Detection of Human Herpesvirus 8 DNA Sequences in Tissues and Bodily Fluids

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Human herpesvirus 8 (HHV-8) has been proposed as a sexually transmitted etiologic agent of Kaposi's sarcoma (KS). In this study, by use of a sensitive polymerase chain reaction assay, HHV-8 DNA was detected in the skin lesions (92%), normal skin (23%), peripheral blood mononuclear cells (PBMC) (46%), plasma (7%), saliva (37%), and semen (12%) but not stool samples from KS patients. The average number of HHV-8 copies per microgram of positive target DNA was 64,000, 9000, 40, 33,000, and 300 for skin, PBMC, plasma, saliva, and semen samples, respectively. Only 1 non-KS donor sample, of saliva, was positive for HHV-8. Sequencing showed 5% divergence among HHV-8 strains. The data suggest that saliva may be more important than semen or stool in the sexual transmission of HHV-8. The relatively high prevalence of HHV-8 in PBMC raises the question as to why there is no evidence for blood-borne virus transmission.

Kaposi's sarcoma (KS) occurs as a mild indolent disease in immunocompetent persons but is an aggressive, lethal disease in immunosuppressed patients, particularly those infected with human immunodeficiency virus (HIV) [1]. Epidemiologic studies suggest that KS is a sexually transmitted disease caused by the newly discovered human herpesvirus 8 (HHV-8) [1, 2]. Subsequently, HHV-8 has been shown to be associated with numerous other tumors, including body cavity lymphomas, angiosarcomas and hemangiomas, premalignant Bowen's disease, malignant squamous cell carcinomas, and actinic keratosis [2].

HHV-8 positivity has been shown to be more closely linked to HIV-positive patients with homosexuality or bisexuality as their major risk factor for HIV than to patients with other risks [2]. Hence, it has been suggested that HHV-8 may be a sexually transmissible virus, particularly via anal intercourse. The fact that HHV-8 can be found in semen samples and in individual spermatozoa and mononuclear cells present in semen has supported this hypothesis [3]. However, in large-scale or smaller matched studies, the prevalence of HHV-8 in semen has been equal to or less than that found in peripheral blood mononuclear cells (PBMC). In this study, we used the polymerase chain reaction (PCR) to detect and quantify HHV-8 DNA sequences in biopsy specimens, blood, semen, saliva, and stool from HIV-positive and -negative KS patients as well as patients and healthy controls without KS.

Materials and Methods

Patient samples. Skin, PBMC, plasma, semen, saliva, feces, and tumor specimens, including some previously analyzed and described elsewhere [4], were obtained from donors with and without KS and with and without HIV infection. One hundred two KS patients and 228 non-KS patients donated at least one type of specimen. Only 10 of the KS patients were HIV-negative, and 7 of these were heterosexual. Only 20 of the 65 HIV-positive, non-KS patients were homosexual. The total number and types of samples are listed in tables 1 and 2.

DNA analyses. DNA from skin, PBMC and whole plasma, semen, and saliva was organically extracted as previously described [4]. DNA was prepared from 10 g of stool as per Deuter et al. [19] with modifications. Briefly, this technique involved homogenizing stool in a lysis buffer, with and without an adsorption matrix of potato flour, which binds bile salts that can inhibit PCR. DNA quantification for all samples was accomplished by spectrophotometry. Amplification of 1 or 4 μg of extracted DNA was done as previously described [4].

Primer sequences derived from the KS330/BAM HHV-8 strain were described previously [4]. Each primer included different non-human, nonviral 21-bp linkers attached to their 5′ end. Before addition of the reaction mixture, the DNA polymerase was incubated for 10 min at room temperature with a 50-fold molar excess of anti-Taq antibody TP1 and a 5-fold molar excess of anti-Taq antibody TP4 (gift of Ortho Clinical Diagnostics Systems, Rochester, NY), which allow for hot start PCR conditions [4]. The reaction mixture was also incubated at room temperature for 5 min

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Informed consent was obtained from all patients or their parents or guardians, and human experimentation guidelines of the US Department of Health and Human Services were followed as were guidelines established by the Institutional Review Board of SUNY Health Science Center at Syracuse.

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Table 1. Prevalence of HHV-8 DNA in different tissues and bodily fluids of Kaposi's sarcoma (KS) patients according to various studies.

Reference	KS lesions	Normal skin	PBMC	Plasma/serum	Semen	Saliva	Feces
Current study	44/48 (92)	6/26 (23)	47/102 (46)	3/43 (7)	4/33 (12)	7/19 (37)	0/11
5	19/22 (86)	1/16 (6)	18/25 (72)	7/25 (28)	0/12	18/25 (72)	—
6[a]	—	—	24/46 (52)	19/75 (25)	—	1/27 (4)	0/18
7[a]	24/26 (92.3)	0/15	4/8 (50)	—	1/1 (100)	—	—
8[a]	8/9 (89)	4/7 (57)	4/8 (50)	—	—	—	—
9	34/34 (100)	0/24	0/12	—	—	—	—
10[a]	38/38 (100)	11/36 (31)	31/37 (84)	—	—	—	—
11	14/14 (100)	6/14 (42.6)	3/14 (21.4)	—	3/14 (21.4)	—	—
12[a]	—	10/15 (67)	—	—	3/15 (20)	—	—
13	—	—	34/98 (35)	—	—	—	—
14	—	—	7/10 (70)	—	—	—	—
15[a]	—	—	—	—	2/14 (14.3)	—	—
16[a]	—	—	—	—	—	25/76 (33)	—
Total[b]	181/191 (95)	38/153 (25)	172/360 (48)	29/143 (20)	13/89 (15)	51/147 (35)	0/29

NOTE. PBMC, peripheral blood mononuclear cells. Data are no./total (%). —, not done.

[a] Nested polylmerase chain reaction analysis.

[b] When comparing total results, all other samples had greater prevalence than feces ($P = .03$ to $<.001$), KS skin lesions had greater prevalence than all other sample types ($P < .001$), PBMC had greater prevalence than saliva or semen ($P < .004$ and $<.001$, respectively), and saliva had greater prevalence than semen ($P = < .001$), by Fisher's exact t test.

before PCR to allow for uracil-*N*-glycosylase (UNG) sterilization [4]. Samples were amplified for 35 cycles as described previously [4]. As a control, specimens were also analyzed for HIV-1 *gag* DNA or RNA via PCR with the primers SK38/SK39 and a commercially available detection assay for the amplified product (Cellular Products, Buffalo, NY) [4]. All samples were also amplified with human β-globin gene primers PCO3 and PCO4 to confirm the presence of amplifiable human DNA [4]. To prevent carryover contamination, all pre- and post-PCR reactions were conducted by separate personnel in different buildings. All positive PCR samples were reamplified with "signature primers" complementary to the linker sequences incorporated into all amplicons. A positive PCR reaction with these signature primers is indicative of carryover contamination [4].

Amplified HHV-8 DNA was detected by Southern blot hybridization as previously described [4]. The sensitivity of the PCR assay was ~10 copies in a background of 1 or 4 μg of total DNA input. This determination was made by amplifying replicate dilutions of an HHV-8–positive KS tumor specimen, whose copy number had been previously determined, in normal human DNA. At a dilution of 10 copies of HHV-8 per aliquot, the replicates were positive ~60% of the time. The HHV-8 DNA copy number of each positive sample was determined by comparing its Southern blot signal with that of a known standard curve.

The 191-bp HHV-8 PCR amplicons from 3 different saliva samples were cloned into the TA cloning vector and sequenced as previously described [4]. Sequences were aligned and phylogenetically compared with other HHV-8 sequences [4]. In all, HHV-8 sequences from 29 of the positive specimens listed in tables 1 and 2 were compared.

Results

All skin, PBMC, plasma, saliva, and semen samples were positive by PCR for human β-globin DNA. Of 11 stool samples, 8 were weakly positive on analysis for human β-globin (data not shown). No difference in results was observed with the additional step of adsorption of bile salts with potato flour. None of the samples from HIV-negative donors was positive for HIV-1 DNA or RNA, while 5%, 5%, 100%, 20%, 35%, and none of the KS lesions, normal skin, PBMC, saliva, semen, and stool samples, respectively, from HIV-positive donors was positive for HIV DNA (data not shown). Seventy-three percent of the plasma samples from HIV-positive donors were positive for HIV-1 RNA (data not shown).

HHV-8 DNA detection was significantly skewed to whether a specimen was from a KS patient (tables 1, 2). Detection rates of 92%, 23%, 46%, 7%, 37%, 12%, and none were observed in KS skin lesions, normal skin, PBMC, plasma, saliva, semen, and stool samples, respectively, from KS patients (table 1). To assess whether the low prevalence of HHV-8 DNA in stool could be secondary to a PCR inhibitor, 1000 copies of control HHV-8 DNA were added to the DNA extracted from stool, and no decrease in PCR amplification was observed compared with the non-admixed control. About 5% of the positive samples above were negative at 1 μg but positive at 4 μg of DNA input (data not shown).

None of the 536 skin, PBMC, plasma, or neoplastic tissue specimens from HIV-positive and -negative donors without KS was positive for HHV-8 DNA (table 2). Saliva samples from 1 non-KS, HIV-1–positive and 23 non-KS, HIV-1–negative donors were found to be negative for HHV-8 DNA when 1 μg of DNA was used in the PCR reaction. However, when 4 μg of DNA was used, 1 of the healthy volunteers was positive for HHV-8. In retrospect, this person had an active case of oral lichen planus. However, DNA extracted from both her normal and lichen planus–involved skin was negative for HHV-8.

None of the HHV-8 PCR-positive samples above was positive when reamplified with signature primers. HHV-8 copy numbers per microgram of sample DNA varied among the

Table 2. Prevalence of HHV-8 DNA in different tissues and bodily fluids of patients without Kaposi's sarcoma according to various studies.

Reference	Normal skin	PBMC	Plasma/serum	Semen	Saliva	Other[a]
Current study						
HIV$^+$	0/10	0/65	0/65	—	0/1	0/38
HIV$^-$	0/1	0/163	0/163	—	1/23 (4)	0/30
5						
HIV$^+$	—	2/20 (10)	0/20	0/10	3/20 (15)	—
HIV$^-$	—	—	—	—	0/24	—
6[b]						
HIV$^+$	—	11/143 (8)	0/22	—	0/24	—
HIV$^-$	—	0/160	—	—	—	—
9						
HIV$^+$	—	—	—	—	—	3/6 (50)
HIV$^-$	0/24	0/12	—	—	—	—
11						
HIV$^+$	—	0/9	—	0/9	—	—
HIV$^-$	—	0/5	—	0/5	—	—
12[b]						
HIV$^+$	—	—	2/9 (22)	3/9 (33)	—	—
HIV$^-$	—	—	—	0/115	—	—
13						
HIV$^+$	—	12/64 (19)	—	—	—	—
HIV$^-$	—	0/11	—	—	—	—
14						
HIV$^+$	—	5/23 (22)	—	—	—	—
HIV$^-$	—	—	—	—	—	—
16[b]						
HIV$^+$	—	—	—	—	—	—
HIV$^-$	—	—	—	—	0/39	—
7[b]						
HIV$^+$	—	—	—	—	—	—
HIV$^-$	0/5	5/45 (11)	—	6/45 (13)	—	0/57
17[b]						
HIV$^+$	—	—	—	—	—	—
HIV$^-$	1/18[c] (6) 4/14 (29) 1/10 (10) 0/4	1/14 (7)	—	35/43 (81)	—	2/10[d] 0/6 0/4 (6) 2/33 (6) 7/16 (44)
18[b]						
HIV$^+$	—	—	—	—	—	—
HIV$^-$	—	—	—	37/63 (59)	—	—
Total[e]						
HIV$^+$	0/10	30/324 (9)	2/116 (1.7)	3/28 (11)	3/45 (7)	3/44 (7)
HIV$^-$	6/76 (8)	6/410 (1.5)	0/163	78/271 (29)	1/86 (1.2)	11/156 (7)

NOTE. PBMC, peripheral blood mononuclear cells. Data are no./total (%). —, not done.

[a] Tumor specimens, lymph nodes, and benign skin lesions.

[b] Nested polymerase chain reaction analysis.

[c] Further stratification (top to bottom): normal skin, foreskin, vulva, glans penis.

[d] Further stratification (top to bottom): kidney, bladder, ureter, uterine cervix, prostate.

[e] In HIV-positive patients, differences between plasma and PBMC, semen, or saliva were all significant ($P < .003$), whereas differences between PBMC and semen or saliva and difference between semen and saliva were not (all $P > .4$). In HIV-negative subjects, prevalence of HHV-8 was significantly greater in semen samples than in all other specimen types ($P < .001$); however, variation in positive semen rates among studies is quite large (0–81%).

different samples as follows: KS skin lesions and normal skin, 1000–100,000 (mean, 64,000); PBMC, 2.5–100,000 (mean, 9000); plasma, 2.5–100 (mean, 40); saliva, 2.5–100,000 (mean, 33,000); and semen, 2.5–500 (mean, 300). All of these were significantly different from each other by χ^2 analyses (P ranged from .03 to <.001). Neither the prevalence in a particular tissue or bodily fluid nor the HHV-8 DNA copy number in positive patients correlated with their peripheral blood CD4 lymphocyte count (data not shown).

Finally, DNA sequencing of the amplified HHV-8 DNA from 3 AIDS and KS patients' saliva samples revealed a few discrete point mutations in each compared with the prototypic KS330/BAM strain (GenBank accession nos. AF042148, AF042149, and AF042150). When phylogenetically compared with other published HHV-8 sequences, the 3 HHV-8 strains obtained from the saliva samples cluster as a group (figure 1). The overall range of divergence among the 31 HHV-8 strains evaluated is between 0 and 5.2% in the 191-bp sequence analyzed.

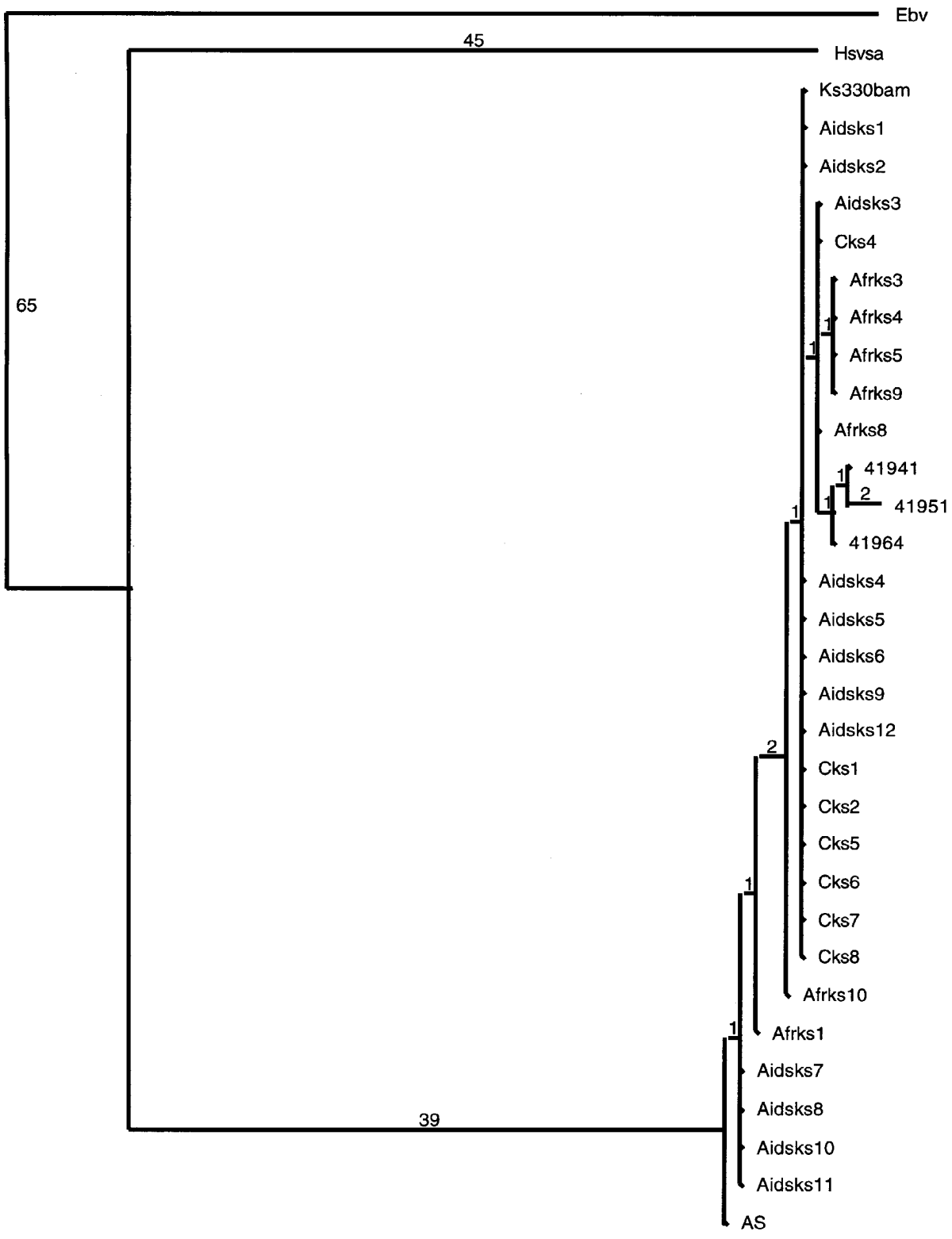

Figure 1. Phylogram generated by parsimony method, based on 191 homologous bases of DNA sequence from 31 different HHV-8 strains. Strains 41941, 41951 and 41964 are from saliva samples from 3 human immunodeficiency virus type 1–positive, Kaposi's sarcoma–positive homosexual men from same clinic in New York City. KS330/BAM strain is considered prototype [8]. Homologous Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS) sequences were used as outgroups. No. of unique bases on each branch is shown. Bootstrap values between EBV and HVS and HHV-8 and between HVS and HHV-8 are 100%. However, bootstrap values among HHV-8 strains are all <50%. As geographic reference, strains identified as AFRKS were detected in samples from Africa. All others except AS30835, which was from Tennessee, are from New York.

Discussion

Data suggesting that HHV-8 is the etiologic agent of KS continue to mount. The epidemiologic data suggest that HHV-8 is a sexually transmissible agent and that KS is a sexually transmitted disease with high penetrance in immunocompromised hosts. Questions remain as to the route(s) of HHV-8 acquisition and specific behavior modification in infected persons to decrease transmission to others, particularly if the latter are immunodeficient.

Conflicting reports exist as to the prevalence of HHV-8 DNA in human tissues and bodily fluids. Most of these reports are based on PCR data, in which variable assay sensitivity or specificity could account for the observed differences. To achieve high sensitivity, some investigators have used nested primers, which necessitates reopening samples in rooms containing previously amplified HHV-8 DNA, almost inviting carryover contamination. In the present study, we have used a highly sensitive assay capable of detecting 10 copies of HHV-8 per aliquot tested 60% of the time. Our data indicate that the HHV-8 copy number can be quite low, however, and high sensitivity requires at least 4 μg of DNA input. Because of the high sensitivity of our assay, we have not felt the need to use nested primer pairs, eliminating this source of false-positive results. Further, our approach allows for the use of "UNG sterilization" to prevent false-positive results. Our signature primer results, the almost total skewing of HHV-8–positive results to samples from KS patients, our HHV-8 sequence data, and our lack of false-positive results in HIV testing all support our impression that contamination is not obfuscating our results.

Our data are consistent with previous reports that HHV-8 DNA is present in >90% of KS tumor lesions, regardless of the form of KS, and that positivity in a tissue or bodily fluid is overwhelmingly skewed to the donor having KS (tables 1, 2). Also, compared with that in other tissues and body fluids, the HHV-8 DNA copy number per microgram of DNA was highest in KS skin lesions. Why the prevalence of HHV-8 was not 100% in KS lesions is unclear, but it may have more to do with sampling error in a specimen than with assay performance.

The epidemiologic data cited above would lead to the hypothesis that HHV-8 would be most prevalent in semen, saliva, or feces in patients with KS compared with PBMC or plasma. However, this is not necessarily the case in our own or others' work, based mostly on unmatched samples (table 1). Detection rates and HHV-8 DNA copy numbers are actually highest in PBMC and saliva samples. Semen and plasma had the lowest positivity rates and copy numbers. No HHV-8 DNA was found in stool specimens.

Comparison of HIV detection rates among the tissues and bodily fluids is interesting. While HHV-8 demonstrated a predilection for skin, HIV-1 did not. HIV DNA was present in all PBMC samples, while RNA was detected in only 73% of plasma samples, presumably secondary to the inhibitory effects of antiretrovirals but also highlighting the fact that, despite some patients having undetectable RNA (limit of detection, 50 copies/mL of plasma), all still had proviral DNA in their PBMC. In contrast, only half of the KS patients had HHV-8 in their PBMC, and a very small minority had positive results in their plasma. HHV-8 was more prevalent in saliva than was HIV and less prevalent in semen, and neither virus was found in stool specimens.

With respect to HHV-8 DNA detection in tissues and bodily fluids from non-KS donors, whether HIV-1–infected or not, our own data would suggest that the frequency is low relative to what would be expected from serology studies [20]. However, some have indicated that the prevalence of HHV-8 DNA could be higher (table 2). Two of these [17, 18] report exceedingly high rates of HHV-8 detection in DNA from semen and the male and female urogenital tract in HIV-1–negative patients (table 2). If correct, these latter two reports make a strong case for a sexual mode of transmission, but their data seem inconsistent with those of others. In part, different detection rates among various investigations could be secondary to different risk factors among the HIV-1–positive population or different geographic locations or ethnic makeup of the study population. Indeed, 20 of our HIV-positive, non-KS patients were homosexual, while 45 belonged to other risk groups. When we analyzed all of the studies in table 2, 28 (13%) of 214 male homosexual HIV-positive, non-KS patients and 2 (2%) of 110 HIV-positive, non-KS patients with other risk factors were positive for HHV-8 DNA in their PBMC ($P < .001$). Also, our own data indicate a degree of divergence among HHV-8 strains. The extent of this divergence among worldwide strains and its impact on detection assay sensitivity is unknown.

Taken as a whole, the data indicate that infection rates and levels of HHV-8 replication are much higher in the KS than in the non-KS population. As has been suggested by others, HHV-8 replication is probably cyclical from latent sites and may be immunologically controlled [6], but from our own and others' data, detection rates and copy number do not necessarily inversely correlate with CD4 cell counts [13, 14]. Whether specific immunoreactivity to HHV-8 is variable among individuals is unknown. The relatively high prevalence and copy number of HHV-8 in saliva compared with semen, found by ourselves and others [5, 16, 21], suggest that oral contact might carry more of a risk for transmission than intercourse. The fact that infectious HHV-8 can be isolated from saliva lends support for this hypothesis [21]. The low prevalence and copy number of HHV-8 DNA in plasma are consistent with the lack of evidence for an association of transfusion of plasma products and KS. However, the higher prevalence and copy number of HHV-8 DNA in PBMC raise the question as to why HHV-8 could not be transmitted via the transfusion of cellular blood products. Further biologic assays and epidemiologic studies are warranted to investigate this question to ascertain whether screening of blood donors for HHV-8 is warranted.

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