests of PCR products

Digests were carried out in a reaction mixture consisting of 5-7.9 µl of PCR product, 0.1 µl bovine serum albumin (BSA), 1 µl Buffer C (Promega), 0.5 µl MboI and 0.5 µl BanII (Promega), plus nuclease-free water to 10 µl if necessary. Digests were heated to 37°C for 1 hr, the optimal temperature for enzyme activity, and at 85°C for 30 min to inactivate the enzymes. Digests were stored at 4°C until they could be separated and visualised on a 2% agarose gel alongside 1 µg of 100 bp ladder (Invitrogen) to estimate the size of resulting bands. Digital images of gels were captured using the Kodak Digital Science DC40 camera (Zhang et al., 2000; Cook et al., 2002a).

2.5 Screening for intrasample K1/V1 sequence differences by denaturing gradient gel electrophoresis

2.5.1 K1/V1 Expand high fidelity PCR

From each person, samples that amplified positively for K1/V1 and for which sequences differed between body compartments were subjected again to nested PCR using same conditions as in Section 2.1.8 except that 0.7 units of the EXPAND High Fidelity PCR System (Roche Diagnostics) were used instead of Taq DNA polymerase. Clones were generated from each K1/V1 PCR product by use of the TOPO TA Cloning System (Invitrogen) as detailed in Section 2.5.2.

2.5.2 TA cloning

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

### 2.5.3 GC-clamp colony PCR

For each colony, another round of PCR was done under conditions identical to the
second-round high-fidelity PCR, except that a "clamping" primer was used in place
of the forward inner sense primer. The clamping primer contains a guanine-cytosine
rich domain with a high melting temperature, to prevent complete denaturation of the
PCR product (Woodward *et al.*, 1994). This primer, K1-1 clamp, has the following
sequence:

\[
5'CGCCCCCGCGCCGGCCGGCTCCGGCGCCCCCGCCCCGGAGTGATTTCAACGC
\]

The sequence for the reverse inner sense primer is as described in Table 2.2. Colony
PCR products were separated and visualised on an agarose gel as described in
Section 2.1.10.
2.5.4 Preparing denaturing gradient polyacrylamide gels

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

2.5.5 Loading and electrophoresis

Clamped colony PCR products (2 µl) generated as described in Section 2.5.3 were mixed with an equal volume of Orange G loading buffer. After all the samples were loaded into the wells, electrophoresis in 0.6X TAE buffer at 60°C and 100 V took place over 18 hr. The electrophoresis apparatus allowed for continuous flow of buffer to maintain a constant temperature of 60°C throughout the run (Igeny PhorU).

2.5.6 Staining of gels

Gels were stained in a solution of 0.5X SYBR Green I (Flowgen) in 200 ml of 0.6X TAE buffer for 20 min to 1 hr. Bands were visualised by ultraviolet transillumination and gel images captured using a Kodak Digital Science DC40 camera.
Chapter 3

Intra-Person Oral HHV-8 Infection
3.1 Introduction

In Africa, HHV-8 commonly is acquired in childhood. Transmission tends to be intrafamilial, and the dominant route is from mother to child as suggested by a seroepidemiological survey of 1337 individuals in a village in French Guiana (Plancoulaine et al., 2000). Extrafamilial nonsexual transmission also has been identified (Cook et al., 2002a; Cook et al., 2002b). Studies among homosexual men in North America implicate the mouth and oropharynx as dominant sites of HHV-8 shedding and saliva as an important vehicle of HHV-8 transmission (Corey et al., 2002). Two reports show that, in Africa, too, HHV-8 has a predilection to be shed in saliva. The first is a study of Egyptian children with acute fever not due to a specific viral exanthem (Andreoni et al., 2002). In 86 children studied, 36 (41.9%) were HHV-8 seropositive, and HHV-8 DNA sequences could be amplified from saliva of 11 (30%) compared to 3 (8.3%) in whom sequences could be amplified from plasma only. Moreover, HHV-8 DNA was detected in saliva of 6 of 50 HHV-8-seronegative children (12%) compared to 2 (4%) in plasma. Thus, children may carry HHV-8 more in saliva than blood. The second report relates to a study conducted in Malawi of asymptomatic family members of patients with KS whose HHV-8 genome detection rate was found to be significantly higher in oral than in blood samples: HHV-8 DNA sequences could be detected in mouth-rinses of 18 (27%) out of 67 family members but not in any blood samples (Cook et al., 2002b).

Saliva or saliva-contaminated objects might facilitate the intra- and extrafamilial spread of HHV-8 in Africa. Assuming that, in these communities there, the main portal of entry of HHV-8 is the mouth, exposure of susceptible persons to the virus is likely to begin early in life. Although it is unclear how immunological responses
generated by the host after first exposure protect against infection from subsequent exposures, it would appear that humoral responses may not be neutralizing, because HHV-8 can actively replicate in people who are seropositive for the virus (Tedeschi et al., 2001). Accordingly, people growing up and living in regions where HHV-8 is hyperendemic are potential hosts to multiple HHV-8 strains. This chapter describes an investigation into the presence of the HHV-8 genome in blood and several different oral compartments, in a group of people residing in Malawi, and an evaluation of the extent to which, in each person, the genomes found in one compartment diverge from those in another.

3.2 Patients, materials, and methods

3.2.1 Patients and samples
The study group included patients with KS (n = 22) and their first-degree relatives (n = 67), a group of subjects described in detail elsewhere (Section 2.1.1 and Table 2.1). Venous blood and the following oral samples: mouth rinse, throat gargle, and palatal exfoliate were obtained. A sampling order of the oral components was imposed to minimize cross-contamination between compartments as described in Section 2.1.3.

3.2.2 Sample processing
After preparation of the blood samples as described in Section 2.1.2, DNA was extracted from the fractionated leukocytes by use of Geneclean III as described in Section 2.1.5. Oral samples were prepared as detailed in Section 2.1.3, and DNA was extracted by use of the QIAamp kit as described in Section 2.1.6.
3.2.3 Length polymorphism and RFLP analyses of DNA amplified from the ORF 73 internal repeat domain

A nested polymerase chain reaction (PCR) protocol was applied to sample extracts to amplify a DNA segment from the IRD of ORF 73, as described in Section 2.1.9. To further evaluate sequence polymorphism within IRD, PCR products were visualised and digested as described in Sections 2.4.2 and 2.4.3.

3.2.4 Sequencing analyses of DNA amplified from ORFs 26 and K1

The 211-bp KS330 was amplified from sample extracts by use of nested PCR, as described in Section 2.1.8. The 246-bp K1/V1 was amplified from sample extracts by nested PCR, as described in Section 2.1.8. To minimize contamination during PCR specific measures were taken as described in Section 2.1.11. PCR products were sequenced, raw DNA sequence data were analyzed and phylogenetic analyses were performed as detailed in Section 2.3. Chromatograms from some samples, e.g., from Patients P and Y, which subsequently showed mixed infection following analysis of their PCR clones, did not yield evidence of mixed infection during direct sequencing (i.e., double peaks over certain base positions were not clearly evident). The inability of direct sequencing to identify mixed infection may be due to the presence of minority sequences that constitute <20% of the total population. Past studies have shown that minority sequences require to be present at >20% of the population for chromatographic peaks to be evident (Ngui and Teo, 1997).
3.2.5 Screening for intrasample K1/V1 sequence differences by denaturing gradient gel electrophoresis

From each person, samples that amplified positively for K1/V1 and for which sequences differed between body compartments were subjected again to nested PCR by use of the EXPAND High Fidelity PCR System as described in Section 2.5.1. Clones were generated from each K1/V1 PCR product as described in Section 2.5.2. For each colony, another round of PCR was done using clamping primer as described in Section 2.5.3. The PCR products were subjected to DGGE as detailed in Sections 2.5.4 and 2.5.5, followed by nucleotide sequencing as described in Sections 2.3.

For the purpose of this study, subgenotypic differences were considered to be significant if the divergence between K1/V1 sequences was set at $\geq 5\%$ to accommodate base incorporation errors due to Taq polymerase error-prone activity. Further indication that differences from sequences that show $\geq 5\%$ base variation are indeed due to the sequences that originate from distinct virus strains arises from very high bootstrap values ($>95\%$) obtained from such sequences.

3.3 Results

3.3.1 Patient and sample characteristics

Table 3.1 summarizes the characteristics of 24 of the 89 people (27%) who were found to carry amplifiable HHV-8 subgenomic DNA in $>1$ sample. Thirteen (54%) of the 24 were male. Patients with index cases of KS are assigned the "i" suffix, and their family members were assigned numerals; letters denote family assignments as described in Section 2.1.1. Ages ranged between 2 and 44 years (mean, 18 years). Seven (29%) patients had KS, 9 (38%) patients were human immunodeficiency virus (HIV)-1-seropositive, and 21 (88%) patients were HHV-8 seropositive. The rate of
HHV-8 subgenomic DNA detectability varied according to the type of sample and the target of amplification within the HHV-8 genome (Table 3.1). In mouth-rinse samples, the detectability rates were 38% for (ORF) 73 internal repeat domain (IRD), 75% for KS330, and 92% for K1/V1 DNA; in throat-gargle samples, the detectability rates were 22% for IRD, 48% for KS330, and 70% for K1/V1 DNA; in palatal-exfoliate samples, the detectability rates were 0% for IRD, 38% for KS330, and 50% for K1/V1 DNA; and, in blood samples, the detectability rates were 4% for IRD, 17% for KS330, and 21% for K1/V1 DNA. The detectability rates according to the number of amplifiable subgenomic DNA segments were as follows: for all 3 segments, 33% for mouth-rinse samples, 22% for throat-gargle samples, 4% for blood samples, and 0% for palatal-exfoliate samples; with at least 2 segments, 42% for mouth-rinse samples, 26% for throat-gargle samples, 21% for palatal-exfoliate samples, and 13% for blood sample; and, with at least 1 segment, 46% for palatal-exfoliate samples, 22% for throat-gargle samples, 21% for mouth-rinse samples, and 4% for blood samples.

3.3.2 IRD length polymorphism detected by RFLP analysis

Figure 3.1 illustrates electrophoretic banding patterns of IRD PCR products derived from 5 people from whom products could be amplified from >1 sample. Length polymorphisms of the products are displayed in the upper panel, whereas RFLPs are shown in the lower panel. Intraperson length polymorphism and RFLP were absent in all patients except 1, T2, in whom the IRD amplicons generated from his mouth-rinse and throat-gargle samples were polymorphic for length, both without and with BanII and MboI restriction.
Table 3.1 Patient's demographic and virological characteristics. Anti-HHV-8 serostatus had been previously determined by anti HHV-8 IgG immunofluorescence assay (Cook et al. 2002b). Anti-HIV status had previously been determined by anti-HIV particle assay (Cook et al. 2002b). ND, not done. Samples from individuals whose K1/V1 sequences were further studied (summarized in Figures 3.4, 3.5, and 3.6, and 3.7) are boxed. Sites affected by KS in patients with KS: Ci, abdomen and limbs; Ei, legs and palate; Fi, foot; Ki, leg; Qi, foot; Yi, arm; Zi, neck.
Figure 3.1 Gel-electrophoretic length polymorphisms of internal repeat domain amplicons without (A) and with (B) restriction by MboI and BanII. DNA marker bands (1Kb) for (A) and (100 bp) for (B) are shown on the left. Letters after patient identifiers: g, gargle; r, rinse.
3.3.3 Differences in KS330 consensus sequences

For 16 people, KS330 DNA could be amplified from at least 2 samples, and, for 6 people (Ci, Fi, Ki, T2, W4 and X1), KS330 DNA was amplifiable from 3 samples. The sequences, which are deposited in GenBank (accession nos. AY219429–AY219458), cluster with ORF 26 sequences of known African HHV-8 strains (Poole et al., 1999). Identical KS330 sequences were observed in the samples from 9 people (B5, Ci, E6, Fi, G2, K1, W2, X1, and Z2). Non identical sequences were found in samples from 4 subjects (Ei, E4, Qi, and W1). Identical and non identical sequences were observed in samples from 3 subjects (Ki, T2 and W4). A phylogenetic tree displaying the variation in consensus KS330 sequences among the samples is shown in Figure 3.2. For all subjects with non identical sequences in samples, intraperson divergences were <3%, and the sequences were genotypically identical except for one person, Ei, the sequence from the blood sample was 4.6% divergent from that from the throat-gargle sample, and their genotype assignments were different: genotype B/C for the blood sequence and genotype B for the throat-gargle sequence.

3.3.4 Differences in K1/V1 consensus sequences

For 24 people, K1/V1 DNA could be amplified from at least 2 samples. K1/V1 sequences were identical between at least 2 samples from 8 people (E6, H2, Ki, K1, P1, X1, Zi and Z2); for 1 person, Zi, sequences in 3 samples (mouth rinse, palatal exfoliate, and blood) were identical. Nonidentical sequences were found in samples from 16 people, with intraperson divergences ranging from 0.6% (between the mouth-rinse and throat-gargle samples of E4) to 28.9% (between the palatal-exfoliate and throat-gargle samples of Y1). A phylogenetic tree displaying the
Figure 3.2 Predicted phylogenetic distribution of consensus KS330 sequences derived from blood and oral samples from 16 people. Bootstrapping for 1000 replicates is noted as a percentage at major branch points. Horizontal line at bottom left represents 1% nt substitution for that horizontal branch length. Letters after patient identifiers: b, blood; g, gargle; p, palate; r, rinse.
variation in consensus K1/V1 sequences among the samples is shown in Figure 3.3. The sequences primarily cluster with those reported from African patients with KS (Poole et al., 1999; Cook et al., 2002b) and have been deposited in GenBank (accession nos. AY219483-AY219535). Seven of the 16 people with nonidentical intraperson K1/V1 sequences yielded samples that carried sequences belonging to 2 genotypes. These were the palatal-exfoliate and blood samples from Fi (A2 and B, respectively); mouth-rinse and throat-gargle samples from T3 (B and A2, respectively); mouth-rinse and throat-gargle samples from W2 (B and A2, respectively); throat-gargle and palatal-exfoliate samples from Y1 (A2 and B, respectively); and mouth-rinse and palatal-exfoliate samples from Y2 (A2 and A5, respectively). For Ki, the identical sequences from her blood and mouth-rinse samples (genotype A5) were different than those from her palatal-exfoliate sample (genotype A2), whereas for P1, the identical sequences from her mouth-rinse and throat-gargle samples (genotype A5) were different to those from her palatal-exfoliate sample (genotype B). For 1 other person, I2, K1/V1 sequences belonged to 3 genotypes (mouth rinse, A1; throat gargle, B; and palatal exfoliate, C2). For 3 people whose samples were found to carry nonidentical sequences of the same genotype (all genotype B), intraperson/intersample sequences were >5% divergent: Cir/Cig (5.4%), T2g/T2r (5.4%), and Yir/Yip (5.9%).

3.3.5 Clonal analysis of intraperson/intersample K1/V1 sequence differences

High-fidelity PCR of sample extracts from 9 people (Fi, G2, I2, Ki, P1, T2, W2, X1, and Yi) yielded K1/V1 DNA. Clones were generated from which differences in nucleotide sequence could be analyzed by the combined DGGE-sequencing approach. Figure 3.4 is a composite of representative DGGE gel pictures showing
Figure 3.3 Predicted phylogenetic method distribution of consensus K1/V1 sequences derived from blood and oral samples from 24 people. Samples from individuals whose K1/V1 sequences were further studied (summarized in Figures 3.4, 3.5, 3.6, and 3.7) are boxed. Bootstrapping for 1000 replicates is noted as a percentage at major branch points. Horizontal line at bottom left represents 10% nt substitution for that horizontal branch length. Letters after patient identifiers: b, blood; g, gargle; p, palate; r, rinse.
Figure 3.4 Composite of 9 representative DGGE gel photographs. Each gel accommodates, for a given individual, K1/V1 DNA amplified from up to 15 K1/V1 clones generated per sample. Dots at bottom indicate polymerase chain reaction products that underwent sequencing; numerals indicate lane positions in the gel.
migration distances achieved by amplicons generated from clonal inserts; each gel accommodates, for a given person, K1/V1 DNA derived from up to 15 clones/sample. All PCR products that yielded minority banding positions and some products representing the majority were sequenced (denoted by dots in Figure 3.4; numerals correspond to lane positions in the gel illustrated). Figure 3.5 is a nonrooted phylogenetic tree depicting the extent of intersample K1/V1 nucleotide sequence diversity. Sequences derived from the PCR clones have been deposited in GenBank (accession nos. AY220915-AY220981). Figure 3.6 shows an alignment of a stretch of amino acid sequences predicted from the nucleotide sequences studied. DGGE and nucleotide sequencing analyses confirm findings from direct K1/V1 PCR sequencing that >1 genotype was carried by Fi, I2, Ki, P1 and W2. Analysis of clones from minority sequences permitted intrasample K1/V1 variability to be further characterized from individual samples. For the mouth-rinse sample from I2, the existence of a group of minority sequences comprising I2r 5 and 8 became evident, with both being 6.4% divergent from the majority sequence I2r4; all 3 sequences belonged to genotype B. In addition, a small population of closely related genotype A1 sequences (I2r 1, 3, 13, and 14) was identifiable.

For Ki and P1, the presence of minority sequences belonging to genotypes not evident after direct sequencing could be revealed. In the mouth-rinse sample from Ki, the genotype of the minority sequences Kir 8/10 and 9 (B) was different than that of the majority sequence Kir 4 (A5). Similarly, from her palatal-exfoliate sample, the genotype of a set of related minority sequences Kip 17, 23, and 27 (A2) and of a unique sequence, Kip 29 (B), were different than that of the dominant sequence, Kip 19 (A5). The 2 genotype B mouth-rinse nucleotide sequences, Kir 8/10 and 9, were
Figure 3.5 Radical unrooted phylogenetic tree of sequences amplified from PCR clones with K1/V1 inserts. Sequences are derived from blood and oral samples from 9 people. Horizontal line at top left represents 10% nt substitution for that horizontal branch length. Letters after patient identifiers: b, blood; g, gargle; p, palate; r, rinse.
Figure 3.6 Alignment of amino acid sequences predicted from K1/V1 sequences depicted in Figures 3.5. The variable 1 (V1) region is indicated at top. Dots indicate residues occupying positions aligned to residues of Fip 3/8/14 at top. Positions at which residues that have undergone intraperson/intersample and intraperson/intrasample changes are highlighted.
5.0% and 5.4% divergent, respectively, from Kip 29 (Figure 3.5). There were, thus,
a total of 4 different groups of sequences that originated from Ki's mouth-rinse and
palatal-exfoliate samples. From the palatal-exfoliate sample of P1, an additional
 genotype (A5) could be assigned to a population of clones bearing the P1p 16/24/29
sequence, contrasting with the genotype (B) assigned to the majority sequence (P1p
17).

For 2 people, X1 and Yi, direct sequencing of K1/V1 from their samples generated
sequences assignable as genotype B, but, for each person, clonal analysis revealed
carriage of minority sequences belonging to another genotype. For the throat-gargle
sample from X1, this was A5 (assigned to X1g28); and for the palatal-exfoliate
sample from Yi, it was A2 (assigned to sequences typified by Yip 17/28, 20, 21/26
and 27/29). X1's mouth-rinse sample carried the following minority sequences:
X1r6; and a closely related group of sequences, X1r 3/11, 9, and 13, which were all
5.0% divergent from X1r6.

For T2, although direct sequencing yielded genotype B sequences from both his
mouth-rinse and throat-gargle samples, analysis of the clones generated revealed that
each sample carried its own distinct set of sequences: T2r 9 and 15 from the mouth-
rinse sample, and T2g16/18/24, 20, and 22 from the throat-gargle sample; the latter
sequences were 5.9%, 6.4%, and 6.4% divergent, respectively, from T2r 9. The intra-
and intersample distribution of genotypes and subgenotypically distinct sequences
recovered from the 9 people described here is summarized in Figure 3.7.
<table>
<thead>
<tr>
<th>Person</th>
<th>No genotypes</th>
<th>No sequences with ≥5% divergence</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Sample</td>
<td>Per sample</td>
</tr>
<tr>
<td>Fi</td>
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</tr>
<tr>
<td></td>
<td>blood</td>
<td>1(B)</td>
</tr>
<tr>
<td>G2</td>
<td>rinse</td>
<td>1(A5)</td>
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<tr>
<td></td>
<td>gargle</td>
<td>1(A5)</td>
</tr>
<tr>
<td>I2</td>
<td>rinse</td>
<td>2 (A1, B)</td>
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<tr>
<td></td>
<td>gargle</td>
<td>1(B)</td>
</tr>
<tr>
<td></td>
<td>palate</td>
<td>1(C2)</td>
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<td>rinse</td>
<td>2 (A5, B)</td>
</tr>
<tr>
<td></td>
<td>palate</td>
<td>3 (A2, A5, B)</td>
</tr>
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<td>rinse</td>
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</tr>
<tr>
<td></td>
<td>palate</td>
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</tr>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
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<td>1(B)</td>
</tr>
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<td></td>
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</tr>
<tr>
<td></td>
<td>palate</td>
<td>2 (A2, B)</td>
</tr>
</tbody>
</table>

**Figure 3.7** Intrasample and intraperson distribution of genotypically and subgenotypically distinct K1/V1 sequences.
Possible linkages between the KS330 and K1/V1 genotypes (as determined by direct sequencing) were analyzed (Table 3.2): The KS330 B/C genotype was linked to the K1/V1 genotype B in 15 patients and to the K1/V1 genotype in 20 patients. The KS330 genotype B was linked to the K1/V1 genotype B in 7 patients, to the K1/V1 genotype A2 in 2 patients and to the K1/V1 genotype A5 in 6 patients.

3.4 Discussion

Multiple HHV-8 infection rarely has been considered. A study of different HHV-8 DNA loci in 63 KS and PEL patients samples, showing that 13 had intertypic recombinations provided evidence of putative recombinant HHV-8 genomes (Poole et al., 1999), assuming dual infection in at least a single host cell. Most studies of humans who have developed KS (Stebbing et al., 2001; Meng et al., 2001; Zong et al., 2002) or multiple HHV-8-associated lesions (Codish et al., 2000) have not shown intraperson variation in HHV-8 subgenomic sequences amplified by PCR from blood and lesional tissues.

Gao et al. (1999) reported that, of 27 KS lesional samples studied, 2 were dually infected. Otherwise, there are no reports of mixed HHV-8 infection. This paucity may be due to a combination of factors. First, samples from homosexual men favor hosts who are exposed to HHV-8 later in life, rather than early in childhood, and favor HHV-8 strains transmitted via sexual routes, rather than those transmitted nonsexually within the household or community. Second, previous studies have been largely confined to patients with KS or patients who are at risk of developing KS. When sequence polymorphism of subgenomic HHV-8 DNA in the blood is sought from such people, the tendency is for none to be found, particularly when consensus
### Table 3.2 Linkage analysis of KS330 and K1/V1 genotypes.

<table>
<thead>
<tr>
<th>Patient sample</th>
<th>KS330 genotype</th>
<th>K1/V1 genotype</th>
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<td>B5r</td>
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<td>B</td>
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</tr>
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<td>B/C</td>
<td>B</td>
</tr>
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</tr>
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</table>
sequencing is applied, because the high HHV-8 genome load (Tedeschi et al., 2001) favors the generation of sequences reflecting the dominant variants. When sequence polymorphism in KS lesional tissues is sought, it is also likely that none will be found, not because of the high viral genome load (Lallemand et al., 2000; Bezold et al., 2001) but because KS is largely a clonal tumor (Rabkin et al., 1997; Judde et al., 2000). Third, in previous studies examining for HHV-8 genomic polymorphisms, the PCR products were directly amplified from the samples (Gao et al., 1999; Poole et al., 1999; Codish et al., 2000; Stebbing et al., 2001; Meng et al., 2001; Zong et al., 2002); in doing so, the existence of minor virus populations would have been revealed only if they constituted a relatively large fraction (>20%) of the entire population (Ngui and Teo, 1997). When consensus sequencing protocols are applied, the sequence information obtained will not differentiate minority sequences from the sequence of the dominant variant.

The present study focuses on whether intrahost HHV-8 subgenomic sequence polymorphism exists among and within oral and blood samples of Malawian individuals with and without KS and the extent of such polymorphism. This group of HHV-8–infected people is a subset of a population whose characteristics and possible routes of HHV-8 acquisition have been described elsewhere (Cook et al., 2002a; Cook et al., 2002b). Although the report by Cook et al. (2002b) focused on interperson HHV-8 subgenomic sequence variation, the present study focuses on intraperson variation. Accordingly, sequence polymorphism data from 2 other oral components, in addition to the mouth-rinse sample (which principally permits characterization of HHV-8 carried in the nonkeratinized oral compartment), are presented here: the throat-gargle sample, to reflect HHV-8 carried predominantly in
the pharyngeal compartment, and the palatal-exfoliate sample, which has consistently been shown in HIV-coinfected people to be the most common site of development for KS (Flaitz et al., 1997; Gorsky and Epstein, 2000), suggesting that HHV-8 might be particularly tropic for this anatomical site. These 2 other oral samples were collected at the same time as the blood and mouth-rinse samples.

The study presented in this chapter is limited by several factors. The first is the variability in PCR amplification yields from the various samples. This variability depends on the type of sample examined. That mouth-rinse and throat-gargle samples yielded higher amplification rates than did blood and palatal-exfoliate samples probably reflects active HHV-8 replication in the oral epithelium other than the hard palate, the high load of shedding into the oral cavity (Pauk et al., 2000), and the possible lower replicative activity of the virus systemically and in the palatal mucosa. Blood samples yielded HHV-8 subgenomic amplicons in a substantial proportion of patients with KS but not at all in those without KS. This result confirms previous data (Cook et al., 2002b) showing that, in HHV-8 infected African people not affected by KS, the virus is not shed into the circulation to a significant extent and that the principal site of viral shedding is the mouth. Variability in amplification rates also depended on the region within the HHV-8 genome to be amplified. Higher yields from KS330 and K1/V1, rather than from the IRD, are due to the much longer amplicon that needs to be generated to permit subsequent analyses.

A further limiting factor was the difference in the degree to which the 3 approaches permitted evaluation of subgenomic HHV-8 sequence differences. Analysis of length
polymorphisms in IRD amplicons was the least efficient approach to discriminating between HHV-8 variants, because of its dependence on the number of repeat sequences in the IRD and on the presence of restriction sites, both of which are few. Less finite are the individual nucleotide positions that differ between viral variants. Direct sequencing studies of a highly variable region, such as K1/V1, yield data that discriminate between variants better than one that is more conserved, such as KS330. KS330 sequence polymorphism generally does not provide sufficient resolution for inferences of HHV-8 genotype and strain variation to be made. Nevertheless, KS330 sequence studies potentially provide useful information on genotype differences if variations are sufficiently wide and, furthermore, generate data that control for artifactual sequence differences due to PCR-induced nucleotide misincorporation.

Direct sequencing studies have an important limitation in that they are able to produce consensus sequence data that will not allow sequences of minority variants to be identified and characterized. Accordingly, the combined DGGE nucleotide sequencing protocol was developed and applied to K1/V1 amplicons generated from clonal inserts. This approach, presents the third limiting factor. To reduce the degree to which PCR induces nucleotide misincorporation, which may lead to false representations of natural intraperson/intersample sequence polymorphism, high-fidelity PCR was applied to sample extracts. High-fidelity PCR was, however, relatively inefficient in generating the amplicons. This explains why, of extracts from the 24 people from whom intersample K1/V1 sequence differences could be evaluated by direct sequencing, K1/V1 amplicons were generated from sample extracts from only 9 people.
Evidence is provided here of multiple HHV-8 infection in a substantial proportion (60%) of the 24 Malawian people studied. The evidence was derived from intraperson genotype and subgenotype sequence differences found among and within samples. Genotypic differences are clear between the blood and oral samples from 3 patients with KS: for Ei, from whom KS330 sequences were genotypically distinct; and for Fi and Ki, from whom K1/V1 sequences were genotypically distinct. For 4 other patients with KS, sequence variations in KS330 or K1/V1, or both, in blood and oral samples were either absent (for Zi) or not different enough to permit genotypic or subgenotypic discrimination (for Ci, Ei, and Qi). Because HHV-8 genomic sequences carried in blood and oral samples from these 7 patients with KS could be either genotypically identical or different, conclusions cannot be drawn about the selective tropism of HHV-8 strains. Current evidence from other studies suggests that HHV-8 exhibits broad tropism (Dupin et al., 1999; Pauk et al., 2000; Blackbourn et al., 2000).

Evidence for the oral carriage of multiple strains of HHV-8 can be found for 16 people using direct sequencing data on KS330, K1/V1 and IRD length polymorphism or combination of them, 4 (Ci, Ei, Ki and Yi) with KS and 12 (B5, E4, I2, G2, P1, T2, T3, W1, W2, W4, Y1, and Y2) without KS. In 8 people, intersample genotype differences are clearly discernible in oral samples: for T2, from IRD length polymorphism findings; and for I2, Ki, P1, T3, W2, Y1, and Y2, from direct sequencing data on K1/V1. Of particular interest are the 3 samples from I2, each of which yielded K1/V1 sequences belonging to a different genotype. Analyses of data from PCR clones revealed genotypic differences in individual samples from I2 (mouth rinse), Ki (mouth rinse and palatal exfoliate), P1 (palatal exfoliate), W2
(throat gargle), X1 (throat gargle), and Yi (palatal exfoliate). Sequences belonging to 3 different genotypes were found in the palatal-exfoliate sample from Ki. For 2 people whose oral samples yielded genotypically identical but distinct HHV-8 variants, clonal analyses further revealed subgenotypic differences: for T2, in mouth-rinse and throat-gargle samples; and for Yi, in palatal-exfoliate sample. For 2 other people whose oral samples yielded genotypically identical HHV-8 variants, analysis of PCR clones also revealed subgenotypic differences: for G2, in mouth-rinse sample; and for X1, in the mouth-rinse and throat-gargle samples. Whether, for these 4 people, the distinct but closely related variants originated from founder strains that underwent genetic drift after infection or were introduced at different exposure time points cannot yet be determined.

HHV-8 DNA could be detected in oral samples from 3 anti–HHV-8–seronegative people (I2, K1, and Y2). All 3 subjects were children and were anti–HIV-1 seronegative (Table 3.1). It is likely that the samples were obtained at the early phase of primary HHV-8 infection (before anti–HHV-8 is mounted to a detectable extent), but follow-up testing of the anti–HHV-8 serostatus would need to be done to monitor for HHV-8 seroconversion. That the K1/V1 amplicons obtained from these children were not due to PCR-related contamination is suggested by the uniqueness of K1/V1 sequences derived from I2's mouth-rinse, throat-gargle, and palatal-exfoliate samples, and Y2's mouth-rinse and palatal-exfoliate samples (Figure 3.3). The sequence derived from K1's mouth-rinse and palatal-exfoliate samples was identical to that from the blood and mouth-rinse samples from Ki, his mother, a result that is consistent with the dynamics of intrafamilial transmission (Cook et al., 2002b).
Although there is constant mixing of cellular components and secreted fluids from various anatomical compartments in the mouth and the oropharynx, the mouth-rinse, throat-gargle, and palatal-exfoliate samples may be considered to hold cells and fluids that originate from different compartments. This study determined that each of the compartments sampled can harbor distinct HHV-8 strains, suggesting that different strains may preferentially persist and replicate in different cell types. Why this is so remains unclear. The roles played by sequential exposure to HHV-8, differences in inoculation routes, inter- and intraspecies viral interference, difference in anatomical site susceptibility to dissemination of HHV-8 from other body sites, systemic and local immune responses, and coexisting oral diseases require further investigation.

The findings in regard to possible KS330-K1/V1 linkages show that the KS330 B/C genotype tends to be associated with the K1/V1 genotypes B and A5 almost equally. Furthermore, the KS330 genotype B was linked almost equally to the K1/V1 genotypes B and A5 (Table 3.2). Such findings do not suggest that there is linkage disequilibrium between these combinations, merely that KS330 genotypes B/C and B, and K1/V1 genotypes B and A5 are equally prevalent in the population studied. To determine if these associations are due to past recombination events, it would be necessary to study larger populations to assess if the linkages observed hold true in the presence of rarer genotypes identified.

The present study was confined to examination of HHV-8 carried in the blood and the mouth. Sampling of other body sites might reveal yet more evidence of multiple HHV-8 carriage. Multiple oral infection by other herpesviruses, e.g., CMV (Bar et...
al., 2001) and EBV (Triantos et al., 1998) has been reported, but mainly in people who have been immunosuppressed. The present findings show that apparently healthy people in regions where HHV-8 is hyperendemic can be multiply infected by HHV-8.
Chapter 4

Inter- And Intra-Person Urinary HHV-8 Infection
4.1 Introduction

The previous chapter describes an investigation into how HHV-8 spreads in a hyperendemic geographical setting (Malawi). Polymorphisms in HHV-8 subgenomic sequences amplified from oral and blood samples of patients with KS and their family members were characterised. It was revealed that in up to 60% of the study individuals, HHV-8 in oral but not blood samples were multitypic. The diversity of HHV-8 shed in urine is unknown, however.

Urine is another body fluid into which HHV-8 is shed. Cattani et al. (1999) studied the HHV-8 genoprevalence in central and southern Italy and found that of 24 urine samples collected from KS patients only 2 (8.3%) tested positive for HHV-8 DNA. In a study conducted by Pauk et al. (2000) of 101 urethral swabs from American men who had sex with men, 2 tested (2%) positive for HHV-8 DNA, one from an HIV-seropositive and the other from a seronegative person. These studies implicate the genitourinary tract as a possible but rare site of HHV-8 infection and persistence. Whether urinary HHV-8 shedding is that rare in Africa is not known. This chapter describes an investigation into the prevalence of HHV-8 shedding in urine, and into whether, like oral fluid, urine can carry multitypic HHV-8. This study evaluated, in particular, the extent of sequence variation in PCR amplicons derived from ORF K1 of the HHV-8 genome. Where possible, it also compared the spectrum of urinary HHV-8 sequences with that of the sequences of orally shed HHV-8 derived from the same individual.
4.2 Materials and methods

78 people from whom mid-stream urine could be collected (19 Malawian patients with KS and 59 of their first-degree relatives without KS), a subset of the study group detailed in Section 2.1.1, were studied. Urine samples were processed as described in Section 2.1.4. Nucleic acid in the cellular fraction of urine was extracted as described in Section 2.1.7. Nested PCR was applied to the extract to amplify the 211-bp KS330 DNA segment of ORF 26 as detailed in Section 2.1.8. Nested PCR was also applied to the extract to amplify the 246-bp K1/V1 DNA segment of ORF K1, as detailed in Section 2.1.8. For sequence polymorphism studies, DNA extracts from the urine and rinse samples were subjected to nested PCR using the EXPAND High Fidelity PCR System to amplify K1/V1 DNA as described in Section 2.5.1. Clones were then generated from each K1/V1 PCR product as described in Section 2.5.2. Another round of PCR was carried out using GC-clamp primers as described in Section 2.5.3. Finally, the PCR products were subjected to DGGE followed by nucleotide sequencing, raw DNA sequence data analyzed and phylogenetic analyses performed as described in Sections 2.3 and 2.5.4.

4.3 Results

4.3.1 Patients and samples

From urine samples of 78 people, HHV-8 DNA (KS330 or K1/V1) was amplifiable in 2/20 (10%) patients with KS (Ni, Ui) and 4/58 family members (B5, G2,I1,P1) (7%) (overall rate: 7.7%) (Table 4.1). B5, G2 and P1 had previously been identified to carry amplifiable K1/V1 DNA and KS330 DNA in the mouth rinse (Table 3.1). It is noted that B5, G2 and I1 were seronegative, while Ni, P1 and Ui were seropositive for HIV-1 (Table 2.1).
<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/gender</th>
<th>HHV-8 KS330</th>
<th>HHV-8 ORFK1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mouth rinse</td>
<td>urine</td>
</tr>
<tr>
<td>B5</td>
<td>7y/M</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>G2</td>
<td>23y/M</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>I1</td>
<td>12y/M</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ni</td>
<td>30y/F</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>P1</td>
<td>28y/F</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ui</td>
<td>32y/F</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

**Table 4.1** Detectability results of KS330 or ORF K1/V1 DNA for mouth rinse and urine samples of individuals from whom at least one sample yielded amplifiable HHV-8 DNA.
In the assessment to determine the spectrum of KS330 and K1/V1 sequences in the urine of these individuals, the sequence of their rinse samples were also included for analysis. Samples follow the identification system described in Section 2.1.1.

4.3.2 Polymorphism of KS330 sequences
For two people G2 (male, 23 y) and P1 (female, 28 y), KS330 DNA could be amplified from their urine samples. These sequences were identical to those of their corresponding mouth rinses. All sequences belonged to the B genotype.

4.3.3 Polymorphism of K1/V1 sequences
Five individuals who carried urinary K1/V1 DNA were identified as B5 (male, 7 y), G2, II (male, 12 y), Ni (female, 30 y) and Ui (female, 32 y). B5 and G2 direct sequences were non-identical from those in their corresponding mouth rinses. The sequence present in urine sample of B5 (B5u) belonged to A5 genotype while that in rinse (B5r) belonged to B genotype, and G2r belonged to A5 genotype while G2u belonged to A2 genotype.

Figure 4.1 is a composite of representative DGGE gel pictures showing migration distances attained by the K1/V1 amplicons (DNA bands were revealed following SYBR Green I staining and ultraviolet transillumination). All PCR products yielding minority banding positions and some products representing the majority were sequenced (denoted by dots in Figure 4.1; numerals correspond to lane positions in the gel illustrated).
Figure 4.1 Composite of 3 representative DGGE gel photographs. Each panel represents one gel. Each gel accommodated, for a given study individual, K1/V1 amplified from up to 15 K1/V1 clones generated per sample. PCR products that underwent sequencing are indicated by dots at bottom; numerals indicate lane in the gel.
The extent of intra- and inter-sample K1/V1 nucleotide sequence diversity is depicted in Figure 4.2. Sequences have been deposited in GenBank (Accession Numbers AY328315-AY328357). An amino acid alignment to illustrate the extent of intra- and inter-sample K1/V1 sequences diversity is shown in Figure 4.3, and the total number of genotypes per study individual is summarized in Figure 4.4.

The sequences of the K1/V1 clones originating from the urine samples of the 5 individuals belonged to single genotypes (B5 to genotype A5; G2 and I1 to genotype A2; and Ni and Ui to genotype B). The ranges of intra-sample nucleotide divergence were as follows: 0.9%-1.9% for B5, 0%-0.9% for G2, 0% for I1, 0%-0.9% for Ni, and 0.5%-3.4% for Ui. For B5, the monotypy and the narrow divergence of the variants in the urine contrasted with the multitype as well as the wide intra-typic divergences found in his rinse. K1/V1 sequences in the rinse segregated to 3 genotypes (A2, A5 and B) (Figures 4.2, 4.3 and 4.4). Within the genotype A2 cluster, divergences were 0.9%-3.9%, and within the genotype B cluster, they were 0.9%-14.5%.

4.4 Discussion

The study described in this chapter reveals that the rate of HHV-8 DNA detection in urine samples of the patient group to be low: only 6 out of 78 (7.7%) tested positive for HHV-8 DNA. Four individuals (B5, I1, Ni and Ui) (5.14%) were positive for K1/V1, one (P1) (1.3%) positive for KS330, and one (G2) (1.3%) positive for both K1/V1 and KS330.
Figure 4.2 Radical unrooted phylogenetic tree of sequences amplified from PCR clones with K1/V1 inserts. Sequences were derived from urine and mouth rinse (u = urine; r = rinse). Bootstrapping for 1000 replicates is noted as a percentage at major branch points. Horizontal line at top left represents 10% nucleotide substitution for that horizontal branch length.
**Figure 4.3** Alignment of amino acid sequences predicted from K1/V1 sequences depicted in Figure 4.2. The variable 1 (V1) region is indicated at top. Dots indicate residues occupying positions aligned to residues of IIu 3/8/13 at top. Positions at which residues have undergone intraperson/intersample and intraperson/intrasample changes are highlighted.
### Table 4.4 Intrasample and intraperson distribution of K1/V1 genotypes.

<table>
<thead>
<tr>
<th>Person</th>
<th>Sample</th>
<th>No. of genotypes</th>
<th>Per sample</th>
<th>Per person</th>
</tr>
</thead>
<tbody>
<tr>
<td>B5</td>
<td>Rinse</td>
<td>3(B,A2,A5)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>1(A5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>Rinse</td>
<td>1(A5)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>1(A2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I1</td>
<td>Urine</td>
<td>1(A2)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Ni</td>
<td>Urine</td>
<td>1(B)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Ui</td>
<td>Urine</td>
<td>1(B)</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4.4** Intrasample and intraperson distribution of K1/V1 genotypes.
This low rate of detection is comparable to that reported from Italy and America (Cattani et al., 1999; Pauk et al., 2000), and contrasts with the high rate of urinary shedding of CMV. Shen et al. (1993b) for example, reported that of 63 Taiwanese children enrolled in kindergarten, 27 (43%) shed CMV into urine. The extent of environmental contamination by urinary HHV-8 in African locales may nonetheless be significant, considering that sanitary conditions there are poor and the rate of childhood HHV-8 infection is high.

Polymorphism studies were confined to K1/V1 DNA. The data from Chapter 3 have shown that the IRD ORF73 and KS330 region do not yield sufficiently discriminating sequence polymorphism results for strain-to-strain HHV-8 differentiation. Accordingly sequence polymorphism in the KS330 DNA amplified from urine samples was not further investigated here.

K1/V1 intra-sample nucleotide divergences in urine ranged from 0% for I1 to 3.4 for Ui. This narrow intra-typic divergence reflects either natural genetic drift or PCR-induced misincorporation, and is consistent with sequences in each sample having originated from a single founder variant. Urinary HHV-8 was found to be monotypic: K1/V1 DNA of B5 belonged to A5 genotype, G2 and I1 belonged to A2 genotype, and Ni and Ui belonged to B genotype. It has already been noted that HHV-8 in oral fluid may be multitypic: thus, K1/V1 DNA sequences amplified from the mouth rinse of B5 belonged to three genotypes (B, A2, A5) (Figure 4.4). The monotypy of urinary HHV-8 contrasts with that situation, and may reflect fewer opportunities for direct inoculation into, or smaller variety of sites supporting HHV-
8 persistence in, or poorer disposition towards preferential infection of selected variants in the genitourinary than the oral mucosa.

The genotype of HHV-8 shed in urine was also observed to be different from that shed into the mouth. For G2, the genotype of the single K1/V1 sequence obtained from the mouth (A5) was different from that of the urinary cluster of sequences (A2), again suggesting segregation. All of the K1/V1 sequences in B5's rinse, except for one sequence, B5r13, clustered separately from the urinary sequences (Figure 4.2). Such findings suggest segregation of HHV-8 variants in each of the two body compartments. The segregation is not complete, however, as is evidenced from the observation in B5 that the B5r13 sequence, which is closely related to the urinary sequences, is also present in the mouth. The tendency for different genotypes to be preferentially shed into different body fluids can be a consequence of: i. the low rate of HHV-8 hematogenous carriage (none of the five individuals studied here carried amplifiable HHV-8 DNA in their blood, as has been previously determined (Cook et al., 2002b)), ii. the tendency for HHV-8 to persist locally at or near the site of infection; iii. the restriction in host cells permitting latent but not lytic infection (Bechtel et al., 2003).

The possibility cannot be excluded, however, that the monotypy or multitypy found in the samples may be related to the sampling process itself, as HHV-8 may be present in urine or oral fluid at such low levels as to be distributed in a Poisson manner. These differences in the spectra of K1/V1 sequences from B5's oral fluid and urine could be explained by Poisson sampling.
Chapter 5

Inter-And Intra-Person Cytomegalovirus Infection
5.1 Introduction

In African countries, CMV infection is acquired early in childhood. Seroprevalence studies show >80% of African children to be CMV-seropositive by their 4\textsuperscript{th} birthday. A study conducted by Krech and Tobin of CMV seroprevalence in young children from ages 4 m to 4 y in 19 countries showed that African cities like Ibadan in Nigeria and Entebbe in Uganda yield seroprevalences of >90% (Krech and Tobin, 1981). In another study in Kumba City in Cameroon, 23 children out of 26 (88.5%) ranged between 4-6 y were seropositive for CMV (Stroffolini \textit{et al.}, 1993). By contrast, young children aged between 4 m-4 y in Oxford and Los Angeles showed seroprevalences of 3% and 15%, respectively (Krech and Tobin, 1981). A study in Italy conducted between 1987 and 1989 showed that the seroprevalence of CMV reached up to 55% by age 6 y (de Mattia \textit{et al.}, 1991). A recent study reported by de Ory \textit{et al.} (2004) showed that the CMV seroprevalence of children aged 2-5 y in the autonomous region of Madrid, Spain was 43%. Such contrasts reflect greater opportunities for CMV transmission in African than industrialised countries.

African people infected by CMV intermittently shed the virus into urine and saliva (Bello and Whittle, 1991; Bello, 1992), so these fluids act as major vehicles of transmission. In order to ascertain better the routes by which CMV may be acquired in an African context, identities and genotypic clusterings in DNA sequences derived from hypervariable domains of the CMV genome carried by the same Malawian population described in the preceding chapters was investigated.

This chapter describes sequence variations in 2 newly identified hypervariable regions in the CMV genome: glycoprotein gpUL 73 (gN) (Pignatelli \textit{et al.}, 2003) and
glycoprotein gpUL 74 (gO) (Paterson et al., 2002). Where possible, the urinary sequences of CMV were compared with corresponding sequences derived from mouth rinses.

5.2 Methods and materials

The study group comprised the same group of 19 KS patients, and 59 of their first-degree relatives as described in Section 2.1.1. Mouth rinse samples were collected as described in Section 2.1.3, and urine was collected as described in Section 2.1.4. DNA was extracted from both oral and urine samples as described in Sections 2.1.6 and 2.1.7. A 304-bp fragment of the gN-coding region of CMV was amplified from sample extracts using nested PCR, as described in Section 2.2.1. A 433-bp fragment of the gO-coding region was also amplified by nested PCR, as described in Section 2.2.2. Measures were taken to minimise the contamination (Section 2.1.11). PCR products were sequenced, raw DNA sequence data analysed, and phylogenetic analyses performed as described in Section 2.3. Certain gO genotypes were investigated if they were in linkage disequilibrium with gN genotypes. Data from three people with mixed infection (B5, Ri, and R2) were excluded from the statistical analysis.

5.3 Results

CMV DNA could be amplified from 41 people (53%) in either the gN or gO region in at least one sample, from 14 people (18%) in both domains in at least one sample, and from 13 (17%) in either domain in both samples. Their characteristics are summarised in Table 5.1. Twenty-one (51%) of the 41 people were seropositive for HIV-1, 36 (88%) were HHV-8-seropositive, and 16 (40%) were KS patients.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/gender</th>
<th>CMV gO PCR (mouth rinse)</th>
<th>CMV gN PCR (mouth rinse)</th>
<th>HIV sero status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ai</td>
<td>7.5 y/F</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>A2</td>
<td>10 y/M</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>Bi</td>
<td>4 y/M</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B1</td>
<td>20 y/F</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
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<td>B5</td>
<td>7 y/M</td>
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<td>+</td>
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<td>Ci</td>
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<td>Di</td>
<td>4 m/M</td>
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<td>-</td>
</tr>
<tr>
<td>E1</td>
<td>31 y/F</td>
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<td>-</td>
<td>+</td>
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<td>-</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
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<td>2 y/M</td>
<td>-</td>
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<td>+</td>
<td>-</td>
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<tr>
<td>I2</td>
<td>11 y/F</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Mi</td>
<td>33 y/M</td>
<td>+</td>
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<tr>
<td>M1</td>
<td>3 y/M</td>
<td>+</td>
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<tr>
<td>M3</td>
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<tr>
<td>Ni</td>
<td>30 y/F</td>
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<tr>
<td>Pi</td>
<td>30 y/M</td>
<td>-</td>
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<tr>
<td>Qi</td>
<td>24 y/M</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
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<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>25 y/F</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>R2</td>
<td>12 y/M</td>
<td>-</td>
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<tr>
<td>Ti</td>
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<td>T3</td>
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<tr>
<td>Zi</td>
<td>1 y/9 m/M</td>
<td>+</td>
<td>-</td>
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</tbody>
</table>

**Table 5.1** Characteristics of CMV-infected Malawian KS patients and their family members.
Forty-six gN sequences recovered were found to belong to four genotypes: gN1 (65.2%), gN3(a) (28.3%), gN3(b) (4.3%) and a previously undescribed genotype, (2.2%), to which was assigned gN3(c). Twenty-five gO sequences could be assigned to six genotypes: gO1(a) (44%), gO2(b) (16%), gO4 (16%), gO1(b) (12%), gO2(a) (8%) and gO1(c) (4%). The gN and gO nomenclatures follow those of Pignatelli et al. (2003) and Mattick et al. (2004) respectively.

Figure 5.1 shows the extent of diversity of gN DNA sequences, and Figure 5.2 an alignment of aminoacid sequences derived from the study samples. Identical nucleotide sequences were found: in the B family, in the mouth rinse and urine samples of a boy (Bi) (4 y), the mouth rinse and urine of his sister (B1) (20 y) and the urine only of his brother (B5) (7 y); in family E, among the mouth rinse of a woman (Ei) (31 y) and that of her daughter (E2) (9 y) and the urine sample of her daughter (E4) (6 y); in family F, between the urine of a woman (F1) (35 y) and both mouth rinse and urine samples of her son (F2) (7 y); in family G, between the mouth rinse of the mother (Gi) (34 y) and the mouth rinse and urine of her son (G2) (23 y); in family R, between the mouth rinse of a man (Ri) (41 y) and the urine of his son (R2) (12 y); in family T, between the urine of a boy (T3) (9 y) and the mouth rinse and urine of his brother (T4) (4 y); family U, between the mouth rinse of a boy (U1r) (14 y) and the mouth rinse of his sister (U3) (8 y); and in family X, between the mouth rinses of a woman (Xi) (41 y), her daughter (X2) (18 y) and her son (X3r) (11 m).

Intra-family divergences of gN sequences were observed in families B, E, R, T, U and W (Figures 5.1 and 5.2). Nucleotide divergence of 0.3% was found between
Figure 5.1 Predicted phylogenetic distribution of consensus gN sequences derived from mouth rinses and urine samples. Bootstrapping for 1000 replicates is noted as a percentage at major branch points. Horizontal line at bottom left represents 10% nucleotide substitution for that horizontal branch length. Sequences have been deposited in GenBank (Accession Numbers AY326987-AY327032). Letters after patient identifiers: r = rinse; u = urine. Samples from individuals whose gN sequences were further studied are boxed as follows:

- intra-individual samples with multitypic sequences
- intra-individual samples with monotypic but divergent sequences
- intra-individual samples with identical sequences
Figure 5.2 Alignment of amino acid sequences predicted from gN sequences depicted in Figure 5.1. Dots indicate residues occupying positions aligned to residues of A2u at top. Samples from individuals whose gN sequences were further studied are boxed as for Figure 5.1.
Figure 5.2 Alignment of amino acid sequences predicted from gN sequences depicted in Figure 5.1. Dots indicate residues occupying positions aligned to residues of A2u at top. Samples from individuals whose gN sequences were further studied are boxed as for Figure 5.1.
B5r and Bir/Biu/B1r/B1u, all belonging to gN1 genotype. Two genotypes were found in family E: gN3(a) for Eir/E2r/E4r/E4u and gN1 for E3u, with nucleotide divergence between the 2 genotypes of 14%. Within family R, nucleotide divergence ranged from 1% (between Rir and R1r, and R1r and R2u) to 22.4% (between R1r and R2r), with the sequences segregating to 4 reported genotypes: gN1 for Rir, R1r and R2u, gN3(a) for R3u, gN3(b) for R2r, and gN3(c) for Riu. Nucleotide divergence of 0.3% was found between Tir and T3u/T4r/T4u, all belonging to gN1 genotype. Two genotypes were found in family U: gN3(b) for Uir and gN3(a) for U1r and U3r, with a nucleotide divergence of 21.2% between the two genotypes. Nucleotide divergence of 1.3% was found between W4r and W6r, both of which could be assigned to gN3(a).

Figure 5.3 shows the extent of diversity of gO DNA sequences, and Figure 5.4 an alignment of amino acid sequences derived from the study samples. Identical gO nucleotide sequences could be recovered from 3 families: family B, among Bir, Biu, and B5r; family F, among Fiu and F1u; and family T, among T3u, T4r and T4u.

Intra-family divergences of gO sequences were revealed in families B, E, F, M and R. Within family B, nucleotide divergence ranged between 33.8% (Bir/Biu/B5r and B1u) to 55.9% (Bir/Biu/B5r and B5u), with the sequences belonging to 3 genotypes: gO1(a) for Bir/Biu/B5r, gO1(b) for B1u and gO4 for B5u. In family E, there was a divergence of 0.5% between Eir and E4u, the sequences belonging to genotype gO4. Within family F, the divergence was 45% between Fiu/F1u (genotype gO2(b)), and F2u (genotype gO1(a)).
Figure 5.3 Predicted phylogenetic distribution of consensus gO sequences derived from mouth rinses and urine samples. Horizontal line at bottom left represents 10% nucleotide substitution for that horizontal branch length. Sequences have been deposited in GenBank (Accession numbers AY326961-AY326986). Samples from individuals whose gO sequences were further studied are boxed as in Figure 5.1.
Figure 5.4 Alignment of amino acid sequences predicted from gO sequences depicted in Figure 5.3. Dots indicate residues occupying positions aligned to residues of Aiv at top. Samples from individuals whose gO sequences were further studied are boxed as in Figure 5.1.
Mir and M1r in M family showed a divergence of 44.8%, the sequences belonging to genotypes gO2(b) and gO1(c), respectively. In the R family, a 43.2% divergence was found between Riu (genotype gO1(b)), and R2u (genotype gO2(a)) (Figures 5.3 and 5.4).

For 11 people, DNA from gN region could be amplified from >1 sample. gN sequences were identical in two samples from 7 people (Bi, B1, F2, G2, Ni, T4 and Zi). Non-identical sequences were found in samples from 4 people, with intra-person divergences of 0.3%, 0.3%, 11.6% and 21.1% for E4, B5, Ri and R2, respectively. For two individuals (Ri and R2), non identical intra-person gN yielded samples that carried sequences belonging to 2 genotypes: gN1 and gN3(c) in Ri, and gN3(b) and gN1 in R2 (Figure 5.1).

For 5 people, DNA from gO region could be amplified from >1 sample. gO sequences were identical in 3 (Bi, G3, and T4). Non identical sequences were found in samples from 2 people, with intra-person divergences of 65% and 56% for Ai and B5, respectively. For 2 individuals (Ai and B5), non identical intra-person sequences belonged to separate genotypes: gO2(b) and gO4 for Ai, and gO1(a) and gO4 for B5 (Figure 5.3).

The gO and gN genotype combinations analyzed were: gO1(a)/gN1 (n=6), gO1(b)/gN1 (n=1), gO1(b)/gN3(a) (n=1), gO4/gN3(a) (n=2), gO2(b)/gN1 (n=1) ) Table 5.2).
<table>
<thead>
<tr>
<th>Patient</th>
<th>CMV gO PCR</th>
<th>CMV gN PCR</th>
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<tbody>
<tr>
<td></td>
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<td>urine</td>
</tr>
<tr>
<td>Bi</td>
<td>gO1(a)</td>
<td>gO1(a)</td>
</tr>
<tr>
<td>B1</td>
<td>–</td>
<td>gO1(b)</td>
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<tr>
<td>Ci</td>
<td>gO1(b)</td>
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<td>gO1(a)</td>
<td>gO1(a)</td>
</tr>
<tr>
<td>Xi</td>
<td>gO1(a)</td>
<td>–</td>
</tr>
<tr>
<td>Zi</td>
<td>gO1(a)</td>
<td>–</td>
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</tbody>
</table>

Table 5.2 Show linkage/recombination of different genotypes of gN and gO regions from same sample.
5.4 Discussion

The CMV gN and gO genes are among a disparate group of herpesvirus genes that exhibit wide nucleotide sequence polymorphisms. These genes include those encoding latent membrane protein-1 of EBV (Miller et al., 1994), Epstein-Barr nuclear antigen-2 (Ling et al., 1993), ORF K1 of HHV-8 (Zong et al., 1999) and UL11 of CMV (Hitomi et al., 1997). The gN protein, which binds with the gM protein to form a virus envelope complex, glycoprotein complex II (gCII), is able to induce neutralizing antibodies (Mach et al., 2000). The gN protein exhibits inter-genotype amino acid sequence variability of up to 50% (Pignatelli et al., 2001). gCII is able to bind heparin of the cell surface, suggesting that it has a role in facilitating virus attachment to the host cell (Kari and Gehrz, 1992). The gO protein is a constituent of a heterotrimeric complex, glycoprotein complex III (gCIII), comprising gO, gH (encoded by UL75) and gL (encoded by UL115); this complex has fusogenic properties thought to be responsible for cell-to-cell spread (Paterson et al., 2002). There is no evidence yet to show that the gO protein elicits neutralizing antibody responses, although the gH protein is known to do so (Rasmussen et al., 1984). The inter-genotype amino acid sequence variation in the gO protein is reported to be up to 46% (Rasmussen et al., 2002), but it was found in this study that the amino acid divergence can reach up to 65.9% (between Eir and Mir Figure 5.4).

Several other CMV protein-encoding genes also show strain-dependent differences, but the extents of their variability are not comparable to those encoding gN and gO. The most widely studied gene is UL55, which encodes gB. Dimers of covalent linked gB protein constitute glycoprotein complex I (gCI), which is responsible for virion entry and cell-to-cell spread (Navarro et al., 1993). The gB protein is a major
target for neutralizing antibodies and for T-cell cytotoxic responses (Hopkins et al., 1996; Alberola et al., 2000). Amino acid variation in gB is nevertheless not >15% (Chou, 1992a). The UL144 gene encodes a homologue of the herpes simplex mediator, a member of the tumour necrosis factor receptor (TNFR) family. It is a type I transmembrane glycoprotein and is expressed early after infection; its function is unknown (Benedict et al., 1999). Variation in the UL144 protein is up to 21% (Lurain et al., 1999). The US9 and US28 genes show variability of about 4% at both nucleotide and amino acids levels (Rasmussen et al., 2003). The glycoprotein encoded by US9 may be a determinant of replication and cell-to-cell dissemination of CMV through its association with cytoskeleton proteins along lateral cell membrane such as actin and cadherin (Maidji et al., 1998). The US28 gene encodes the CC chemokine receptor, which can modify the environment of the CMV-infected cell by sequestering extra cellular CC chemokines that influence the inflammatory response and the infiltration by lymphocytes, monocytes and basophils. Thus, the cytotoxic potential of CD8+ lymphocytes and natural killer (NK) cells has been observed in vitro to be decreased (Bodaghi et al., 1998). The US28 product appears also to play a role in promoting smooth muscle cell migration (Streblow et al., 2001). It and the rhesus CMV homologue RhUS28.5, are localised to the envelope of infectious virions, and may therefore play a role in viral entry (Penfold et al., 2003).

A highly variable gene, UL11, encodes a protein which is expressed on the surface of CMV infected cells; its function is unknown but it exhibits >40% amino acid variability (Hitomi et al., 1997).

The nature of selection pressures acting to generate plasticity in the gN and gO genes is enigmatic. While gN is known to be able to induce neutralizing antibodies (Mach
et al., 2000), and its amino acid sequence variation may therefore be attributed to pressures acting on the gene to evade neutralisation, such variability is nonetheless substantially greater than that for gH (up to 4% amino acid sequence variation) (Chou, 1992b) and gB (up to 15% variation) (Chou, 1992a), which are major targets of neutralising antibodies (Alberola et al., 2000). Furthermore, there is no evidence that the gO protein elicits neutralizing antibody responses. Hypervariability may perhaps be related to the pressure acting on the gene to effect T-cell cytotoxic responses. It has been reported that the extreme hypervariability in a stretch of the ORF K1 protein of HHV-8 is associated with domains that are targets of intense cytotoxic responses (Stebbing et al., 2003). Whether the CMV gN and gO proteins similarly carry epitopes eliciting T-cell responses is unknown.

There may be other selective forces operating that have yet to be defined. They may relate to tropism, virulence and host immune status, which would lead to an accumulation of specific viral variants in selecting environments. Thus, Meyer-König et al. (1998) observed that gB2 and gB3 preferentially infect monocytes and lymphocytes. A recent study showed that the gB2 CMV strain exhibits tropism for neurons (Tarrago et al., 2003). Several reports have also suggested an association of different gB genotypes with pathogenicity; a study in Hungary showed gB1 to be dominant in congenital infections (Lukacs et al., 2001) and another study by Woo et al. (1997) in Hong Kong reported the dominance of gB1 strains in CMV-infected premature babies. Fries et al. (1994) and Hebart et al. (1997) demonstrated a correlation between the gB genotypes and severity of CMV disease in bone marrow transplant recipients.
The study described in this chapter has exploited the hypervariability of the gN and gO genes to investigate extra-and intra-household, and intra-individual CMV variability in an African community setting in Malawi. In families with identical gN or gO sequences there were some in which identity was observed between mother and child: for gN sequences, between Ei and her daughters (E2 and E4), F1 and her son (F2), Gi and her son (G2), Ti and her sons (T3 and T4), Xi and her daughter (X2) and son (X3); and for gO sequences, F1 and her son (Fi). CMV sequence identities were also observed between members of the family. Thus, identical gN sequences could be found between a father (Ri) and son (R2), and between siblings: Bi and his sister (B1) and brother (B5), and U1 and his sister (U3), and identical gO sequences between Bi and his brother (B5), and between T3 and his brother (T4).

Both vertical and horizontal CMV infection probably account for CMV gN and gO sequence identity among members of the same household. Children may acquire infection vertically in utero, perinatally, or during breast-feeding (Bryant et al., 2002; Revello et al., 2002). Horizontal infection is facilitated in close-living conditions, since CMV is often shed in saliva and urine (Pass et al., 1986; Adler, 1989). While in the USA and Japan epidemiological studies have identified day care centres as main sources of infection which then spreads from the children to their parents (Pass et al., 1986; Kashiwagi et al., 2001), the sources and vehicles of intra-household horizontal CMV infection remain to be identified in the African context. A study in the Gambia of 178 women and their babies followed up for 9-15 m, and in mothers and siblings of 22-infected infants showed that titres of CMV in urine were comparable to those in saliva, both in the infants (10^3 and 10^{2.7} TCID_{50}/ml respectively) and mothers (10^{1.7} and 10^{1.4} TCID_{50}/ml respectively). Eighty percent of
babies in this study generated more specimens yielding CMV over a longer time compared to 20% of the mothers. Furthermore, 36.6% of siblings excreted CMV in either urine or saliva compared to 6.1% of mothers. Thus, infected children are significant reservoirs of infection. The spread of CMV may be facilitated through bed-wetting, as families tend to sleep together (Bello, 1992). The tendency for members in African communities to engage in traditional behavioural activities based on saliva exchange may also facilitate the spread of CMV (Wojcicki, 2003). The activities include frequent kissing of infants by mother and other family members and hand-to-mouth eating from the same bowl.

Some families were observed to carry multiple genotypes. The gN sequences recovered from family R belonged to 4 genotypes: the gO sequences from family B to three genotypes, gN sequences recovered from family E and U to two genotypes, and gO sequences from family F, M and R to two genotypes. The multitypic sequences recovered from each of these families are consistent with extra-household transmission having taken place. How CMV extraneous to the household may lead to infection remains undefined. Contact with urine and saliva carrying CMV or objects contaminated with such fluids is one possibility. Transmission relating to sexual activities may also contribute. CMV has been observed to be shed into semen: Aynaud et al. (2002) showed that in 111 semen samples taken from HIV-seronegative Parisian male partners of women with histologically-detected human papillomavirus (HPV)-associated genital lesions, CMV DNA could be detected in 7 (6.3%). CMV can also be shed from the cervix: Collier et al. (1995) evaluated 1481 heterosexual women attending a sexually transmitted disease clinic at Seattle, from 951 who were CMV seropositive cervix swabs were collected and showed that 86
(9.4%) were culture-positive for CMV. In a similar study, Mostad et al. (1999) investigated the prevalence of CMV shedding in 311 cervical swab samples collected from women attending a sexually transmitted diseases clinic in Mombasa, Kenya, and detected CMV DNA in 183 (59%).

The study described in this chapter has also provided evidence for intra-host sequence polymorphism in CMV originating from mouth rinses and urine of 5 people (Ai, B5, E4, Ri and R2) and 4 (Ai, B5, Ri and R2) could be identified to be carrying 2 genotypes each. All except Ri were children. Two individuals, Ai and Ri, were HIV-1 seropositive. Present studies have shown that HIV-infected people are particularly prone to mixed infections. Verbraak et al. (1998) studied CMV DNA sequences from aqueous humour and peripheral blood leukocytes of 13 AIDS patients with CMV retinitis in the Netherlands and found that in 7, sequences obtained from the eye and blood were different. In another study of samples taken from various tissues (including lung, stomach, adrenal gland and liver) obtained at autopsy of 12 AIDS patients in London, UL4 DNA were amplified from 10, of which 5 were found to carry >1 genotype (Bar et al., 2001). The presence of different sequences in the same compartment in HIV-infected patients also has been reported by Fidough-Houhou et al. (2001) who showed that in 98 HIV-seropositive patients in Paris, CMV gB DNA could be amplified from saliva samples in 37 patients, of whom 5 (13.5%) showed mixed gB strains.

The finding that 3 of the 5 people, B5, E4, and R2, who were HIV-seronegative, substantiates other studies, which imply that immunocompetent children and adults are also prone to multiple infection. Past studies comparing the RFLP patterns of
Chapter 5 Inter- And Intra-Person Cytomegalovirus Infection

CMV DNA derived from cell culture isolates of immunocompetent adults have revealed mixed CMV infection. Chandler et al. (1987) who studied 34 cultured CMV isolates over a 14 m period from the cervix, urine and throat of 8 randomly selected healthy women attending a sexually transmitted disease clinic in Seattle showed that 2 shed different strains from the same site and another 2 shed different strains simultaneously from different body sites. Mixed CMV infection also has been reported by Collier et al. (1989) in an immunocompetent homosexual American patient who was infected by 4 different strains, 2 isolated from urine and 2 from semen. Shen et al. (1993a) amplified gB DNA from serial urine samples obtained from an immunocompetent child in Taiwan and documented mixed infection. Lasry et al. (1996) examined the CMV excretion in 439 urine samples from 93 healthy children <1 y in 6 day care centres in Paris by amplifying and sequencing the gB gene, and revealed mixed infection in 2 children. These various studies suggest that in western countries, mixed CMV infection can occur in immunocompetent people but is very rare, whereas in those who are immunocompromised, the incidence is higher.

The findings in regard to gO/gN linkages agree with those of Mattick et al. (20040 who found that gOl(a) tends to be associated with gN1, and gOl(b with gN3a. A study involving a larger sample set may confirm that there is linkage equilibrium between these combinations. Furthermore, the study reveals new linkages: gO1(b) with gN1,gO4 with gN3(a), and gO2(b) with gN1. These various linkages are suggestive of past recombination between CMV genes (Table 5.2).
Multiple CMV carriage may be due to reactivation of endogenous CMV strains or to super-infection by new strains from exogenous sources. That the latter possibility may apply is suggested by the finding that for 2 individuals carrying mixed infection studied here, sequence identity could be identified in another member of the family: the gN sequence derived from the mouth rinse of Ri was identical to that in urine of his son, R2; and the gO sequence was identical between the mouth rinse of Bi and mouth rinse and urine of his brother B5.
Chapter 6

Conclusions and Suggestions for Further Work
6.1 Introduction

People living in African regions are prone to be repeatedly exposed to HHV-8 and CMV. The current study involved samples collected from people living in Malawi, a region of high HHV-8 and CMV endemicity. Family groups in which one member had been affected by KS were selected to investigate if the hosts living in such a region carry multiple HHV-8 or CMV strains. In the study, several methods were utilised to investigate the molecular epidemiology of HHV-8 and CMV in samples obtained from different anatomical body compartments. The samples investigated were mouth rinse, gargles, palatal exfoliates, blood cells and urine.

6.2 Intra-individual oral HHV-8 infection

Multiple HHV-8 infection rarely has been considered (Gao et al., 1999). Most studies of humans who have developed KS (Stebbing et al., 2001; Meng et al., 2001; Zong et al., 2002) or multiple HHV-8-associated lesions (Codish et al., 2000) have not shown intraperson variation in HHV-8 subgenomic sequences amplified by PCR from blood and lesional tissues.

To effect such a study, sequence variation was sought in DNA segments derived from HHV-8 ORFs 73, 26 and K1 using restriction fragment-length polymorphism analysis, nucleotide sequencing, PCR cloning and denaturing gel gradient electrophoresis. DNA was amplified by PCR from mouthrinses, throat gargles, palatal exfoliates and blood. Twenty-four people were identified who carried amplifiable HHV-8 DNA in >1 sample; 9 (38%) were seropositive for HIV-1, 21 (88%) were anti-HHV-8-seropositive and 7 (29%) exhibited KS. Amplification rates were variable depending on the region within the HHV-8 genome to be amplified.
Highest yields were obtained after amplification from K1/V1, followed by KS330 and the IRD. Mouth-rinse and throat-gargle samples yielded higher amplification rates than did blood and palatal-exfoliate samples, probably reflecting active HHV-8 replication in the oral epithelium, and the possible lower replicative activity of the virus systemically and in the palatal mucosa. The palate was especially investigated since it is the site at which oral KS most often develops. Blood samples yielded HHV-8 subgenomic amplicons in a substantial proportion of patients with KS but not at all in those without KS. This finding confirms previous data (Cook et al., 2002b) showing that, in HHV-8-infected African people not affected by KS, the virus is not shed into their circulation to a significant extent and that the principal site of viral shedding is the mouth. It supports the emerging consensus that oral fluid and saliva are important vehicles for HHV-8 shedding and transmission.

To examine for intra-individual HHV-8 diversity, the principal approach used was PCR of K1/V1 combined with DGGE nucleotide sequencing of K1/V1 amplicons generated from clonal inserts. This was to permit sequences of minority variants to be identified and characterized. Such an approach was necessary to overcome the limitation imposed by consensus sequencing, which would represent the sequence of the dominant variant only. The data so obtained were amalgamated with data accrued from RFLP analysis of the IRD and consensus sequencing of KS330.

For 3 KS patients, intraperson genotypic differences, arising from nucleotide sequence variations in KS330 and K1/V1, were found in blood and oral samples. Evidence for the oral carriage of multiple strains of HHV-8 could be found for 16 people out of 24 (>60%), 4 with KS and 12 without KS. For 1 patient with KS and
for 7 people without KS, intra-person genotypic differences, originating predominantly from K1/V1, were found in oral samples. For the 2 patients with KS and for 4 individuals without KS, intra-sample carriage of distinct ORF K1 sequences could be discernible in oral samples. Furthermore, subgenomic sequences could be identified in oral samples for 2 patients with an identical genotype and for 2 patients with identical genotype but with distinct variants. These various data therefore reveal the presence of multiple HHV-8 infection inter- and intra-compartmentally.

It would be important to determine whether multiple HHV-8 carriage reflects simultaneous coinfection by >1 HHV-8 strain, reactivation of latent strains, or superinfection. If it reflects superinfection, then such transmissions may not be prevented by vaccination. Nonetheless, although the immune system might not prevent superinfecting virus from initiating replication in the host, it might, after vaccination, prevent the further spread of the initial infecting strain (following reactivation) and of the subsequent superinfecting strains. In view of the relentless march of HIV/AIDS in Africa, where HHV-8 is also hyperendemic, initiatives to develop vaccines against HHV-8 would be beneficial.

These findings also confirm the predominance of the mouth as a source of HHV-8 shedding. They are indicative of the mouth as an important, if not the principal reservoir of HHV-8 and of oral fluid as the prime vehicle for its transmission.

**Further work**: The coexistence in a given host of >1 HHV-8 strain, particularly if the strains are genotypically different, potentially confounds molecular
epidemiologic analyses of HHV-8 transmission. Strains that persist in the host as minority variants will not be revealed if appropriate methods are not applied, and such variants will be missed in the course of tracking the spread of HHV-8. Accordingly, future studies aiming to track the transmission of particular HHV 8 variants should take into consideration transmission by minority variants if consensus sequencing at first reveals non identical variants between the transmitter and recipients of a transmission event.

The approach adopted in this study can be applied to other population groups. The mode of transmission in HHV 8-endemic regions in the Mediterranean deserves attention. There are already some indications that oral transmission of HHV 8 may be more significant than sexual transmission (Vieira et al., 1997; Pauk et al., 2000; Marcelin et al., 2004). Confirming the phenomenon of multiple HHV 8 infection within and among anatomical compartments in the given host in the Mediterranean context should clarify better how the virus spreads there.

In HHV-8 hyper-endemic area it may be useful to examine tissue specimens from various organs, e.g. lung, brain, spleen, liver, etc, obtained from individuals who died from KS or any other causes, in order to investigate HHV-8 sequences present in those tissues. Studies may also be done to determine if there is any link between multiple HHV-8 infection and the progression of HHV-8-associated diseases.

6.3 Inter- and intra-person urinary HHV-8 infection

As an extension of the study of polymorphism in HHV-8 subgenomic sequences amplifed from blood and oral samples, urine was collected from the same study
The extent of HHV-8 urinary sequences variation derived particularly from K1/V1 was examined and compared with sequences derived from oral samples of the same individual.

Both direct sequencing and PCR combined with DGGE analysis of K1/V1 clones technique were used. Variation in the KS330 region was investigated, but was found not to yield sufficient polymorphism to permit strain-to-strain HHV-8 differentiation.

The rate of HHV-8 DNA detection in urine samples of the patient group was low: only 6 out of 78 (7.7%) tested positive for HHV-8 DNA. Four individuals were positive for K1/V1, one positive for KS330, and one positive for both K1/V1 and KS330. All K1/V1 intra-sample sequences obtained from 5 urine samples showed no or narrow intra-typic divergence, and were all monotypic.

The genotype of HHV-8 shed in urine was observed to be different from that shed into the mouth in two patients. One of the patients from whom urinary HHV-8 was characterised showed HHV-8 multityp (up to three genotypes) in his mouth rinse sample. Segregation of HHV-8 variants in two body compartments is suggested from these data. Nonetheless, that the monotypy or multityp found in our samples may be related to the sampling process itself cannot be excluded, as HHV-8 may be present in urine or oral fluid at such low levels as to be distributed in a Poisson manner.

**Further work:** How important urine might be in contributing to HHV-8 endemicity would require further investigation. Other than studying larger sample sizes and in
different geographical regions, the assessments of the urinary viral load by, e.g., real-time PCR quantification techniques, would be useful. Confirming monotypy or multityp of urinary HHV-8 in the various anatomical compartments in larger sample sizes of study individuals may shed light on whether specific HHV-8 variants have a predilection to infect and persist in specific tissue types.

6.4 Inter-and intra-person cytomegalovirus infection

CMV is another herpes virus which is shed in body fluids like urine and saliva. From the same Malawian group, the routes by which CMV may be acquired, and the genotypic and intra-genotypic diversity were investigated both inter-and intra compartmentally. Two newly identified hypervariable regions in the CMV genome, gN and gO, were studied. Both urine and mouth rinse samples were examined, and, where possible, the urinary sequences of CMV were compared with corresponding sequences derived from mouth rinses.

CMV DNA could be amplified from 41 people (53%) in either the gN or gO region in at least one sample, from 14 people (18%) in both domains in at least one sample, and from 13 (17%) in either domain in both samples. Forty-six gN sequences recovered were found to belong to four genotypes, and 25 gO sequences could be assigned to six genotypes. Identical gN or gO sequences were found in 8 families. Identity was observed between mother and child for 5 families. Four families showed CMV sequence identities between other members of the family.

Both vertical and horizontal CMV infection probably account for CMV gN and gO sequence identity among members of the same household. Six families were
observed to carry multiple genotypes. The multotypic sequences recovered from each of these families are consistent with extra-household transmission having taken place. However, CMV extra-household transmission remains undefined. Contact with urine and saliva carrying CMV or objects contaminated with such fluids is one possibility. Transmission relating to sexual activities may be another.

Evidence was provided for intra-host sequence polymorphism in CMV originating from mouth rinses and urine of 5 people, with 4 of them carrying 2 genotypes. Multiple CMV carriage may be due to reactivation of endogenous CMV strains or to super-infection by new strains from exogenous sources. Four of the 5 were children and 3 of the five were HIV-seronegative, and presumably immunocompetent. Most previous studies have been focused on multiple infection in immunocompromised hosts. The findings for this study are significant in that multiple infection in the context of immunocompetent people living in a hyper endemic area is revealed.

Further work: The multiplicity of routes by which CMV spreads in the intra- and extra-household contexts, and the vulnerability of immunocompetent people to multiple CMV infection potentially influence the efficacy of vaccination against CMV. Continuing studies into how CMV genotypes circulate in different communities could help in the evaluation of the efficacy of CMV vaccines in the prevention of primary and congenital infection. Again, the DGGE technique as applied here may also be applied to future studies of CMV molecular epidemiology.

The study also shows that the degree of nucleotide sequence polymorphism in hypervariable regions of CMV (gN and gO) do not match that of HHV-8 ORF K1.
Consequently, the molecular epidemiology of CMV could not be done in the refined manner as was carried out for HHV-8. It would therefore be worthwhile to search for sub-genomic regions of CMV to identify regions that exceed gN and gO in hypervariability. Such research may also be extended to study the ‘micro’ molecular epidemiology of the other herpes viruses. This form of investigation may help to understand better some longstanding questions in herpes viruses pathogenesis: is recurrent herpes labialis and genitalis due to reactivation of one particular HSV-1 or HSV-2 strain rather than many strains? Similarly, is herpes zoster a result of reactivation of one particular strain or >1 strains? With the advent of widespread vaccination against VZV, how might chickenpox or herpes zoster that develop in a vaccinee be determined not to be due to the vaccine strain? Is oral hairy leukoplakia due to reactivation of single EBV strain or from superinfection of many strains? If the latter, which anatomical compartments sequester these various strains?
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Appendix 1

Publications and conference presentations
Appendix 1: Publications and conference presentations

Publications


Conference presentations


Appendix 1: Publications and conference presentations

Teo CG, Beyari MM, Cook RD, Hodgson TA, Porter SR, Borgstein E, Molyneux EM. Multiple human herpes virus 8 infection (Poster presentation) 10th Conference on Retroviruses and Opportunistic Infection, Boston, USA, 10th -14th February 2003.

Beyari MM, Hodgson TA, Cook RD, Kondowe W, Molyneux EM, Scully CM, Teo CG, Porter SR. Multiple human herpes virus 8 infection (poster presentation) Annual Meeting of the British Society for Oral Medicine, Dundee, 9th -10th May 2003.

Appendix 2
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**Table 1**

Tables 1-5 DNA detectability for different regions of HHV-8 and CMV for different samples obtained from all patients (with Kaposi's sarcoma and their families members) in this study. Detestability results between brackets for mouth rinses obtained from (Cookb et al., 2002). ND, Not done.
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Table 5
Appendix 3

Reproduction of publications reprints generated from work done for this thesis
Multiple Human Herpesvirus–8 Infection

Mohammed M. Beyari,¹,² T. A. Hodgson,¹ R. D. Cook,¹,² W. Kondowe,³ E. M. Molyneux,³ C. M. Scully,¹ C. G. Too,¹ and S. R. Porter¹
Genotypic Profile of Human Herpesvirus 8 (Kaposi’s Sarcoma-Associated Herpesvirus) in Urine

M. M. Beyari,1,2* T. A. Hodgson,1 W. Kondowe,3 E. M. Molyneux,3 C. M. Scully,1 S. R. Porter,1 and C. G. Teo2
Inter- and Intra-Person Cytomegalovirus Infection in Malawian Families

Mohammed M. Beyari,1,2* T.A. Hodgson,1 W. Kondowe,3 E.M. Molyneux,3 C. Scully,1 S.R. Porter,1 and C.G. Teo2

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Inter- and Intra-Person Cytomegalovirus Infection