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

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

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

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

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

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

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

Ethical approval was obtained prior to commencing this

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All ORF K1/V1 DNA sequences have been deposited in GenBank, accession numbers AF398120–AF398140 and AF451301–AF451321.
### Table 1. Characteristics and virological status of Malawian KS patients and their family members

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

<table>
<thead>
<tr>
<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>
</tr>
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<tbody>
<tr>
<td>Age/sex</td>
<td>Site of KS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A,‡§</td>
<td>7 y/F</td>
<td>Abdomen, limbs</td>
<td>No</td>
<td>A2 (10 y/M)‡, A3 (7 m/M)</td>
<td>A1 (34 y)‡§</td>
</tr>
<tr>
<td>B,‡</td>
<td>4 y/M</td>
<td>Cervical</td>
<td>Yes</td>
<td>B1 (20 y/F)‡, B2 (13 y/M)‡, B3 (12 y/M)‡, B4 (10 y/M)‡, B5 (7 y/M)‡, B6 (11 m/M)</td>
<td>B7 (40 y)‡</td>
</tr>
<tr>
<td>C,‡‡</td>
<td>8 y/M</td>
<td>Abdomen, limbs</td>
<td>No</td>
<td>C2 (14 y)‡, C3 (12 y/M)‡</td>
<td>C1 (45 y)‡</td>
</tr>
<tr>
<td>E,‡</td>
<td>31 y/F</td>
<td>Legs, palate</td>
<td>No</td>
<td>E1 (5 y/F)‡, E2 (9 y/F)‡, E3 (6 y/F)‡, E4 (6 y/F)‡, E5 (13 y/M)‡, E6 (10 y/M)‡</td>
<td>F2 (7 y/M)‡</td>
</tr>
<tr>
<td>F,‡</td>
<td>2 y/M</td>
<td>Foot</td>
<td>No</td>
<td>G1 (32 y)‡‡</td>
<td>F1 (35 y)‡§</td>
</tr>
<tr>
<td>G‡</td>
<td>34 y/F</td>
<td>Foot, leg, palate</td>
<td>No</td>
<td>G2 (23 y/M)‡‡, G3 (19 y/F)‡, G4 (15 y/M)‡, H1 (13 y/M)‡, H2 (12 y/M)‡, H3 (9 y/M)‡, H4 (8 y/M)</td>
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<tr>
<td>H‡</td>
<td>35 y/F</td>
<td>Leg</td>
<td>Yes</td>
<td>J1 (22 y)‡</td>
<td>J1 (12 y/M)‡</td>
</tr>
<tr>
<td>I‡</td>
<td>36 y/F</td>
<td>Leg</td>
<td>Yes</td>
<td>J2 (3 y/M)</td>
<td>K1 (12 y/M)‡</td>
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<tr>
<td>J‡‡</td>
<td>28 y/M</td>
<td>Leg</td>
<td>No</td>
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<td>N1 (18 y/F)</td>
</tr>
<tr>
<td>K,‡‡</td>
<td>30 y/F</td>
<td>Leg, foot</td>
<td>No</td>
<td>M4 (32 y)‡</td>
<td>N1 (18 y/F)</td>
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<tr>
<td>L‡</td>
<td>33 y/M</td>
<td>Leg</td>
<td>Yes</td>
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<td>P2 (2 y/M)</td>
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<tr>
<td>M‡</td>
<td>30 y/F</td>
<td>Leg, palate</td>
<td>No</td>
<td>Q1 (21 y)‡§</td>
<td></td>
</tr>
<tr>
<td>N‡</td>
<td>30 y/M</td>
<td>Leg, palate</td>
<td>Yes</td>
<td>P1 (28 y)‡§</td>
<td></td>
</tr>
<tr>
<td>P‡</td>
<td>30 y/M</td>
<td>Leg</td>
<td>Yes</td>
<td>R1 (25 y)‡‡</td>
<td>R2 (12 y/M)‡, R3 (9 y/M)‡</td>
</tr>
<tr>
<td>Q‡‡</td>
<td>24 y/M</td>
<td>Foot</td>
<td>No</td>
<td>S2 (37 y)‡</td>
<td></td>
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<tr>
<td>R‡</td>
<td>41 y/M</td>
<td>Leg</td>
<td>Yes</td>
<td>T1 (37 y)‡§</td>
<td>T2 (11 y/M)‡, T3 (9 y/M)‡, T4 (4 y/M)‡</td>
</tr>
<tr>
<td>S‡</td>
<td>40 y/M</td>
<td>Leg</td>
<td>Yes</td>
<td>S1 (37 y)</td>
<td>T2 (11 y/M)‡, T3 (9 y/M)‡, T4 (4 y/M)‡</td>
</tr>
<tr>
<td>T‡</td>
<td>33 y/F</td>
<td>Leg, foot</td>
<td>Yes</td>
<td>U1 (44 y)‡, T3 (9 y/M)‡, T4 (4 y/M)‡, U2 (12 y/F), U3 (8 y/F)</td>
<td>T2 (11 y/M)‡, T3 (9 y/M)‡, T4 (4 y/M)‡</td>
</tr>
<tr>
<td>U‡</td>
<td>32 y/F</td>
<td>Leg, foot, gingiva</td>
<td>No</td>
<td>W1 (36 y)‡‡</td>
<td>W2 (20 y/M)‡‡, W3 (19 y/M)‡, W4 (13 y/F)‡, W5 (6 y/M)‡, W6 (7 m/F)‡ §</td>
</tr>
<tr>
<td>W‡‡</td>
<td>43 y/F</td>
<td>Leg, palate</td>
<td>Yes</td>
<td>W1 (36 y)‡‡</td>
<td></td>
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<tr>
<td>X‡</td>
<td>41 y/F</td>
<td>Chest, palate</td>
<td>Yes</td>
<td>X1 (44 y)‡‡</td>
<td>X2 (18 y/F)‡, X3 (11 m/M)</td>
</tr>
<tr>
<td>Y‡</td>
<td>32 y/M</td>
<td>Arm</td>
<td>Yes</td>
<td>Y1 (38 y)</td>
<td>Y2 (8 y/F), Y3 (5 y/F), Y4 (3 y/F)</td>
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<tr>
<td>Z‡‡</td>
<td>21m/M</td>
<td>Cervical</td>
<td>No</td>
<td>Z1 (5 y/F)‡</td>
<td>Z2 (22 y)‡‡</td>
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</table>

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

Study from the Eastman Dental Institute (UK), University College London (UK) and the University of Malawi. Consecutive patients of the Central Hospital, Blantyre, Malawi, with presumptive cutaneous, oral and histologically confirmed nodal KS were invited to join the study. At presentation, all patients were offered palliative treatment with intravenous vincristine (2 mg/m²). Regardless of whether treatment was administered, patients were requested to return within a week with all available household members. During the return visit, blood and mouth rinse samples were taken from patients and from each family member. HIV-1 seropositivity was confirmed by an IgG capture particle adherence test (Parry et al., 1995), and HHV-8 seropositivity by the HHV-8 IgG IFA Kit (Advanced Biotechnologies Incorporated). This IFA utilizes the KS-1 cell line as a source of HHV-8, expressing both latent and lytic HHV-8 antigens on its cell surface. This highly specific and sensitive assay is appropriate for use in epidemiological studies (Chantyne et al., 1998).

DNA was extracted from the immunomagnetically selected CD45⁺ leukocyte fraction of blood, as described previously (Leao et al., 2000). Study participants were asked to rinse with...
PBS and spit the rinse into a universal centrifuge tube. These samples were subsequently spun to separate the pellet from the cell-free fraction. DNA was extracted from 150 μl of mouth rinse pellets by a guanidium thiocyanate–silica procedure (Boom et al., 1990).

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

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

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

Thirty-nine (91%) of the 22 index cases were HIV-1 seropositive and all were HHV-8 seropositive. Of the relatives, 16 (23%) were HIV-1 seropositive and 46 (69%) were HHV-8 seropositive. Overall, there was a positive association between HHV-8 and HIV-1 seropositivity in the total number of study subjects \( (P = 0.004) \) (\( \chi^2 \) test).

Of the 22 index patients, 5 (23%) were K1/V1 DNA positive in blood and 4 (18%) in oral rinses (Table 1). HHV-8 DNA negativity in the blood in index cases was significantly associated with prior vincristine therapy \( (P = 0.001) \) (\( \chi^2 \) test). In the samples of 67 family members of patients with KS, K1/V1 DNA was positive in the oral rinse of 18 (27%) but not in any of the blood samples. ORF K1/V1 DNA was detected in the oral sample in the absence of HHV-8 seropositivity in four of the family members (B3, K1, R3 and X2).

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

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

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

Identical K1/V1 DNA sequences were recovered in families E, G, K and Z. Identical sequences were found in family E, between the mother (E1) and one of her sons (E6), in families K and Z, between the mothers (K1 and Z1) and their sons (K1 and Z2), and in family G between a father (G1) and son (G2). Common sequences recovered from these family members indicated that intra-familial transmission of HHV-8 may have occurred. Transmission between mothers and their children has been previously reported (Plancoulaine et al., 2000), and this may have occurred in families E, K and Z. In family G, a father and son shared the same sequence. The mother’s sequence (G1) was not available for study and the possibility that she may have acted as a source of infection for both her spouse and her son cannot be excluded. In addition, sequences recovered from families G, K and Z, all ORF K1 A5 subtypes, were very similar and it is possible that a regional HHV-8 variant is circulating in this population.

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

Within family B, there were two sequences recovered from B5 clones, designated B5(I) and B5(II). Subject B5 might have been dually infected with two HHV-8 variants, one A4 subtype virus [B5(I)] and one B1 subtype virus [B5(II)].
Fig. 1. Predicted phylogenetic distribution of a 213 bp segment of HHV-8 ORF K1/V1 (a) and a 462 bp segment of ORF K1/V2 (b) amplified from KS patients and their family members (in bold) in a background of GenBank sequences representing major ORF K1 subtypes. Sequences were recovered from oral samples unless otherwise indicated. Linear unrooted phylogenetic dendograms were generated using PHYLIP, DNADIST and NEIGHBOR programs. Bootstrapping for 1000 replicates is noted as a percentage at major branch points. The genetic distance size scale for 0.1 (10% divergence) is indicated. *Clones B5(III), T2(VI), E4(II), W4(IV), W2(V), B3(V), E4(III), E4(I), B3(IV), B3(I) and W4(V) have an identical sequence to T2(V).
Recombination in HHV-8 viral strains recovered from African patients suggests dual infection may sometimes occur (Zong et al., 2002). We were not able to consistently amplify the B5(I) sequence, and contamination from an external source cannot be ruled out.

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

The disparate K1/V1 and K1/V2 sequences found between T2 and T3 demonstrated that these siblings were unlikely to have acquired HHV-8 infection from one another. ORF K1/V1 sequences recovered from X1 and his daughter, X2, belonged to different genotypes (A2 and B1, respectively) indicating no transmission linkage between each other. ORF K1/V1 sequences compared between W4, her father, W1, and brother, W2, differed by one or two amino acids. Nevertheless, intrafamilial HHV-8 transmission via the mother (W1), whose ORF K1 sequence was not available, is possible. In family E, recovered ORF K1/V1 sequences revealed minor nucleotide variations between E4, E5 and EiE6. However, only E4 carried a divergent amino acid sequence (Fig. 2) and transmission from the mother, Ei, was possible in this family.

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

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

These data also substantiate previous observations of early non-sexual HHV-8 acquisition in children living in endemic populations (Mayama et al., 1998; Gessain et al., 1999; Plancoulaine et al., 2000). The higher rate of recovery of HHV-8 sequences from oral compared with blood samples suggests that oral secretions potentially act as vehicles of non-sexual horizontal spread. Thus, the control of HHV-8 infection...
and the KS epidemic cannot rely on community-wide environmental sterilization and disinfection. Reducing the size of the reservoir of infection by anti-viral treatment of infected persons is also impractical. The optimal route to control of KS in endemic regions would be through vaccination.

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References


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