Frequent Detection of Kaposi’s Sarcoma–Associated Herpesvirus (Human Herpesvirus 8) DNA in Saliva of Human Immunodeficiency Virus–Infected Men: Clinical and Immunologic Correlates

David M. Koelle, Meei-Li Huang, Bala Chandran, Jeffrey Vieira, Michael Piepkorn, and Lawrence Corey

The prevalence, quantity, temporal pattern, and clinical and immunologic correlates of shedding of Kaposi’s sarcoma (KS)–associated herpesvirus (KSHV; or human herpesvirus [HHV]-8) DNA in saliva were studied. KSHV DNA was detected in saliva from 18 (75%) of 24 human immunodeficiency virus (HIV)-positive patients with KS and from 1 of 1 HIV-negative patient with KS, 3 (15%) of 20 HIV-positive patients without KS, and none of 24 controls. KSHV DNA levels ranged from $10^{2.4}$ to $10^6$ copies/mL and were lower than levels for Epstein-Barr virus but comparable to those for HHV-6. Detection of KSHV DNA in saliva was not associated with oral KS or decreased peripheral blood CD4 cell counts. KSHV DNA was not detected in semen. Resistance of KSHV DNA from saliva to DNase treatment was consistent with the presence of virions. These data suggest that KSHV can replicate in the oropharynx and that salivary contact could contribute to KSHV transmission.

A new herpesvirus, named Kaposi’s sarcoma (KS)–associated herpesvirus (KSHV) or human herpesvirus (HHV)-8, has been identified in KS tumor tissue from patients from all KS risk groups. KSHV infection appears to be necessary for the development of KS [1–4]. Many human immunodeficiency virus (HIV)–infected persons appear to be infected with KSHV but do not have clinical KS [5–7]. The route(s) of KSHV transmission and acquisition are unknown, although epidemiologic and serologic data suggest that sexual transmission may occur. The incidence of KS is higher among homosexual and bisexual men than among patients infected with HIV by parenteral blood exposure [8]. Among gay and bisexual men, KS has been linked to sexual practices involving fecal-oral contact, a high number of sex partners, and a history of sexually transmitted diseases [9–11]. Preliminary serologic analyses suggest that in the United States, KSHV is relatively restricted to adult gay or bisexual men. Patients with KS have the highest serologically defined KSHV infection rates (80%–100%), followed by HIV-infected gay and bisexual men without KS (28%–30%) and patients of sexually transmitted disease clinics (13%), with most studies measuring a lower rate (<5%) among HIV-negative blood donors [5–7, 12]. One study found that the seroprevalence in the general adult population may be as high as 25% [13], and further refinement of serologic methods is needed. These epidemiologic and serologic data are consistent with a sexual route of KSHV transmission.

KS also occurs in an endemic pattern among older persons in the Mediterranean region and in an aggressive infantile pattern in parts of Africa. Seroprevalence rates in Italy and parts of Africa are higher than in the United States, regardless of the test methodology [6, 7, 13]. Thus, it is possible that nonsexual transmission of KSHV also occurs. Body fluids and sites potentially involved in KSHV transmission have not been clearly identified. Studies of the prevalence of KSHV in semen have yielded conflicting results, ranging from the ubiquitous presence of low levels of KSHV DNA in the semen of most HIV-infected and some HIV-seronegative persons [14, 15] to the absence of detectable KSHV DNA in the semen of patients with AIDS-associated KS [16, 17]. Fecal specimens have also been negative for the presence of KSHV [18]. Bloodborne transmission of KSHV appears to be infrequent (despite the presence of KSHV within circulating B lymphocytes [17]), as evidenced by the fact that injection drug users and hemophiliac patients with AIDS have relatively low incidences of KS [8].

Among the herpesviruses that infect humans, KSHV is most similar to Epstein-Barr virus (EBV) [13]. Both viruses are B...
lymphotropic and have latent and lytic life cycles in B lymphocytes [17, 19]. EBV undergoes lytic infection in oropharyngeal epithelial cells; persistent salivary shedding of EBV is well documented, and contact with saliva is responsible for EBV transmission [20, 21]. Other HHVs, including cytomegalovirus, herpes simplex virus (HSV)-1, and probably HHV-6 and HHV-7, can also be transmitted in saliva. We hypothesized that KSHV may also be capable of replication in the oropharynx and, therefore, undertook a study of KSHV DNA levels in saliva from patients with or at risk for KS. The levels of serum antibodies to KSHV-containing cell lines, induced to lytic KSHV replication, and clinical and immunologic variables were also measured to determine the relationships between these factors and the shedding of KSHV DNA in saliva. We have begun characterization of the KSHV DNA in saliva by assessment of its sensitivity to digestion with DNase.

Materials and Methods

Patients and specimens. Persons with KS were sought from community health care providers. HIV-infected patients without KS were enrolled from ongoing studies of HIV and herpesviruses. Analysis of peripheral blood cell T cell subsets was performed by the University of Washington Hematopathology Laboratory. HIV serology was done using EIA and immunoblot analysis. The single HIV-seronegative patient with KS was confirmed to be HIV uninfected by use of both serologic assays and a peripheral blood mononuclear cell (PBMC) polymerase chain reaction (PCR) assay for HIV DNA (Amplicor; Roche, Nutley, NJ).

All patients underwent a detailed skin and mucous membrane examination and a standardized interview. Acid-citrate-dextrose–anticoagulated blood, whole exfoliated saliva, and 4-mm punch biopsies of normal skin and KS lesions were obtained. Saliva, whole semen, and plasma were frozen at −70°C until analysis. Selected patients had parotid saliva collected by use of small, buccal collection cups (Quality Biological, Gaithersburg, MD) or by daily saliva collection in clinic or at home (with storage at 4°C for ≤5 days). Plasma was collected by centrifugation of acid-citrate-dextrose blood (400 g, 10 min). PBMC isolated by ficoll-hypaque centrifugation using heparin-free wash solutions were frozen (−70°C) in 150 μL of PBS. Saliva was also collected from 8 healthy HIV-seronegative adult volunteers, and stored salivary DNA from 16 healthy adults from a study of HHV-6 was also analyzed [22].

Specimen input into PCR reactions was normalized for cellularity or volume. For saliva, plasma, and semen, 200- to 400-μL samples were submitted for DNA extraction. For PBMC, 3 × 10⁶ cells were used. One-fifth to one-tenth of fresh skin biopsies were used for DNA extraction. For formalin-fixed, paraffin-embedded tissue, 3–9 serial 10- to 20-μm sections were deparaffinized [23] before DNA isolation.

PCR analysis. DNA was isolated by phenol-chloroform extraction after overnight digestion at 50°C with 100 μg/mL proteinase K, 0.5% SDS, 25 mM EDTA, 100 mM NaCl, and 10 mM TRIS (pH 8.0) [24]; precipitated with sodium acetate (0.25 M), glyccogen (100 μg/mL), and 2 vol of ethanol; and resuspended in 200 μL of 10 mM TRIS (pH 8.0). Ten microliters (5%) of the DNA was used for each PCR reaction, and semiquantitative results (see below) were adjusted accordingly. HHV-6 and human β-globin primers, PCR conditions, assay sensitivity, and product detection and quantitation have been described [22, 23].

For KSHV-specific PCR, primer pairs were KS-1 and -2, amplifying the KS330Bam fragment of ORF 26 [1], and primer pairs KS-A and -B (CGTCGGTCGTGTCGTGTGAT and GCATAAGTACCGGAGAAGGGAGGAAAGC; GAACCAGAGCGGCGAGAAAGC) were used to amplify a 118-base pair region of EBER1 [25]. For varicella-zoster virus (VZV)–specific PCR, primers VZV3782 and VZV3960 (TCCGTTCTGGTCTGGTTGGA and CGCCGGGCGTCGCCGTATACCTT) were utilized to amplify a 199-base pair of ORF 62 [26].

Each 100-μL PCR mixture contained 50 mM KCl, 1.5 mM MgCl₂, 2 U AmpliTaq (Perkin-Elmer, Foster City, CA), 200 μM each dNTP, and 0.83 μM each primer. KS-1 and -2 reactions contained 15% glycerol; other primer pairs were used with 10% glycerol. PCR conditions for KS-1 and -2 were 96°C for 3 min, 35 cycles at 98°C (30 s), 53°C (30 s), and 72°C (30 s), and 72°C for 5 min. Reaction conditions for KS-A and -B, EBV, and VZV were identical except for annealing temperatures (50°C for KS-A and -B, 54°C for EBV and VZV).

PCR product detection utilized liquid hybridization with 32P-labeled probes. KSHV-specific PCR products were detected with probes KS-P [1] and KS-P2 (CAACCCCTGGGGCTTTCCGACG), specific for PCR amplimers generated by KS-1 and -2 and KS-A and -B, respectively. EBV PCR product was detected with probe EBER1-P (CCACAGACAGCTCTCCACACCG). VZV PCR product was detected with probe VZV3899 (ACCACCCCGCGCC-CTGGTGTTCG). PCR products (7 μL) and probe (10⁶ cpm) were heated in 25 μL of 1.2 M NaCl, 100 μM each dNTP, and 44% formamide at 97°C for 5 min. Ten microliters of cooled hybridization reactions were analyzed on 6% acrylamide gels, dried, and autoradiographed.

To quantitate KSHV PCR assays, a standard was prepared by cloning KS-1 and -2 PCR product into TA vector (Invitrogen, San Diego) and quantitated by ultraviolet absorption. Specimen DNA and log₁₀ dilutions of standard DNA (10⁷–10⁹ copies) were amplified using the same PCR master mix. The intensity of the specimen band at the predicted molecular weight was visually compared to the standard curve. Specimens were rerun in log₁₀ dilutions (in 10 mM TRIS, pH 8.0) when initial samples contained >10⁹ DNA copies/mL. Results are expressed as KSHV DNA copies per milliliter of sample for plasma and saliva and per 10⁶ cells for PBMC. For tissue, 2 × 10⁶ β-globin DNA copies were assigned the value of 1 × 10⁶ cells. In preliminary experiments, PBMC number measured by hemocytometry and quantitative β-globin PCR were concordant. EBV DNA was quantitated as described for KSHV. DNA from Raji cells containing an average of 50 EBV genomes/cell [27] was used for the standard curve. Sensitivity of the KSHV, EBV, and VZV PCR reactions was routinely <10 copies of viral DNA.

To ensure that negative results were not due to nonspecific inhibition of PCR, we constructed an internal positive control, KSfly2, that would be amplified in PCR using KS-1 and -2 primers.
KS-fly2 DNA is identical to the KS330Bam233 PCR product except that the KS-P probe sequence is replaced by a 21-bp *Drosophila* species DNA sequence [28]. Fifty copies of KS-fly2 DNA were included in each PCR reaction using KS-1 and -2 primers. Each KS-1 and -2 PCR product was hybridized with a 32P-labeled probe specific for the *Drosophila* species DNA sequence [28], electrophoresed, and autoradiographed. DNA from specimens exhibiting inhibition was repurified and reamplified. All negative KS-1 and -2 PCR results in this study required detection of KS-fly2 DNA. To detect failure to isolate DNA, cellular specimens were analyzed by PCR for human β-globin [28]. To monitor false-positives, specimen sets were processed in parallel (from DNA isolation through autoradiography) with aliquots of HSB2 cells [23]. PCR reactions without DNA were also included in each PCR run.

Saliva fractionation and assessment of viral DNA susceptibility to DNase. Saliva (5 mL) was liquefied with 1 mM dithiothreitol for 10 min at room temperature, centrifuged (800 g) for 10 min, and filtered (0.8 µm). The cell-free salivary fluid was ultracentrifuged at 90,000 g for 60 min, and pelleted material was resuspended in 100 µL of one-third-strength PBS. DNA was extracted from the supernatant and pellet, and 5% was used for PCR with KS-1 and -2 primers. To investigate the DNase sensitivity of the viral DNA, 10 µL of the resuspended pellet fraction was spiked with 3 × 10^4 copies of VZV DNA and treated for 1 h at 37°C with 10 U of RQ DNase (Promega, Madison, WI) in 100 µL of buffer containing 10 mM NaCl, 2 mM MgCl₂, 10 mM TRIS-HCl (pH 8.0), and 1 mM dithiothreitol. The DNAase was inactivated with 25 mM EDTA and 2 vol of lysis buffer (6 M guanidinium thiocyanate, 37.5 M sodium citrate, 0.75% N-lauroylsarcosine, 0.15 M β-mercaptoethanol). DNA was extracted and used for KSHV-, EBV-, and VZV-specific PCR reactions as described above. For control reactions, VZV DNA (3 × 10^4 copies) or infectious EBV strain B95.8 [29] (2 µL of cell culture supernatant) was digested with DNase in the absence of saliva.

Detection of anti-KSHV antibodies. HIV-8 positive and EBV-negative body cavity B cell lymphoma cell line BCBL-1 [18] and the EBV-negative B-cell line BJAB [30] were grown in RPMI 1640 medium with glutaMAX (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. Cells (10^5) in 10 mL of medium were induced with 20 ng/mL phorbol ester (TPA; Sigma, St. Louis). Cells were collected, washed in PBS (pH 7.4), spotted on slides (inner diameter, 5 mm; 10 circles/slide), air dried under ultraviolet light, and fixed in acetone (−20°C for 10 min). Fixed slides were incubated for 30 min at 37°C with 2-fold dilutions of patient sera, beginning at 1:10.

After incubation, slides were washed rigorously by being dipped 15 times each in three beakers of PBS. Slides were then incubated for 30 min at 37°C with a prestandardized dilution of fluorescein isothiocyanate-conjugated goat anti-human IgG (Hyclone, Ogden, UT). After being washed, slides were counterstained with a 1:20,000 dilution of Evan’s blue (Sigma) for 5 min at room temperature, washed, mounted with 50% (vol/vol) glycerol in PBS, and examined by use of a fluorescence microscope. The titer was read as the maximal dilution of serum that gave a characteristic diffuse cytoplasmic pattern of fluorescence on induced BCBL-1 cells. Sera with a titer of ≥1:40 against TPA-induced BCBL-1 cells were scored as positive for antibodies to KSHV. All sera were negative for reactivity with the BJAB control B-cell line at a dilution of 1:40.

**Statistical methods.** Comparison of detection of KSHV DNA between specimen sites was made with Fisher’s exact test, two-tailed. Specimen sets from which 1 of the comparison specimens displayed inhibition of PCR or was not available were excluded. Comparison of peripheral lymphocyte numbers between groups was performed on log-transformed cell counts with the Mann-Whitney U test, two-tailed. CD4 cell counts of 0 were assigned a value of 1 to permit log transformation. Comparison of titers of anti-KSHV antibodies between groups was performed on log-transformed titers, using Mann-Whitney U test, two-tailed. Anti-KSHV titers and peripheral lymphocyte numbers were correlated after log transformation of both variables and exclusion of data pairs with negative anti-KSHV tests by linear regression; P values are two-tailed. Throughout, analysis was performed using Instat (Graphpad Software, San Diego).

**Results**

**Detection of KSHV DNA in saliva from patients with KS.** Twenty-five homosexual or bisexual male patients with a history of KS were studied; their laboratory and clinical characteristics are shown in table 1. One patient (no. 1) had completed treatment for KS 1 year prior to study, and KS in another patient (2) had spontaneously resolved 7 years before the study. Of the 25 study subjects, 23 had active KS (patients 3–25) and 24 were infected with HIV (patient 10 was HIV negative).

Overall, KSHV DNA was detected in saliva from 16 (70%) of 23 subjects with active KS (patients 3–18). KSHV DNA was present in saliva in 9 (69%) of 13 persons in whom clinical KS lesions were limited to cutaneous sites (patients 3–11 and 19–22) and in 7 (70%) of 10 persons in whom oropharyngeal, laryngeal, or esophageal KS lesions were present (patients 12–18 and 23–25). KSHV DNA was detected in saliva from both HIV-infected patients with resolved KS and the single HIV-negative patient with KS. There was no association between log-transformed peripheral blood CD4 T cell counts and detection of salivary KSHV DNA (*P* = .12, Mann-Whitney U test, two-tailed).

To validate the detection of KSHV DNA in saliva by use of KS-1 and -2 primer pairs, all positive specimens were also tested with KS-A and -B primer pairs. Confirmatory testing was positive in all cases. The patterns of relative intensities of the specific bands on autoradiographs were similar for both primer pairs (figure 1). Levels of salivary KSHV DNA ranged from 10^2 to >10^7 copies/mL in specimens from the HIV-infected patients, and saliva from the HIV-uninfected patient (patient 10) had >10^7 copies/mL.

To evaluate the consistency and pattern of salivary KSHV DNA shedding, samples were collected daily from 4 HIV-infected subjects with salivary KSHV DNA at study entry (patients 1–3 and 8), including the 2 patients with resolved or treated KS. Specimens were evaluated by PCR for KSHV DNA
Table 1. Clinical and laboratory characteristics of HIV-positive study patients with KS grouped by site(s) of KS and detection of KSHV DNA in saliva.

<table>
<thead>
<tr>
<th>Patient characteristic, no.</th>
<th>No. of years HIV-positive*</th>
<th>No. of years KS-positive*</th>
<th>No. of CD4 cells/µl</th>
<th>Site(s) of KS lesion(s)</th>
<th>Titer of anti-KSHV antibody</th>
<th>Log_{10} KSHV DNA in saliva</th>
<th>Log_{10} KSHV DNA in plasma</th>
<th>Log_{10} KSHV DNA in tissues</th>
<th>PBMC</th>
<th>KSHV DNA in normal skin</th>
<th>KSHV DNA in semen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin KS, KSHV DNA in saliva</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>0.5</td>
<td>137</td>
<td>Skin²</td>
<td>320</td>
<td>4</td>
<td>Neg</td>
<td>Pos²</td>
<td>1.9</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>7</td>
<td>528</td>
<td>Skin²</td>
<td>80</td>
<td>6</td>
<td>Neg</td>
<td>Neg⁰</td>
<td>3.8</td>
<td>ND</td>
<td>Neg</td>
</tr>
<tr>
<td>3</td>
<td>9.6</td>
<td>0.3</td>
<td>33</td>
<td>Skin</td>
<td>40</td>
<td>4</td>
<td>Neg</td>
<td>5.3</td>
<td>2.8</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>2</td>
<td>138</td>
<td>Skin</td>
<td>160</td>
<td>5.4</td>
<td>Neg</td>
<td>5.6</td>
<td>Neg</td>
<td></td>
<td>Neg</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>1.3</td>
<td>423</td>
<td>Skin</td>
<td>160</td>
<td>5.7</td>
<td>Neg</td>
<td>Pos²</td>
<td>4.2</td>
<td>ND</td>
<td>Neg</td>
</tr>
<tr>
<td>6</td>
<td>3.0</td>
<td>2.5</td>
<td>377</td>
<td>Skin</td>
<td>160</td>
<td>4.7</td>
<td>Neg</td>
<td>Pos²</td>
<td>4.2</td>
<td>Inhib</td>
<td>Neg</td>
</tr>
<tr>
<td>7</td>
<td>0.1</td>
<td>0.1</td>
<td>60</td>
<td>Skin</td>
<td>320</td>
<td>4.7</td>
<td>2.4</td>
<td>Neg</td>
<td>5.2</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>8</td>
<td>9</td>
<td>0.3</td>
<td>312</td>
<td>Skin</td>
<td>320</td>
<td>&gt;5.7</td>
<td>2.7</td>
<td>Pos²</td>
<td>5.2</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>9</td>
<td>11</td>
<td>1.7</td>
<td>321</td>
<td>Skin</td>
<td>640</td>
<td>&gt;5.7</td>
<td>Neg</td>
<td>Pos²</td>
<td>5.2</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>10</td>
<td>NA</td>
<td>3.0</td>
<td>667</td>
<td>Skin, larynx</td>
<td>160</td>
<td>&gt;5.7</td>
<td>Neg</td>
<td>ND</td>
<td>3.2</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>1.7</td>
<td>0.5</td>
<td>134</td>
<td>Skin</td>
<td>160</td>
<td>4.4</td>
<td>Neg</td>
<td>Pos²</td>
<td>Neg</td>
<td>ND</td>
<td>Neg</td>
</tr>
<tr>
<td>Oral KS, KSHV DNA in saliva</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>11</td>
<td>0.1</td>
<td>34</td>
<td>Skin, larynx</td>
<td>20</td>
<td>5.7</td>
<td>Neg</td>
<td>Neg⁰</td>
<td>2.2</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>4.8</td>
<td>0.6</td>
<td>401</td>
<td>Skin, mouth</td>
<td>320</td>
<td>5.7</td>
<td>3.0</td>
<td>6.3</td>
<td>5.6</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>0.5</td>
<td>0.3</td>
<td>0</td>
<td>Skin, mouth</td>
<td>&lt;20</td>
<td>4.7</td>
<td>4.4</td>
<td>7.0</td>
<td>5.3</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>5</td>
<td>1.5</td>
<td>86</td>
<td>Skin, mouth</td>
<td>80</td>
<td>4.4</td>
<td>Neg</td>
<td>6.0</td>
<td>3.5</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>16</td>
<td>0.1</td>
<td>0.2</td>
<td>40</td>
<td>Skin, mouth</td>
<td>160</td>
<td>&gt;5.7</td>
<td>Neg</td>
<td>3.3</td>
<td>3.8</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>17</td>
<td>11</td>
<td>1.0</td>
<td>0</td>
<td>Skin, esophagus</td>
<td>40</td>
<td>&gt;5.7</td>
<td>2.4</td>
<td>Pos²</td>
<td>3.2</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>Skin KS, no KSHV DNA in saliva</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>9</td>
<td>0.2</td>
<td>19</td>
<td>Skin</td>
<td>320</td>
<td>Neg</td>
<td>&lt;2.4</td>
<td>5.6</td>
<td>1.5</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>7</td>
<td>4</td>
<td>121</td>
<td>Skin</td>
<td>2560</td>
<td>Neg</td>
<td>Neg</td>
<td>ND</td>
<td>Neg</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>9</td>
<td>6</td>
<td>435</td>
<td>Skin</td>
<td>10,240</td>
<td>Neg</td>
<td>Neg</td>
<td>5.6</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>22</td>
<td>11</td>
<td>9</td>
<td>5</td>
<td>Skin</td>
<td>640</td>
<td>Neg</td>
<td>Neg</td>
<td>Pos²</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>Oral KS, no KSHV DNA in saliva</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>1</td>
<td>1</td>
<td>32</td>
<td>Skin, mouth</td>
<td>160</td>
<td>Neg</td>
<td>3.4</td>
<td>6.6</td>
<td>2.8</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>24</td>
<td>1.7</td>
<td>1.7</td>
<td>84</td>
<td>Skin, mouth</td>
<td>&lt;20</td>
<td>Neg</td>
<td>Neg</td>
<td>Pos²</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>25</td>
<td>1.4</td>
<td>0.5</td>
<td>11</td>
<td>Mouth</td>
<td>160</td>
<td>Neg</td>
<td>Inhib</td>
<td>ND</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
</tbody>
</table>

NOTE. PBMC = peripheral blood mononuclear cells; Neg = negative; Pos = positive; ND = not done; NA = not applicable; Inhib = inhibitory for polymerase chain reaction (PCR).

* Minimum time patient was HIV infected or had clinically recognized KS prior to specimen collection.

¹ No clinical KS at time of saliva collection (see text).

² PCR was positive, but quantitation was not performed.

³ PCR on 3 20-µM sections of paraffin-embedded KS tissue; positive for β-globin DNA by PCR.

(figure 2). These studies were done 20–60 days after the initial specimen collection, and KSHV DNA was found in 3 of the 4 patients. In 2 subjects (patients 2 and 8), KSHV DNA was consistently present at high copy numbers (10³–10⁶ copies/mL of saliva) in all samples. Patient 3, who had 10⁴ copies/mL on study entry, had low positive PCR detection on 2 of 5 days. EBV and HHV-6 DNA levels were also determined in patients 1–3 and 8: All had detectable salivary HHV-6 DNA (20/21 specimens), and 3 of the 4 patients had detectable salivary EBV DNA (16/16 specimens). The KSHV DNA levels for patients 2 and 8 were comparable to levels of HHV-6 DNA and at least 10- to 100-fold lower than levels of EBV DNA.
present in patients 7 and 11 for only 1 month before biopsy. Normal skin samples were obtained at the time of the KS lesion biopsies from 17 patients: 16 were evaluable by PCR; 15 were negative for KSHV DNA; and 1 sample had a low copy number (<100/10^6 cells) of KSHV DNA.

KSHV DNA was detected in PBMC from 18 (72%) of 25 subjects (summarized in table 2). The amount of KSHV DNA ranged from 10^1.5 to >10^5.2 copies/10^6 PBMC. KSHV DNA was detected in 7 (29%) of 24 plasma or serum specimens; levels ranged from <10^2.4 to 10^4.4 copies/mL of plasma. The detection of salivary KSHV DNA among patients with KS was associated with the detection of KSHV DNA in PBMC (P = .007) but not plasma (P = 1.0, Fisher’s exact test, two-tailed).

Semen samples were collected from 12 patients with KS. KSHV DNA was not detected in any of the specimens. β-globin PCR confirmed the isolation of DNA from all semen specimens, and none were inhibitory for amplification of KSHV DNA.

**Figure 1.** Detection of KSHV DNA in saliva of patients 1–6. Top, polymerase chain reaction (PCR) results with KS-1 and -2 primer pairs and detection with KS-P probe. Bottom, PCR results with KS-A and -B primer pairs and detection with KS-P2 probe. P = DNA from KS tissue included as positive control. N = DNA from HSB-2 cells included as negative control. Lane nos. correspond to patient nos. in table 1. Representative standard curve used for quantitation of KSHV DNA included in PCR are indicated.

**Figure 2.** Comparison of salivary KSHV (open bars), Epstein-Barr virus (hatched bars), and human herpesvirus 6 (solid bars) DNA levels in daily specimens. Patient nos. are from table 1. To determine viral DNA levels, salivary DNA was diluted when necessary and reanalyzed. Pt. = patient.

**Detection of KSHV DNA in skin, blood, and semen specimens from patient with KS.** Additional specimens analyzed for KSHV DNA by PCR included lesion and normal skin tissue, PBMC, plasma, and semen (table 1). Tissue samples of KS lesions from 22 of the 25 subjects were evaluated, and KSHV DNA was detected in 19 (86%). KSHV DNA levels ranged from 10^3 to 10^6.6/10^6 lesion cells as assessed by semiquantitative KSHV and β-globin PCR performed on 9 specimens. The 3 negative specimens were paraffin-embedded tissue collected 7 years prior to analysis (patient 2) and 2 biopsies of skin lesions (patients 7 and 11). Of note, lesions had been clinically
### Table 2. Summary of detection of KSHV DNA by polymerase chain reaction in HIV-positive and -negative subjects with and without KS.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>With KS</th>
<th>Without KS</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV positive</td>
<td>HIV negative</td>
<td>HIV positive</td>
</tr>
<tr>
<td>Saliva</td>
<td>17 (71)</td>
<td>1</td>
</tr>
<tr>
<td>PBMC</td>
<td>17 (71)</td>
<td>1</td>
</tr>
<tr>
<td>Plasma</td>
<td>7 (29)</td>
<td>0</td>
</tr>
<tr>
<td>Semen</td>
<td>0 (0)*</td>
<td>ND</td>
</tr>
</tbody>
</table>

NOTE: Data are no. (%) of subjects. PBMC = peripheral blood mononuclear cells, ND = not done.

* Specimens for 12 subjects were tested.
† Specimens for 10 subjects were tested.

### Detection of KSHV DNA in specimens from patients without KS.

We also tested specimens from 20 HIV-infected homosexual or bisexual male patients without clinical KS (table 2). Salivary KSHV DNA was detected in single specimens from 3 (15%) of 20 patients, using KS-1 and -2 primer pairs, and was confirmed in each case with KS-A and -B primer pairs. The levels of KSHV DNA were low in saliva specimens from this group (10^{-2.3}–10^{-1.5} DNA copies/mL). KSHV DNA was also detected in PBMC from 2 of 20 patients. Positive saliva and PBMC specimens were from separate individuals, yielding a prevalence of 25% (5 of 20) for KSHV infection among this population, as detected by PCR. KSHV DNA was not detected in semen from a subgroup of 10 subjects, including 2 patients with KSHV DNA in saliva, 4 additional patients seropositive for KSHV, and 4 patients seronegative for KSHV (see below).

### Figure 3.

DNase resistance of salivary KSHV and Epstein-Barr virus DNA analyzed by polymerase chain reaction, using KS-1 and -2 and EBV primers. Ultracentrifuge-pelleted cell-free saliva fluid from patients 8, 15, and 16 was analyzed for KSHV DNA before and after DNase treatment. Exogenous varicella zoster virus DNA was added before DNase treatment as positive control. Control reactions included EBV from productively infected cells and VZV DNA in TRIS buffer.

All semen were positive for amplification of β-globin DNA by PCR, and none were inhibitory of amplification of control KS-fly2 template DNA. KSHV DNA was not detected in single saliva specimens from 24 healthy adults.

The prevalence of KSHV DNA in saliva and PBMC specimens was compared for HIV-positive patients with KS (n = 24) and HIV-positive, KS-negative patients with serologic evidence (see below) of KSHV infection (n = 11). Among these KSHV-infected and HIV-infected subjects, detection of KSHV DNA in saliva was associated with the presence of clinical KS (P = .006, Fisher’s exact test, two-tailed). Detection of KSHV DNA in PBMC was also associated with the presence of clinical KS (P = .009, Fisher’s exact test, two-tailed).

### Table 3. Detection of serum antibodies to KSHV by IFA and KSHV DNA in saliva of HIV-infected men with or without KS by polymerase chain reaction (PCR).

<table>
<thead>
<tr>
<th>Subject characteristic</th>
<th>With KS (n = 24)</th>
<th>Without KS (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSHV seropositive</td>
<td>21 (88)</td>
<td>11 (55)</td>
</tr>
<tr>
<td>Saliva KSHV DNA positive</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>Saliva KSHV DNA negative</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>KSHV seronegative</td>
<td>3 (12)</td>
<td>9 (45)</td>
</tr>
<tr>
<td>Saliva KSHV DNA positive</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Saliva KSHV DNA negative</td>
<td>1</td>
<td>8</td>
</tr>
</tbody>
</table>

NOTE. GMT = geometric mean titer.

* Patients with KS vs. patients without KS (P = .001, Mann-Whitney U test, 2-tailed).
† Patients with KSHV PCR-negative saliva vs. patients with KSHV PCR-positive saliva (P = .07, Mann-Whitney U test, 2-tailed).
KSHV DNA was detected in saliva of 18 of 25 patients with active or resolved KS and 3 of 20 HIV-infected men without KS. Large amounts of KSHV DNA (up to $10^6$ DNA copies/mL) were detected in saliva specimens from patients with KS. Among 4 patients with KSHV DNA in saliva at their initial study visit, KSHV DNA shedding in saliva during a daily sampling protocol was consistent in 2, intermittent in 1, and not detected in another. Thus, KSHV DNA shedding in saliva may be episodic in some patients. The KSHV DNA in saliva was resistant to DNase treatment, consistent with the presence of KSHV virions. We therefore hypothesize that salivary contact may be involved in some cases of KSHV transmission.

The source or sources of KSHV DNA in saliva are unknown. The shedding of KSHV DNA-positive tumor cells or debris into saliva may have contributed to the KSHV DNA detected in some patients. However, among HIV-infected patients with KS, the prevalence and levels of salivary KSHV DNA were similar between patients with and without oral KS lesions. KSHV DNA was also detected in saliva from patients with resolved KS. Eleven of the 18 patients with KSHV DNA in saliva, including 4 with high levels of viral DNA ($\geq 10^5$ copies/mL), did not have oral KS. Several such patients had prolonged (several years) follow-up periods between the diagnosis of cutaneous KS and specimen collection, and none have developed clinically evident oral KS in 3–9 months of follow-up after specimen collection. Moreover, we detected KSHV DNA in saliva of HIV-infected patients without any evidence of KS at enrollment or during subsequent follow-up. Thus, shedding of tumor cells into saliva appears not to be the only source of KSHV DNA in saliva.

Replication of KSHV in normal oropharyngeal cells is another potential source of the KSHV DNA in saliva. KSHV is known to preferentially localize in B lymphocytes in the PBMC [17] and to be present in lymphoid tissue [32]. B lymphocytes in lingual and palatine tonsillar tissue, which bear crypts in contact with the oropharyngeal lumen, might therefore be a source of salivary KSHV. It is also possible that KSHV undergoes replication in oropharyngeal epithelial cells. The related herpesvirus, EBV, undergoes latent and lytic replication in oropharyngeal epithelial cells and is associated with oral pathologic conditions, including oral hairy leukoplakia and nasopharyngeal carcinoma. Regardless of the role of saliva in KSHV transmission, the detection of KSHV DNA in saliva indicates that oropharyngeal cells are permissive for KSHV and that the oropharynx could be a site of KSHV acquisition from other body sites during sexual contact. Further studies are required to determine the cell types with access to the salivary compartment that are permissive for KSHV. The infectious nature of KSHV in saliva is currently under separate study. Transfer of KSHV DNA from saliva to cell cultures has been achieved (Vieira J, et al., unpublished data).

Previous studies to evaluate the presence of KSHV in saliva have been limited. Ambroziak et al. [17] studied the saliva from 4 patients and did not detect KSHV DNA in any specimens. Among 21 patients with KSHV in their PBMC, only 1 had a positive throat swab. Sputum, expected to contain some saliva, was positive in only 1 of 27 patients with KS [19]. Our study differed from these in several respects. We used a PCR assay with a documented sensitivity of 1–10 DNA copies. Moreover, as saliva can often inhibit PCR reactions to give false-negative results [22], we assessed all specimens for inhibition of PCR. To reduce false-negative results, all initial positive KSHV PCR results were confirmed with a second KS-specific primer pair, and repeat specimen collections were obtained from 4 patients. The daily sampling data indicate that some persons display intermittent shedding of KSHV DNA in saliva; therefore, shedding may be missed if only single specimens are analyzed. After our analysis was underway, results of PCR analysis of KSHV DNA in saliva from HIV-positive patients from Houston became available [33]. KSHV DNA was detected with nested PCR in 25 (32.9%) of 76 specimens. The HIV risk factors and clinical KS status of these patients were not presented.

We did not detect KSHV DNA in the saliva of 24 healthy adult donors. Our results are consistent with negative findings for 39 HIV-negative individuals [33]. If KSHV infection is rare in healthy adults in the United States [5–7], our negative findings may simply reflect this fact. If, however, many apparently healthy adults are infected with KSHV [13–15, 32], then these findings could indicate tight immunologic control of KSHV replication in the saliva-accessible compartment among healthy persons.

Increased viral shedding in the saliva of patients with compromised cellular immunity has been documented for HSV-1, HSV-2, and EBV. The possible roles of KSHV-specific cellular immune responses in control of KSHV infection and malignancies associated with KSHV remain unknown. We did not detect a correlation between CD4 cell counts and salivary shedding of KSHV DNA among HIV-positive patients with KS. The increased titers of anti-KSHV antibodies among HIV-positive patients with KS compared with those among HIV-positive

Discussion

KSHV DNA was detected in saliva of 18 of 25 patients with active or resolved KS and 3 of 20 HIV-infected men without KS. Large amounts of KSHV DNA (up to $10^6$ DNA copies/mL) were detected in saliva specimens from patients with KS. Among 4 patients with KSHV DNA in saliva at their initial study visit, KSHV DNA shedding in saliva during a daily sampling protocol was consistent in 2, intermittent in 1, and not detected in another. Thus, KSHV DNA shedding in saliva may be episodic in some patients. The KSHV DNA in saliva was resistant to DNase treatment, consistent with the presence of KSHV virions. We therefore hypothesize that salivary contact may be involved in some cases of KSHV transmission.

The source or sources of KSHV DNA in saliva are unknown. The shedding of KSHV DNA-positive tumor cells or debris into saliva may have contributed to the KSHV DNA detected in some patients. However, among HIV-infected patients with KS, the prevalence and levels of salivary KSHV DNA were similar between patients with and without oral KS lesions. KSHV DNA was also detected in saliva from patients with resolved KS. Eleven of the 18 patients with KSHV DNA in saliva, including 4 with high levels of viral DNA ($\geq 10^5$ copies/mL), did not have oral KS. Several such patients had prolonged (several years) follow-up periods between the diagnosis of cutaneous KS and specimen collection, and none have developed clinically evident oral KS in 3–9 months of follow-up after specimen collection. Moreover, we detected KSHV DNA in saliva of HIV-infected patients without any evidence of KS at enrollment or during subsequent follow-up. Thus, shedding of tumor cells into saliva appears not to be the only source of KSHV DNA in saliva.

Replication of KSHV in normal oropharyngeal cells is another potential source of the KSHV DNA in saliva. KSHV is known to preferentially localize in B lymphocytes in the PBMC [17] and to be present in lymphoid tissue [32]. B lymphocytes in lingual and palatine tonsillar tissue, which bear crypts in contact with the oropharyngeal lumen, might therefore be a source of salivary KSHV. It is also possible that KSHV undergoes replication in oropharyngeal epithelial cells. The related herpesvirus, EBV, undergoes latent and lytic replication in oropharyngeal epithelial cells and is associated with oral pathologic conditions, including oral hairy leukoplakia and nasopharyngeal carcinoma. Regardless of the role of saliva in KSHV transmission, the detection of KSHV DNA in saliva indicates that oropharyngeal cells are permissive for KSHV and that the oropharynx could be a site of KSHV acquisition from other body sites during sexual contact. Further studies are required to determine the cell types with access to the salivary compartment that are permissive for KSHV. The infectious nature of KSHV in saliva is currently under separate study. Transfer of KSHV DNA from saliva to cell cultures has been achieved (Vieira J, et al., unpublished data).

Previous studies to evaluate the presence of KSHV in saliva have been limited. Ambroziak et al. [17] studied the saliva from 4 patients and did not detect KSHV DNA in any specimens. Among 21 patients with KSHV in their PBMC, only 1 had a positive throat swab. Sputum, expected to contain some saliva, was positive in only 1 of 27 patients with KS [19]. Our study differed from these in several respects. We used a PCR assay with a documented sensitivity of 1–10 DNA copies. Moreover, as saliva can often inhibit PCR reactions to give false-negative results [22], we assessed all specimens for inhibition of PCR. To reduce false-negative results, all initial positive KSHV PCR results were confirmed with a second KS-specific primer pair, and repeat specimen collections were obtained from 4 patients. The daily sampling data indicate that some persons display intermittent shedding of KSHV DNA in saliva; therefore, shedding may be missed if only single specimens are analyzed. After our analysis was underway, results of PCR analysis of KSHV DNA in saliva from HIV-positive patients from Houston became available [33]. KSHV DNA was detected with nested PCR in 25 (32.9%) of 76 specimens. The HIV risk factors and clinical KS status of these patients were not presented.

We did not detect KSHV DNA in the saliva of 24 healthy adult donors. Our results are consistent with negative findings for 39 HIV-negative individuals [33]. If KSHV infection is rare in healthy adults in the United States [5–7], our negative findings may simply reflect this fact. If, however, many apparently healthy adults are infected with KSHV [13–15, 32], then these findings could indicate tight immunologic control of KSHV replication in the saliva-accessible compartment among healthy persons.

Increased viral shedding in the saliva of patients with compromised cellular immunity has been documented for HSV-1, HSV-2, and EBV. The possible roles of KSHV-specific cellular immune responses in control of KSHV infection and malignancies associated with KSHV remain unknown. We did not detect a correlation between CD4 cell counts and salivary shedding of KSHV DNA among HIV-positive patients with KS. The increased titers of anti-KSHV antibodies among HIV-positive patients with KS compared with those among HIV-positive
patients without KS suggests that the development of KS may be associated with KSHV reactivation and, consequently, with an increase in antibody levels. The titers of anti-KSHV antibody among HIV-positive patients with KS who shed KSHV DNA in saliva appeared to be marginally lower than titers among nonshedders, although this difference did not reach statistical significance (P = .07). The intermittent nature of KSHV DNA shedding in saliva in some patients (figure 2) and the small sample sizes prevent firm conclusions concerning the relationship between antibody levels and salivary shedding of KSHV among patients with KS.

Epidemic KS among HIV-infected patients in the United States is believed to be caused by a sexually transmitted agent and has been linked in some studies to oral-anal contact [8, 9]. There are several examples of herpes viruses that are transmitted both by salivary and sexual contact (e.g., cytomegalovirus, HSV-1, and HSV-2). Many other infections classified primarily as sexually transmitted diseases, such as Treponema pallidum, Chlamydia trachomatis, and human papilloma virus, can be transmitted or acquired orally. Of importance, the relationship between DNA copy number as assessed by PCR, and the titer of KSHV infectious units in various biologic specimens, as well as the inoculum of KSHV required for infection (by any route), are not known at this time. Therefore, the infectious load of KSHV in saliva cannot be compared with the inoculum of KSHV required for infection at oropharyngeal or other anatomic sites. The biologic plausibility of KSHV transmission by salivary contact can be assessed only if these variables can be accurately estimated through additional research.

The medical implications of salivary shedding of KSHV DNA are thus difficult to evaluate. Both KSHV infection and reduced immune function appear to be necessary for the development of KS. The currently available serologic data [6, 7, 12, 13] indicate that many adults, at least in the United States, are not infected with KSHV and may thus be potentially susceptible to primary infection. Accurate counselling by health care providers concerning kissing and other activities involving oropharyngeal contact is not possible at this time. Improved viral culture, molecular detection, and serologic assays, increased understanding of the pathogenesis of KSHV infection and KS, and focused epidemiologic studies will be required to fully define the routes of KSHV transmission mechanisms.

Acknowledgments

We thank the patients who participated in this study and the health care providers who referred them; Catherine Diamond, Matthew L. Johnson, Heather Parker, Eiko Wakabayashi, Jennifer Moses, Jeanine Frank, and Aimee N. Ekstrom, who assisted with specimen processing and PCR analysis; and Jill Wirtala, who assisted with tissue sectioning.

References