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Distribution of Kaposi Sarcoma–Associated Herpesvirus/Human Herpesvirus 8 in Maternal Saliva and Breast Milk in Zambia: Implications for Transmission

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Background. The seroprevalence of Kaposi sarcoma–associated herpesvirus (KSHV)/human herpesvirus 8 (HHV-8) in sub-Saharan Africa suggests that multiple routes of transmission exist. In the present study, we examined 2 possible routes of mother-to-child transmission, through breast milk and saliva, during the first 6 months after delivery.

Methods. The prevalence of HHV-8 DNA in the breast-milk cells ($n = 75$), milk supernatant ($n = 56$), colostrum ($n = 2$), and saliva cells ($n = 65$) of HHV-8–seropositive mothers who recently gave birth was examined. Polymerase chain reaction analysis was performed for the detection of HHV-8 in cross-sectional samples isolated at 2, 4, and 6 months after delivery.

Results. None of the 75 breast-milk samples but 2 of the colostrum samples that were analyzed contained HHV-8 DNA at a limit of detection of ~ 1 HHV-8 copy/ 10^4 cellular genomes, whereas Epstein-Barr virus DNA and HIV-1 DNA were detected in 16 and 22 samples, respectively. Analysis of 65 saliva cell samples, which were obtained from mothers who also provided milk samples, revealed that 19 of the samples had detectable HHV-8 DNA. Viral DNA was found at all time points, but the presence of viral DNA in saliva was independent of maternal HIV-1 serostatus ($\chi^2 = 0.33$; $P = .57$).

Conclusions. Our findings demonstrate the lack of HHV-8 DNA in the breast milk of seropositive mothers, and they suggest that contact with breast milk is not a likely source of horizontal transmission of virus to infants in sub-Saharan Africa.

Human herpesvirus 8 (HHV-8) was first identified, in 1994, in tissue from a patient with AIDS who had Kaposi sarcoma (KS) [1]. In the United States and Western Europe, HHV-8 infection is uncommon in the general population, but it is considered to be common in

regions of South America, southern Italy, and Africa [2–4]. After the onset of the AIDS pandemic, several sub-Saharan countries, including Zambia, reported a significant increase in the number of cases of KS among both adults and children [5, 6]. In the United States and Western Europe, similar increases in KS were observed, but these increases were observed almost exclusively among HIV-1–infected men. This suggests that different routes of transmission of HHV-8 or different rates of infection may be occurring in these populations, or that different genetic, cultural, or environmental factors influence transmission of HHV-8.

The modes of transmission of HHV-8 remain poorly understood, despite the existence of significant data on virus seroprevalence. In areas in which seroprevalence is low, HHV-8 appears to be predominantly acquired through sexual contact. Infection and seroconversion

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have been associated with male homosexual practices and the number of HIV-1-positive sex partners, and they also have been associated with individuals at risk for STD [7–9]. In the United States, saliva has been implicated as a source for transmission of HHV-8, because viral DNA has been detected in as many as 70% of oral cavity samples in studies involving HIV-1-infected men with KS [10]. Very little is known regarding the presence of HHV-8 in saliva and the mechanisms of transmission of HHV-8 in regions of endemicity. A cumulative increase in the seroprevalence of HHV-8 in children, from birth through adolescence, has been observed in regions of endemicity; this finding suggests that multiple routes, including perinatal transmission, are involved [4, 11]. Perinatal transmission of HHV-8 may occur as a result of breast-feeding, as has been previously suggested for cytomegalovirus (CMV) [12], but it fails to account for the continued acquisition of HHV-8 after breast-feeding has ceased. CMV, as well as other herpesviruses, has been detected in milk and predominantly localizes to the breast-milk supernatant (liquid) fraction [13]. Whether HHV-8 can be detected in breast milk from infected mothers is not known.

We previously documented HHV-8 infection that occurred in Zambian children by 12 months of age, using serological and HHV-8 DNA detection methods [14, 15]. Although children born to HHV-8-infected mothers can be congenitally infected, the frequency of such transmission is apparently low and does not adequately account for the increasing seroprevalence subsequent to gestation and delivery [4, 11, 15]. Therefore, horizontal transmission is a likely route for a large proportion of HHV-8 infections that occur in early childhood.

As part of our continuing cohort study of the epidemiology of HHV-8 infection among Zambian mother-infant pairs, the major goal of the present study was to examine whether transmission of HHV-8 to infants can occur via breast milk. In the present study, we determined the prevalence of virus in breast milk and maternal saliva. Establishing the presence or absence of HHV-8 in these maternal body fluids will extend our understanding of how HHV-8 is spread to children and will thereby enable the development of strategies to prevent transmission of HHV-8 and pediatric KS.

SUBJECTS, MATERIALS, AND METHODS

Subjects and specimen collection. Analyses were performed for specimens obtained from participants in a longitudinal cohort study of transmission of HHV-8 performed at the University of Zambia Teaching Hospital (UTH; Lusaka, Zambia). Approval for the study was obtained from the Ministry of Health of Zambia, the Research and Ethics Committee of the UTH, and the institutional review boards of the University of Nebraska–Lincoln and the University of Miami (Miami, FL). Written, informed consent was obtained from all study partic-

ipants. In the present study, mothers who showed no clinical signs of KS were recruited, at delivery, in the labor ward of the UTH, and they were followed through the first 6 months after delivery. Whole breast-milk and saliva samples were obtained and were spun for 10 min at 500 g, to separate the liquid and cell portions; each sample was stored at -80°C .

Extraction of DNA from breast milk and saliva. Extraction of thawed cell pellets was performed using the Gentra Systems DNA isolation kit with proteinase K (0.1 mg/mL), according to the manufacturer's instructions [14]. For extraction of viral DNA from the liquid portion of breast milk, samples were spun for 5 min at 10,000 g, and 150 μL of supernatant was decanted and used for viral DNA extraction, which was performed as described above. All DNA extractions were segregated from polymerase chain reaction (PCR) amplification and plasmid manipulation and were performed in a dedicated laboratory, with the use of an isolated hood with UV light; stringent conditions and controls were used to minimize contamination between samples.

Conventional HHV-8, Epstein-Barr virus (EBV), and HIV-1 PCR. DNA extracted from the samples was subjected to PCR specific for human β -globin, by use of primers described elsewhere [16]. The β -globin PCR-positive samples were subsequently analyzed for HHV-8 by use of primers for the minor capsid (ORF26) and gB (N-terminus [gBN]) genes, as described elsewhere [1, 15, 17]. Samples were defined as being positive for HHV-8, either by first-round PCR for both ORF26 and gB, with confirmation by Southern blot analysis, or by nested PCR that consistently detected positive samples by both ORF26 and gBN PCR performed in 2 separate amplification reactions. PCR analysis for EBV was performed using primers specific for the EBV thymidine kinase (TK) gene: TK1 (5'-GTGGGATCCA-TGGCTGGATT-3') and TK2 (5'-GCTACCCGGAGAGTTTCC-AGT-3'). Thermal-cycling parameters for EBV-TK PCR were as follows: 1 cycle for 5 min at 95°C ; 38 cycles for 30 s at 95°C , for 30 s at 56°C , and for 30 s at 72°C ; and 1 cycle for 3 min at 72°C . All reactions were subjected to Southern blot analysis by use of a digoxigenin-labeled probe, as described elsewhere [15]. HIV-1 PCR was used to detect a short sequence of the 5' viral long-terminal repeat in all breast-milk samples obtained from HIV-1-infected mothers, as described elsewhere [18].

Real-time PCR for the detection of HHV-8. Real-time PCR for the detection of HHV-8 and human β -globin was used for all samples demonstrated to be positive by conventional PCR, to quantitate the number of copies of HHV-8 and the number of cellular equivalents present in each sample [16, 19]. For the present study, real-time PCR was performed according to the manufacturer's suggested conditions, by use of TaqMan chemistry (Applied Biosystems). The HHV-8 reaction, which used an amplicon within the ORF73 gene, contained 300 nmol/L forward primer (lat-273F), 200 nmol/L reverse primer (lat-

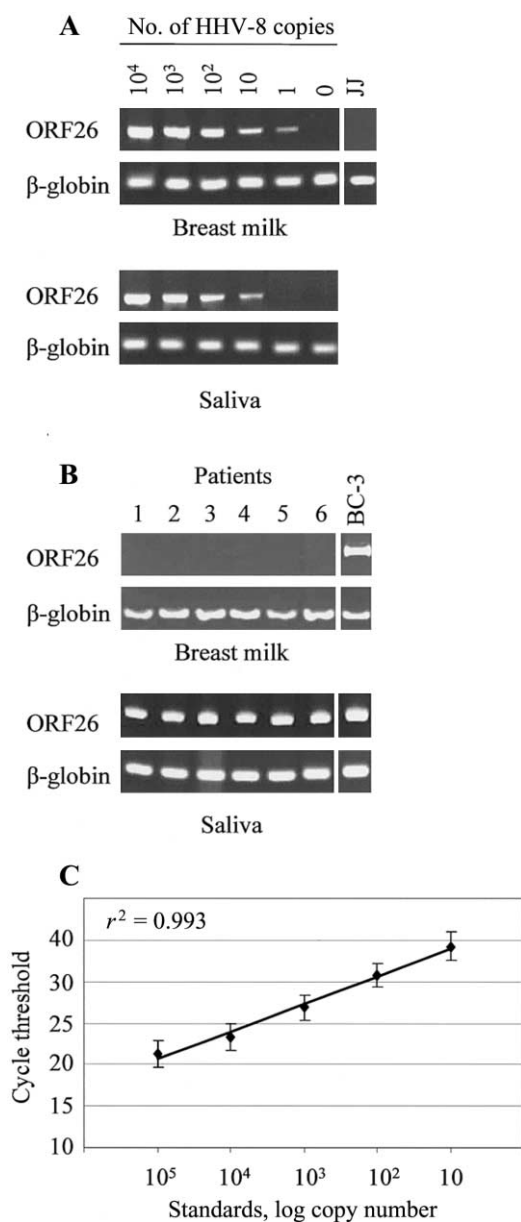


Figure 1. Detection of human herpesvirus 8 (HHV-8) in breast milk and saliva, by use of conventional and TaqMan-based polymerase chain reaction (PCR). *A*, First-round ORF26 PCR and human β -globin-specific PCR were performed on BCBL-1 DNA (10 – 10^5 HHV-8 copies, as determined by real-time PCR) and on spiked donor breast-milk (*top*) and saliva (*bottom*) DNA samples, to determine the sensitivity of detection of HHV-8 in each body fluid. Donor DNA alone (0) and DNA from the Epstein-Barr virus-positive, HHV-8-negative Burkitt lymphoma cell line (Jijoye [JJ]), were run in parallel as controls. *B*, Representative experiments of first-round ORF26 PCR and human β -globin-specific PCR performed on DNA in breast-milk (*top*) and saliva cell (*bottom*) samples obtained from 6 patients (1–6), with BC-3 DNA used as a positive control. *C*, Standard curve for real-time PCR for HHV-8. The standard curve used for determination of the HHV-8 copy number, generated from 5 independent experiments and 15 replicates, is shown. The standards, as log input of HHV-8 copies, are plotted versus the mean cycle threshold, with error bars representing 2 times the SEM. An r^2 of 0.993 indicates a strong linear relationship between input copies and the cycle threshold.

335R), and 200 nmol/L dual-labeled TaqMan probe (lat-294T). The standard curve for HHV-8 was generated using a cloned sequence of the ORF73 gene in the pGEM-T vector as template (pGEMT.lat294T), according to the manufacturer's instructions (Promega). Purified pGEMT.lat294T was linearized by restriction enzyme digestion, and the copy number was calculated from the determination of molecular mass. Serial 10-fold dilutions (10 – 10^5 -fold dilutions) of the plasmid were made in a background of 10^4 human genomes/ μ L (derived from unstimulated peripheral blood mononuclear cells [PBMCs]). A similar standard curve was generated for human β -globin PCR by diluting known copies of unstimulated PBMC DNA in Tris-EDTA buffer. PBMC DNA copy numbers were generated by quantifying the amount of PBMC DNA by use of a spectrophotometer and by multiplying this amount by a conversion factor between the number of genomic copies and the molecular weight (1.2×10^5 genomic copies/ μ g DNA). By use of BioRad iCycler software (version 3.0b; BioRad Laboratories), the cycle threshold (CT) from the PCR amplification of the HHV-8 and β -globin standards was used to generate a curve for each amplicon ($r^2 > 0.98$), allowing for accurate extrapolation of HHV-8 and β -globin copy numbers present in the known samples. For each reaction, the standard curve for HHV-8 and a similar standard curve for the human β -globin gene were run in parallel with saliva samples. Samples were run in triplicate, and the average CT value was calculated and used for determination of the copy number.

Sequence and phylogenetic analysis of HHV-8 K1. PCR amplification of the K1 gene was performed on HHV-8-positive saliva samples, as well as the HHV-8 persistently infected BC-3 and BCBL-1 cell lines, by use of several primer sets. For first-round PCR amplification of K1, primers 2089 and 2088 were used as described elsewhere [20]. Nested PCR was performed on HHV-8-negative samples by use of primers K1A (5'-GGT-TTGCTTTTCGAGGACTATTA-3'; nt 134–155) and K1D (5'-CAGAGCTACGAGTGTGCATAAATA-3'; nt 796–818), both of which were based on the BC-1 genomic sequence. HHV-8-negative samples were further subjected to alternative PCR that used primers K1A and K1B (5'-GCTGACCACAAGTGACTGTGTT-3'; nt 417–438). K1A/D or K1A/B PCR fragments were amplified, and products were gel purified and cloned into pGEM-T vector, according to the manufacturer's recommendations (Promega). Stringent conditions were used to limit PCR contamination, as outlined in the "Conventional HHV-8, Epstein-Barr virus (EBV), and HIV-1 PCR" subsection above, and equivalently amplified BC-3 or BCBL-1 sequences were cloned and sequenced in parallel to serve as controls. Two colonies were sequenced using the ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit and the ABI 373 DNA Sequencer (Applied Biosystems), according to the manufacturer's specified procedures. Sequences were aligned manually by use of

Table 1. Detection of human herpesvirus 8 (HHV-8), Epstein-Barr virus (EBV), and HIV-1, by polymerase chain reaction (PCR) analysis of cross-sectional breast-milk cell samples obtained from HHV-8-seropositive (HHV-8⁺) mothers during the first 6 months after delivery.

HHV-8 ⁺ group (HIV-1 serostatus)	HHV-8				EBV				HIV-1			
	Total	Months after delivery			Total	Months after delivery			Total	Months after delivery		
		2	4	6		2	4	6		2	4	6
1 (HIV-1 ⁺)	0/33	0/24	0/9	nt	15/33 ^a	8/24	7/9	nt	22/33	18/24	4/9	nt
2 (HIV-1 ⁻)	0/42	0/24	0/12	0/6	1/42	0/24	0/12	1/6	nt	nt	nt	nt
Total	0/75 ^{b,c}	0/48	0/21	0/6	16/75	8/48	7/21	1/6	22/33	18/24	4/9	nt

NOTE. Data are no. of positive samples/no. of samples tested. HIV-1⁺, HIV-1 positive; HIV-1⁻, HIV-1 negative; nt, none tested.

^a $\chi^2 = 20.4$; $P < .001$.

^b Does not include the 2 ORF26 PCR-negative colostrum samples obtained from HHV-8⁺ women.

^c Five specimens were found to be positive once, by use of nested ORF26 PCR, but positive status could not be confirmed by PCR performed with a second set of primers.

CLUSTAL W [21], and neighbor-joining trees were constructed using general time reversible distance with phylogenetic analysis using parsimony (PAUP, version 4.0 b10; Sinauer Associates).

Statistical analysis. χ^2 Tests for independence were performed for analysis of PCR detection between populations. The Wilcoxon rank sum test was used for comparisons of the median number of HHV-8 copies.

RESULTS

Detection of HHV-8 copy standards in breast milk and saliva.

To examine the horizontal transmission of HHV-8 from mother to infant via breast milk and/or saliva in Zambia, we first determined the sensitivity of PCR performed on both body fluids. BCBL-1 DNA with known HHV-8 copy numbers, as determined by real-time PCR, was added to equivalent amounts of donor DNA, and the limit of detection in breast milk and saliva was determined by PCR amplification of the ORF26 gene. DNA extracted from donor milk and saliva samples, at a concentration of 100 ng/ μ L ($\sim 10^4$ cellular genomes/ μ L), was then used to serially dilute BCBL-1 DNA in 10-fold increments, creating HHV-8 copy standards from 1 to 10^5 viral copies. By use of the viral copy standards, HHV-8 DNA was consistently detected using conventional PCR with first-round ORF26 primers at 1 viral copy in a background of 10^4 milk cell equivalents and at 10 viral copies in 10^4 saliva cell equivalents (figure 1A). In donor saliva DNA, a faint band was observed with the 1-copy standard, but detection was inconsistent. In addition, HHV-8 PCR could detect the presence of 10 BC-3 cells in whole milk, by use of the same extraction and PCR conditions (data not shown). No PCR product was amplified, under the same conditions, by use of DNA extracted from a related herpesvirus (i.e., EBV)-infected cell line, Jijoye (figure 1A).

Prevalence of HHV-8 in breast milk. Although HHV-8 has been detected in a variety of tissues and body fluids from infected persons, the presence of HHV-8 in breast milk has

remained unexamined. The cross-sectional prevalence of HHV-8 in breast milk from seropositive mothers in Zambia was determined. HHV-8-infected patients were divided into 2 groups: HIV-1-seropositive patients (group 1) and HIV-1-seronegative patients (group 2), as defined elsewhere [14]. DNA extracted from fresh colostrum and frozen breast-milk cell pellets obtained close to the time of birth (for colostrum) or at 2, 4, or 6 months after delivery (for breast milk) was first analyzed for DNA integrity and for the presence of inhibitors, by use of human β -globin primers. Only β -globin-positive samples were used for subsequent detection of HHV-8. PCR analysis of 2 colostrum samples, as well as 33 and 42 breast-milk samples from HIV-1-infected mothers and uninfected mothers, respectively, failed to consistently detect HHV-8 DNA in any sample (table 1). Five milk specimens were sporadically determined to be positive for HHV-8 DNA when they were assayed with nested ORF26 primers, but detection was inconsistent and could not be confirmed using primers from either the gB gene or the K1 gene. Thus, given the sensitivity of the assay (1 copy/ 10^4 cells), these specimens were considered to be negative for

Table 2. Detection of human herpesvirus 8 (HHV-8) and Epstein-Barr virus (EBV) by polymerase chain reaction analysis of cross-sectional breast-milk supernatant samples obtained from HHV-8-seropositive (HHV-8⁺) mothers during the first 6 months after delivery.

HHV-8 ⁺ group (HIV-1 serostatus)	HHV-8				EBV			
	Total	Months after delivery			Total	Months after delivery		
		2	4	6		2	4	6
1 (HIV-1 ⁺)	0/22	0/14	0/7	0/1	7/22 ^a	4/14	3/7	0/1
2 (HIV-1 ⁻)	0/34	0/18	0/10	0/6	0/34	0/18	0/10	0/6
Total	0/56	0/32	0/17	0/7	7/56	4/32	3/17	0/7

NOTE. Data are the no. of positive samples/no. of samples tested. HIV-1⁺, HIV-1 positive; HIV-1⁻, HIV-1 negative.

^a $\chi^2 = 12.4$; $P < .001$.

Table 3. Detection of human herpesvirus 8 (HHV-8) by polymerase chain reaction analysis of cross-sectional saliva cell samples obtained from HHV-8-seropositive (HHV-8⁺) mothers during the first 6 months after delivery.

HHV-8 ⁺ group (HIV-1 serostatus)	Total	Months after delivery		
		2	4	6
1 (HIV-1 ⁺)	6/24 ^a	4/15	2/8	0/1
2 (HIV-1 ⁻)	13/41	6/21	3/12	4/8
Total	19 ^b /65	10/36	5/20	4/9

NOTE. Data are no. of positive samples/no. of samples tested. HIV-1⁺, HIV-1 positive; HIV-1⁻, HIV-1 negative.

^a $\chi^2 = 0.13$; $P = .71$.

^b Twenty-nine percent of samples.

HHV-8. Results of a representative experiment performed with 6 breast-milk samples obtained from HHV-8-seropositive mothers are shown in figure 1B. The same breast-milk samples were also subjected to PCR specific for EBV, which is known to be present in breast milk [13], to ensure that herpesvirus DNA could be amplified in the present analysis. EBV was detected in 16 of 75 breast-milk cell samples, with the presence of EBV dependent on the HIV-1-positive serostatus of the mother, as shown in table 1. Furthermore, PCR amplification of a small fragment of the HIV-1 LTR revealed that 22 of 33 breast-milk samples obtained from patients in group 1 contained HIV-1 DNA (table 1). To determine whether HHV-8 was present in the liquid portion of breast milk, DNA was extracted from the supernatant fractions prepared from whole milk. Fifty-six cognate supernatant samples were available from the breast-milk cell samples analyzed and reported in table 1. None of these samples was found to be positive for HHV-8 by ORF26 PCR, by use of both first-round and nested ORF26 PCR reactions, a finding that is similar to what was observed for the cellular fraction (table 2). On the other hand, EBV-specific PCR revealed 7 positive samples.

Prevalence of HHV-8 in saliva. Because HHV-8 was un-

detectable in breast-milk cells from HHV-8-seropositive mothers, and because breast milk is unlikely to be a source of transmission of HHV-8 to infants, we next examined the prevalence of HHV-8 in cross-sectional saliva cell samples obtained from the same population of seropositive mothers. For DNA from maternal saliva cells obtained at 2, 4, or 6 months after delivery, PCR analysis was performed using the same criteria for positive samples that were mentioned above for breast-milk analysis. Table 3 shows the results of HHV-8 PCR analysis of saliva cell samples obtained from 65 HHV-8-seropositive mothers. The saliva cell samples of 19 mothers were positive for HHV-8 DNA, with positive results noted for 10, 5, and 4 samples obtained at 2, 4, and 6 months, respectively. Furthermore, the probability of a mother having a saliva cell sample that was positive at a single time point was independent of the HIV-1 serostatus of the mother (patients in group 1 vs. patients in group 2; $\chi^2 = 0.33$; $P = .57$). A representative experiment of ORF26 PCR performed on saliva samples obtained from the same 6 mothers who had breast-milk samples analyzed for HHV-8 in figure 1A is shown in figure 1B. To determine whether mothers whose breast-milk samples were negative for HHV-8 had detectable viral DNA in saliva cells, mothers with breast-milk and saliva cell samples obtained at the same follow-up visit had their samples analyzed. A total of 60 mothers with paired specimens were compared with respect to HHV-8 PCR status. At each follow-up time point, HHV-8 was detected in saliva samples, but not in breast-milk samples, obtained from the same patient (table 4).

The HHV-8 infection status of the infants of a subset of the mothers whose saliva samples were analyzed for the presence of HHV-8 ($n = 55$) was then determined at 12 months after delivery. To eliminate the detection of maternal antibodies, the antibody titers of the infants were determined, at different time points after delivery, by use of lytic IFA analysis, as performed elsewhere [14]. Twelve infants had antibody titers determined at birth and at 6 months after delivery, whereas 43 infants had antibody titers determined at birth and at 12 months after

Table 4. Results of human herpesvirus 8 (HHV-8) polymerase chain reaction analysis performed on paired breast-milk and saliva cell samples obtained from HHV-8-seropositive (HHV-8⁺) mothers at different time points after delivery.

HHV-8 ⁺ group (HIV-1 serostatus) ^a	Months after delivery					
	2		4		6	
	Breast milk	Saliva	Breast milk	Saliva	Breast milk	Saliva
1 (HIV-1 ⁺)	0/15	4/15	0/7	2/7	nt	nt
2 (HIV-1 ⁻)	0/20	6/20	0/12	3/12	0/6	3/6
Total	0/35	10/35	0/19	5/19	0/6	3/6

NOTE. Data are no. of positive samples/no. of samples tested. HIV-1⁺, HIV-1 positive; HIV-1⁻, HIV-1 negative; nt, none tested.

^a $N = 60$.

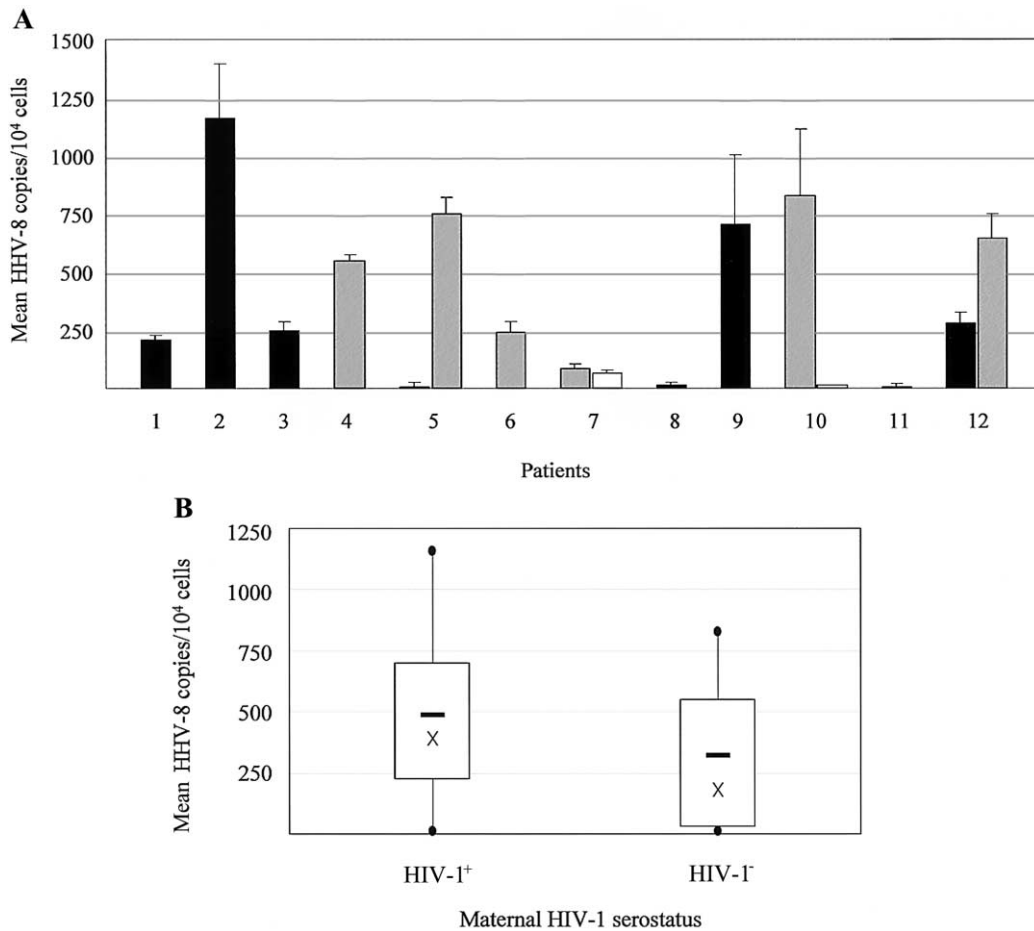


Figure 2. Real-time polymerase chain reaction (PCR) analysis of the 16 saliva cell samples obtained from 12 patients who were found to be positive for human herpesvirus 8 (HHV-8) by conventional PCR. *A*, The nos. of human β -globin and HHV-8 copies were calculated per sample, and the mean nos. of HHV-8 copies/ 10^4 cells analyzed are shown for triplicate reactions, with error bars representing the SD. Patients 5, 7, 10, and 12 had real-time PCR performed at 2 time points. The time points when the saliva specimens were obtained are indicated. *B*, Box-and-whisker plots of the number of HHV-8 copies in saliva cell samples obtained from HIV-1–infected mothers and uninfected mothers. Minimum and maximum values (●), as well as the mean (—) and median (×) values, are shown. Black bars denote samples obtained at 2 months; gray bars, samples obtained at 4 months; and white bars, samples obtained at 6 months. The box denotes the range of values between the first and third quartiles. $P = .26$, 1-sided Wilcoxon rank sum test of medians.

delivery; only infants who had increasing antibody titers from birth to either 6 or 12 months of age were considered to be infected. In the subset of 55 mother-infant pairs, 14 of the mothers were found to be positive for HHV-8 DNA in saliva, by PCR, whereas 41 were found to be negative for HHV-8 by PCR. IFA analysis revealed that 14 of 55 infants were seropositive for HHV-8 by 12 months after delivery. Of these 14 seropositive infants, 3 were born to mothers who had detectable HHV-8 DNA in their saliva cells, and 11 were born to mothers who had no HHV-8 DNA in their saliva at the time that saliva cells were obtained. The probability of an infant being seropositive for HHV-8 was independent of the HHV-8 PCR status of their mother's saliva, as tested at 1 time point ($\chi^2 = 0.16$; $P = .68$).

Quantification of HHV-8 in saliva. Knowing that several mothers in our study group had detectable HHV-8 in saliva,

we next sought to determine the number of copies of HHV-8 in saliva cells, by use of established real-time PCR procedures. By use of plasmid copy-number standards from 10 to 10^5 copies of the ORF73 gene/reaction, a high correlation was observed between input plasmid copies and the CT value ($r^2 = 0.993$); this finding indicates that detection of HHV-8 was linear over 4 orders of magnitude (figure 1C). By use of real-time PCR, BCBL-1 and BC-3 cells were found to contain ~ 26 and 13 HHV-8 copies/cell, respectively (data not shown). The relative cellular and viral copy numbers/sample were determined, and samples that contained >5000 cellular equivalents and ≥ 10 copies of HHV-8 were reported as the number of HHV-8 copies/ 10^4 cellular equivalents. Insufficient quantity of several of our saliva samples prevented analysis of all mothers found to be positive for HHV-8 by conventional PCR. Sixteen samples

BCBL1	CNDTRLWRLTKPTLTIDITCNFTCQEVSQSGHRQSIWITWHA	A
BC3	-----L---Q---VSNLI---S--G-F---H-L----YP	C
1609	--G---L-I-ASN--VSSLA-----MTT--PTH----E-YT	B
1166	--G---H-I-ASN--VSSF-----MTT--PTH----E-YT	B
081	--G---H-I-ASN--VSSL-----MTT--PTH----Q-YT	B
1241	--G---H-I-ASN--VSSL-----MTT--PTH----Q-YT	B
111M	--G---H-I-ASN--VSSL-----MTT--PTH----Q-YT	B
G71_Gambia	--G---I-ASN--VSSL-----MTT--PTH----E-YT	B
G91_Gambia	--G-Q-L-I-ASN--VSAM-----MTT--PTH----E-YT	B
Ug52_Uganda	--G---H-I-ASN--VSSL-----MTT--PTH----E-YT	B
UgD1_Uganda	--G-Q-R-IRGSN--VLL-----MTA--PTH----E-YT	B
Mou_Congo	--G---I-Y-I-ASN--VSSL-----MTT--PTH----E-YT	B
Eti_FG	--G---H---ASNI--VSSL--D---MTT--PTH----E-YT	B
Sir_CAR	--G---L-I-ASNI--VSSL-----MTT--PTH----E-FT	B
Ali_Senegal	--G---L-I-ASN--VSSL-----MTT--PTH----E-YT	B
Kok_CAR	--G---I-ASN--VSS-----S---MTP--PTH----E-YS	B
Ug81_Uganda	--G---H-I-TSN--VLL-----MTT--PTH----E-YT	B
431_Zaire	--G-Q-H-I-ASN--VSSL-----MTT--PTH----Q-YT	B
Lov_FG	-----DQSF-VAT-----N-	A5
Na46_Cameroon	-----DQSF-VAS-----N-	A5
Kos_CAR	-----RDQSF-V-A-----N-	A5
Ngo_CAR	-----RDQSF-V-A-----N-	A5
Gbo_CAR	-----RDQSF-V-A-----N-	A5
Sar_CAR	-----DQSF-VATL-----N-	A5
Lad_FG	-----DKSF-VAT-----N-	A5
Ema7_Spain	--N---L---ERRVILVT-A-----	A
Ukb12_UK	--N---F---TIFSTVT-A-----	A
IaP1_Italy	--N---L---TK---YTFTM-----S-T-	A
S1_Sweden	-----KR---T-T-----	A
Ug111_Uganda	--N---F---EK--PGVN-A-----E--P-N-----	A
BC1_USA	--N---L---ERRVIL-T-A-----R-	A
Ife10_Italy	--N---F-M-ET--FAVR-A-----	A
Seb_FG	--N---L---TT-T-SRVT-A-----	A
K127_USA	-----F---ER--FPVT-P-----V-----	A
ts55_USA	--N---F---ER-VFPVT-A-----	A
Min_Mauritania	--N---L---TT--YRVT-A-----	A
Bud_Turkey	--N---F---ER--FSVT-A-----	A
BBG1_France	-----L---QQ-F-VVTLI---S-----H-L----YP	C
Ukb22_UK	-----F---QQ---VVDLI---S--G-----H-L----YP	C
Ukma1_UK	-----F---QQ-W-VHTLI---S--G-----H-L----YP	C
GK18_Greece	-----L-V-QG---V-TLI---S--G-----Y-L----Y-	C
Bri_France	-----L---QG-F-VHNLI---S--G-Y---Y-L---Y-	C
Asm72_USA	-----F---HD-F-VVNFV---S--G-----H-L-M-YG-	C
E65_Algeria	-----L---QQ-F-VVTLI---S--G-----H-L----YP	C
E37_France	-----L---QQ-F-VVTLI---S--G-----H-L----YP	C
Dia_Senegal	-----L-P-QQ-V-VVTLI---S--G-----H-L----YP	C
Gk17_Greece	-----F---QD-F-VVNFV---S--G-----H-L-MA-Y-	C
Au1_Australia	--G---F-I-G---LFTL-G--S--DE--ISH-----E-YP	D
Zks3_NZ	--G-L-M-YHRT---LMNLAA-W---N---ISH---Q-YT	D
Tks10_Taiwan	--G---L-I-GA---PSL-G---DH--LSH---QRYP	D

Figure 3. K1 amino acid alignment and phylogenetic analysis of saliva samples. *A*, Amino acid alignment of the first variable region of K1 (variable region 1 [VR1]; aa 52–92) from BCBL-1, BC-3, and saliva samples from patients 1609 and 1166; 3 peripheral blood mononuclear cell samples obtained from patients with KS in Zambia and analyzed in our laboratory (sequences 08M, 1111, 1241; unpublished data); and 46 other HHV-8 strains representing phylogenetic clades A–D (obtained from GenBank). *B*, Phylogenetic tree of the VR1 nucleotide sequence (123 nucleotides) generated by neighbor joining with phylogenetic analysis using parsimony (PAUP, version 4.0 b10; Sinauer Associates), for all samples in panel *A*, with the BC-1 sequence used as an outgroup. Subtypes and variants are indicated to the right of each sequence. Arrows denote the 2 saliva samples from the present study. CAR, Central African Republic; FG, French Guiana; NZ, New Zealand; UK, United Kingdom.

obtained from the 12 mothers who were found to be positive for HHV-8 by conventional PCR (8 mothers with samples from 1 time point tested and 4 mothers with samples from 2 time points tested) met the aforementioned criteria for quantitative analysis. These samples are shown in figure 2*A*. A large variation in the mean number of HHV-8 copies/10⁴ cells was observed (range, 4–1185 copies/10⁴ cells). Of the mothers who had samples from 2 time points tested, 3 had samples that showed variations between time points. Two mothers had samples with

high copy numbers at one time point but very low copy numbers at the other time point, whereas 1 mother had only a small difference between time points.

To test whether HIV-1 infection increases the HHV-8 burden in saliva cells, the number of HHV-8 copies/10⁴ cells was examined in both HIV-1-infected mothers and uninfected mothers (figure 2*A*) at all time points. The median, the mean, and the first and third quartile ranges are shown in figure 2*B*, for HIV-1-infected mothers and uninfected mothers. Mothers who

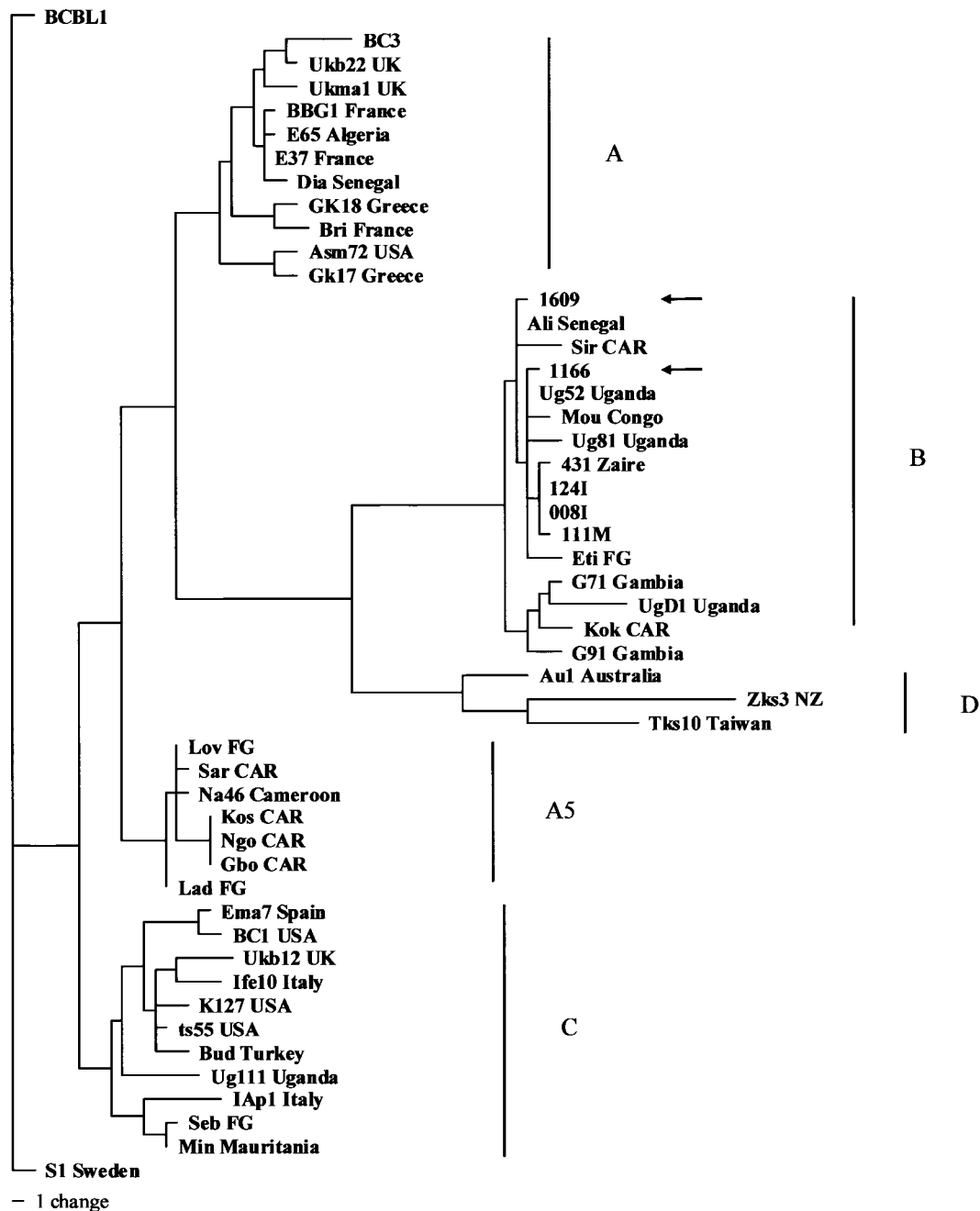


Figure 3. (Continued.)

were infected with HIV-1 appeared to have a higher median number of HHV-8 copies/ 10^4 cells, but the Wilcoxon rank sum test of medians did not reveal a significant difference between HIV-1-infected and uninfected mothers ($P = .26$, by 1-sided Wilcoxon rank sum test), on the basis of evaluation of a small number of samples ($n = 16$).

Sequence analysis of the K1 gene of HHV-8-positive patients. The HHV-8 glycoprotein K1 has been shown to contain regions of high variability (variable region 1 [VR1] and variable region 2 [VR2]), and these regions have been used to

segregate HHV-8 strains into distinct clades [22]. Because of the high degree of variability present in the VR1, we used this region to determine whether the HHV-8 sequences detected in the saliva samples evaluated in the present study were related to other known African isolates. In addition, we wanted to exclude the possibility of PCR contamination by laboratory BCBL-1 or BC-3 HHV-8 sequences. Figure 3A shows the amino acid alignment of the VR1 of K1 (aa 52–92) for 2 saliva samples that were found to be positive for HHV-8 (samples 1609 and 1166), consensus sequences from PBMC samples isolated from

3 Zambian patients with KS analyzed at the University of Nebraska–Lincoln (sequences 08I, 124I, and 111M), and 43 other published VR1 sequences comprising all clades and variants of HHV-8 based on K1 [22–24]. The saliva sequences are distinct from each other at 3 amino acid positions, and they differ from the BCBL-1 and BC-3 sequences used in our laboratory. Furthermore, they show similarity to, but are distinct from, clade B K1 sequences from Africa, including Ug52, UgD1, Mou, Ali, 431, and G91, and the other Zambian sequences (sequences 08I, 124I, and 111M). Phylogenetic analysis of the VR1 nucleotide sequence from the same 2 saliva samples in the present study, along with the same K1 sequences used in the amino acid alignment, is shown in figure 3B. Similar to the amino acid alignment, the neighbor-joining analysis indicates that the 2 saliva isolates recovered from Zambian mothers are closer to clade B African sequences than to those from other geographic regions.

DISCUSSION

The seroprevalence of HHV-8 in several regions of the world supports the concept that multiple factors influence transmission and that multiple modes of transmission may exist [9, 14, 25]. Our previous work on mother-to-infant transmission of HHV-8 suggested that in utero or intrapartum transmission was not a major contributor to the occurrence of HHV-8 infection in the first year of life [14, 15]. Continued work has also shown that many infants have seroconversion to an HHV-8–positive status during the first year after delivery; therefore, the primary purpose of the present analysis was to determine whether HHV-8 could be detected in maternal fluids with potential for virus transmission, such as breast milk and saliva.

A number of herpesviruses, including human herpesvirus 7, EBV, CMV, and herpes simplex virus 1, have been detected in breast milk, supporting the possibility of maternal-to-infant transmission via this fluid [26–29]. Studies of infants who were breast-fed by CMV-seropositive mothers have reported rates of infection of 37%–76%, compared with 4% for infants breast-fed by seronegative mothers [27, 30, 31]. CMV DNA was more frequently detected in breast-fed infants than in bottle-fed infants [31, 32]. Moreover, 2 studies reported the absence of viral DNA in the breast milk of CMV-seronegative mothers, with the result that none of the infants of these mothers were infected after birth [27]. Both CMV and HHV-8 are known to infect monocytes, macrophages, and epithelial cells, both of which are found in the cellular components of colostrum and mature milk [33–36]. Thus, extrapolation of findings from studies of CMV suggest that HHV-8 might also be found in breast milk and might infect cells within breast milk.

In the present study, we examined a number of cross-sectional breast-milk samples obtained from infected mothers within the first 6 months after delivery, but we failed to detect

HHV-8 DNA at limits of detection of 1 HHV-8 copy/10⁴ cells. Spiking experiments performed in donor breast milk suggest that HHV-8–infected cells can be readily detected in breast milk, if present. In the present study, failure to detect HHV-8 could have been the result of there being too few cells present in our specimen to allow for sufficient detection of cell-associated HHV-8 DNA, as has been suggested for CMV [13]; however, a subset of 9 β -globin–positive breast-milk samples contained a mean of 14,725 cellular equivalents, as determined by real-time PCR analysis. This indicated that sufficient numbers of cells and target DNA were present in the breast-milk samples, unless the number of infected cells present was below the limit of detection of our assays. In support of this concept, we detected very weak HHV-8 nested PCR signals in a few breast-milk specimens, but such detection was inconsistent and could not be confirmed using a second set of primers. It is also possible that, by first collecting samples at 2 months, we missed the peak level for the detection of viral DNA in breast milk. To exclude this possibility, we examined colostrum obtained from 2 HHV-8–seropositive mothers. Colostrum is known to contain the highest number of cells during lactation. Despite the high number of cellular DNA equivalents in the colostrum samples, both of the samples were PCR negative for HHV-8. There is an additional possibility that breast milk, which contains several potential antiviral components (immunoglobulin, lactoferrin, vitamin A, lipids, and prostaglandins), could eliminate free virus or virus-infected cells from breast milk [37]. Nevertheless, our results suggest that HHV-8–infected cells may only rarely, if ever, be present in the breast tissue and milk, and that breast milk is not a likely route of transmission of HHV-8 to infants.

Despite the failure to detect HHV-8 in breast-milk samples, EBV and HIV-1 DNA were readily detected in the same samples analyzed for HHV-8. These results are similar to those of previous studies of EBV and HIV-1 in breast milk, which reported prevalences of 46% for EBV and 56%–78% for HIV-1 [28, 38]. Of interest, the presence of EBV DNA in breast milk at the time of testing was dependent on the mother being infected with HIV-1 at the time of delivery. The significance of HIV-1 infection of the mother, with regard to the presence of EBV DNA in breast milk, needs to be investigated further.

We hypothesized that maternal saliva could be an important mediator of transmission because HHV-8 is not present in detectable quantities in breast milk and infection appears to occur early in life by means other than in utero or intrapartum means. HHV-8 is frequently detected in samples obtained from the oral cavity, regardless of the population from which they are obtained; this finding suggests that HHV-8 could be transmitted via saliva [35, 39–41]. Indeed, horizontal transmission has been suggested previously among men who have sex with men (MSM) and within families. It is known that HIV-1–

coinfected individuals and patients with KS all have elevated levels of HHV-8 in the oral cavity, compared with levels of HHV-8 in the PBMCs, plasma, semen, and skin; this suggests that the oral cavity may be a site of active viral reactivation and replication [10, 35, 40]. Cook et al. [42] reported that HHV-8 DNA was more frequently detected in saliva than in PBMCs of family members of a patient with KS. Moreover, that similar viral sequences were detected in the saliva of some family members supports the concept of transmission via the oral cavity.

In the present study, examination of HHV-8–infected mothers during the first 6 months after delivery of their infants indicated that 29% of the mothers were positive for HHV-8 in the cells from their saliva. This finding is in agreement with previous studies of HIV-1–infected men in the United States [10, 35]. The HHV-8 serostatus of the infant at 12 months after delivery was independent of the presence of HHV-8 in the saliva cells of the mother at a single time point. Three infants were born to mothers with detectable viral DNA, whereas 11 infants were born to mothers with undetectable viral DNA in saliva cells at the time of sampling. Unfortunately, none of the 3 mothers with HHV-8–infected children have sufficient material left for quantification of viral copy numbers in saliva cells by use of real-time PCR, which would allow detection of a correlation between virus burden and infection of the infants of these mothers. The lack of a dependent association between the presence of HHV-8 DNA in the saliva cells of a mother and the occurrence of HHV-8 infection in her infant is not unexpected. The level of HHV-8 in the oral cavity has been shown to vary over time in a cohort of MSM in the United States [35]. In fact, testing of available longitudinal samples from the mothers evaluated in the present study suggests that the presence of HHV-8 in saliva is variable between time points. Furthermore, the time at which saliva from the mothers was obtained for analysis may not correlate with the time at which transmission of HHV-8 to their infants occurred. Different sources and routes of infection, other than mother-to-infant sources and routes (transmitted by other household members), likely exist in this population, as has been suggested for similar populations [25, 42]. In a study of patients with KS, the prevalence of HHV-8 antibodies was higher among the spouses and children of the patients than among age-matched control subjects [42, 43].

Although the presence of HHV-8 in saliva cells was independent of the HIV-1 infection status of the mother, there appeared to be a higher median number of HHV-8 copies/ 10^4 cells in HIV-1–infected mothers versus uninfected mothers. However, by use of the Wilcoxon rank sum test of medians, there was no significant difference in the median level of HHV-8 copies/ 10^4 cells between HIV-1–infected mothers and uninfected mothers. Although this finding is surprising, given that previous studies of HIV-1–infected men and individuals with

KS have observed an elevated level of HHV-8 in HIV-1–infected men, the lack of a difference observed in the present study is likely the result of the small number of samples available for analysis and the fluctuation of the HHV-8 load in saliva with time. Nevertheless, the present study supports what other studies have suggested for regions where HHV-8 is endemic—namely, that exposure through infected saliva is likely responsible for the observed age-specific increase in seroprevalence through adolescence [4, 11].

We report the first postdelivery study of breast milk for the presence of HHV-8 DNA in a region of Africa where HHV-8 is endemic. Because this study is a cross-sectional analysis of specimens obtained from individual mothers, rather than a longitudinal analysis, it is possible that the presence of HHV-8 in breast milk at other time points could have been missed. Nevertheless, the results of the present study suggest that breast milk does not frequently contain HHV-8 and is not a main mode of transmission of HHV-8. However, frequent detection of HHV-8 in saliva suggests that contact with saliva that contains virus may be a prominent mode of transmission of HHV-8 to infants and children in regions of endemicity.

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ERRATUM

In an article in the 15 June 2004 issue of the *Journal* (Brayfield BP, Kankasa C, West JT, et al. The distribution of Kaposi's sarcoma-associated herpesvirus/human herpesvirus type 8 in Zambian maternal saliva and breast milk: implications for transmission. *J Infect Dis* 2004; 189:2260–70), the “A” and “B”

subtype designations in figure 3B were mislabeled and reversed. The subtype “A” in the upper group of sequences in the figure should be changed to “C,” and the subtype designation “C” in the lower group of sequences should be changed to “A.” The authors regret this error.