

Detection of Kaposi Sarcoma–Associated Herpesvirus DNA in Saliva and Buffy-Coat Samples from Children with Sickle Cell Disease in Uganda

Sam M. Mbulaiteye,¹ Ruth M. Pfeiffer,¹ Eric A. Engels,¹ Vickie Marshall,² Paul M. Bakaki,³ Anchilla M. Owor,³ Christopher M. Ndugwa,³ Edward Katongole-Mbidde,³ James J. Goedert,¹ Robert J. Biggar,¹ and Denise Whitby²

¹Division of Cancer Epidemiology and Genetics, National Cancer Institute, Department of Health and Human Services, Rockville, and ²Viral Epidemiology Section, AIDS Vaccine Program, Science Applications International Corporation–Frederick, National Cancer Institute at Frederick, Frederick, Maryland; ³Makerere University Medical School and Mulago Hospital, Kampala, Uganda

Among 233 children, Kaposi sarcoma–associated herpesvirus (KSHV) DNA was detected in 43% of children seropositive for both K8.1 and orf73, in 29% of children seropositive for K8.1 only, in 14% of children seropositive for orf73 only, and in 7% of children seronegative for both K8.1 and orf73; among 228 mothers, KSHV DNA was detected in 27%, 25%, 4%, and 1%, respectively. KSHV DNA was detected more frequently and at higher levels in saliva than in buffy-coat samples and in children than in mothers. In both children and mothers, detection in saliva was associated with detection in peripheral blood. Detection was associated with K8.1 seropositivity, younger age, and high household density, indicating the importance of in-household person-to-person transmission, likely via saliva.

Kaposi sarcoma–associated herpesvirus (KSHV; also called “human herpesvirus 8”) is the causative agent of Kaposi sarcoma (KS) [1]. KSHV seroprevalence and risk for KS are highest in sub-Saharan Africa, are intermediate in Mediterranean countries, and are lowest in the United States and northern Europe. In the United States and northern Europe, KSHV infection is rare in children; however, it is common in children in Africa, where spread predominantly occurs via horizontal non-sexual modes [1].

The route of KSHV transmission is uncertain, but salivary transmission has been suggested [2]. KSHV DNA is more frequently detected by polymerase chain reaction (PCR) in saliva than in other body fluids from

asymptomatic subjects, both in African and other populations in which prevalence is high [2]. Transmissible KSHV virions are found in saliva from homosexual men in the United States [3], and, in longitudinal studies, intermittent shedding is typically found [4]. Among febrile Egyptian children, KSHV DNA was detected in 33% of 36 saliva samples but in only 16% of 31 serum samples [5]. Elsewhere in Africa, KSHV DNA was detected in ~20% of peripheral-blood samples from children [6], blood donors [7], and pregnant women [8].

These studies were small and did not evaluate detection in saliva. We evaluated the prevalence and determinants of KSHV excretion in children with sickle cell disease and their mothers from Uganda, where KSHV and KS are endemic.

SUBJECTS, MATERIALS, AND METHODS

Our subjects were 600 Ugandan children with sickle cell disease (but without KS) enrolled at the Sickle Cell Clinic, Mulago Hospital, Kampala, during 2001–2002 [9]. Their mothers, if available, were also enrolled. The mothers gave written, informed consent for themselves and their children; children ≥ 7 years old gave witnessed assent. The present study was approved by the insti-

Received 27 February 2004; accepted 26 April 2004; electronically published 10 September 2004.

Presented in part: 6th International Workshop on KSHV/HHV8 and Related Rhadinoviruses, Glen Cove, New York, 18–22 July 2003.

Financial support: National Cancer Institute, Department of Health and Human Services (contracts N02-CP-91027 and N01-CO-12400).

Reprints or correspondence: Dr. Sam M. Mbulaiteye, 6120 Executive Blvd., Executive Plaza S., Rm. 8006, Rockville, MD 20852 (mbulait@mail.nih.gov).

The Journal of Infectious Diseases 2004;190:1382–6

© 2004 by the Infectious Diseases Society of America. All rights reserved. 0022-1899/2004/19008-0003\$15.00

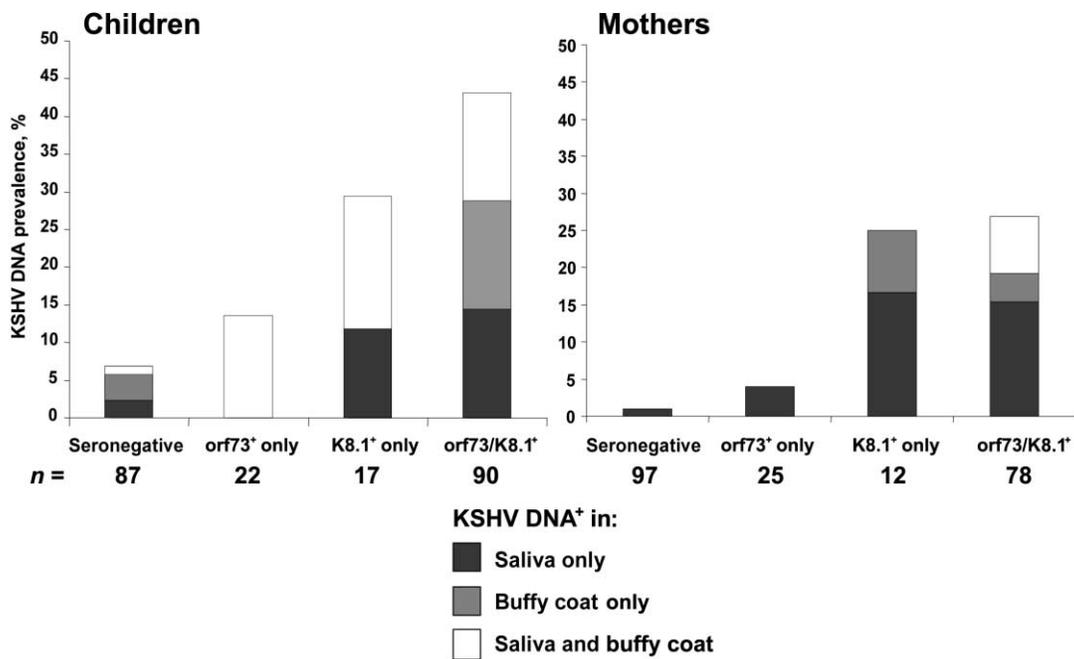


Figure 1. Frequency of detection of Kaposi sarcoma–associated herpesvirus (KSHV) DNA in saliva and buffy-coat samples from Ugandan children and mothers, stratified by KSHV-antibody serostatus (seronegative for both K8.1 and orf73, seropositive for orf73 only, seropositive for K8.1 only, and seropositive for both K8.1 and orf73).

tutional review boards of the National Cancer Institute and the Uganda National Council for Science and Technology.

Information was collected on age, sex, household density, location of residence, source of drinking water, and history of illness during the preceding week. Venous blood and whole saliva samples from subjects were processed and were stored at -80°C until testing. KSHV serologic testing was based on EIAs for K8.1, a KSHV structural glycoprotein expressed during lytic infection, and for orf73, a nuclear antigen expressed during latency [9]. For the present study, all children who were KSHV seropositive or indeterminate by either test ($n = 183$) and a random sample of 50 seronegative children were selected, as were their mothers. DNA was extracted from saliva and buffy-coat samples by use of the QIAamp DNA extraction kits (Qiagen-Operon), according to the manufacturer’s instructions. Dedicated rooms and equipment were used for the preparation of reagents, extraction of DNA, and PCR amplification. KSHV K6 and human endogenous retrovirus–3 cell TaqMan PCR assays were run by use of the ABI Prism 7700 sequence detection system (Applied Biosystems), as described elsewhere [10–12]. Cell-free KSHV genome copy numbers in saliva are expressed as copies per milliliter of saliva, and cell-associated virus copy numbers in buffy-coat samples are expressed as copies/ 10^6 peripheral-blood cells (PBCs). The K6 TaqMan PCR assay can detect 1 KSHV genome copy in 150 ng of input DNA and has a linear dynamic range of 5 logs. K1 PCR and genotyping were performed as described elsewhere [13].

For analysis, subjects were grouped into 4 categories, on the basis of whether they were seropositive or seronegative (considering indeterminate as seronegative) by the 2 KSHV serologic assays. Odds ratios (ORs) and 95% confidence intervals (CIs) for associations between individual variables and detection of KSHV DNA were estimated by use of logistic regression models, controlling for serostatus category and age. Associations between child or mother serostatus and detection of KSHV were estimated by use of generalized estimation equations [14]. Mean values were compared between groups by use of Student’s *t* test. Virus levels were log-transformed before performing the *t* test. Two-sided $P < .05$ was considered to be statistically significant.

RESULTS

The median age of the children was 8.5 years (range, 0–16.0 years), and 54% were male. The median age of the mothers was 34.3 years (range, 19.4–59.6 years).

KSHV DNA was detected in 43% of saliva or buffy-coat samples from 90 children seropositive for both K8.1 and orf73, in 29% of saliva or buffy-coat samples from 17 children seropositive for K8.1 only, and in 14% of saliva or buffy-coat samples from 22 children seropositive for orf73 only (figure 1). K8.1-seropositive, KSHV DNA–positive children had significantly higher K8.1 antibody levels than did K8.1-seropositive, KSHV DNA–negative children (mean OD, 2.11 vs. 1.78

[$P < .001$]). However, orf73 antibody levels were similar in orf73-seropositive, KSHV DNA-positive children and orf73-seropositive, KSHV DNA-negative children (mean OD, 1.14 vs. 1.05 [$P = .16$]).

KSHV DNA was detected in 27% of saliva or buffy-coat samples from 78 mothers seropositive for both K8.1 and orf73, in 25% of saliva or buffy-coat samples from 12 mothers seropositive for K8.1 only, and in 4% of saliva or buffy-coat samples from 25 mothers seropositive for orf73 only (figure 1). As with the findings for children, K8.1-seropositive, KSHV DNA-positive mothers had significantly higher K8.1 antibody levels than did K8.1-seropositive, KSHV DNA-negative mothers (mean OD, 2.21 vs. 1.89 [$P = .004$]). However, in contrast to the findings for children, among orf73-seropositive mothers, orf73 antibody levels were significantly higher in those who were positive for KSHV DNA than in those who were negative for KSHV DNA (mean OD, 1.42 vs. 1.20 [$P = .04$]).

Among seropositive children, detection of KSHV DNA in saliva and buffy-coat samples was inversely related to age, although the trend was statistically significant for detection of KSHV DNA in buffy-coat samples only ($P < .005$). Detection of KSHV DNA in saliva samples was associated with detection in buffy-coat samples (OR for children, 8.9 [95% CI, 3.6–21.6]; OR for mothers, 24.9 [95% CI, 6.1–102]). The proportions of children and mothers with detectable KSHV DNA in saliva samples only were the same (12% for both). However, compared with the rates among mothers, KSHV DNA was detected among children more frequently in both saliva and buffy-coat samples (15% vs. 6% [$P = .06$]) and in buffy-coat samples only (10% vs. 3% [$P = .02$]). KSHV DNA was detected 1.5-fold (95% CI, 0.52–4.39-fold) more frequently in saliva samples and 2.8-fold (95% CI, 1.05–7.38-fold) more frequently in buffy-coat samples from children with KSHV DNA-positive mothers than in those from children with KSHV DNA-negative mothers.

Six of 87 seronegative children had detectable KSHV DNA in saliva or buffy-coat samples, compared with only 1 of 102 seronegative mothers (7% vs. 1% [$P = .06$]; figure 1). Of the children, 1 had detectable KSHV DNA in both saliva and buffy-coat samples (the mother was positive in her saliva sample only), 2 had detectable KSHV DNA in saliva samples only, and 3 had detectable KSHV DNA in buffy-coat samples only. Three of the KSHV DNA-positive children had mothers who were seropositive for both K8.1 and orf73, 2 of whom also had detectable KSHV DNA in their saliva samples; the remaining 3 children had seronegative mothers who were negative for KSHV DNA. Full-length K1 was successfully amplified from the saliva of the child concordantly positive in saliva sample and buffy-coat sample, but full-length K1 was not successfully amplified from samples from the other seronegative subjects, most of whom were positive below a quantifiable level. The K1 sequence obtained from this child was a unique A5 subtype

sequence that differed from K1 sequences obtained from 20 other samples from the seropositive mothers and children (data not shown).

KSHV DNA was detected but could not be quantified in 4 saliva and 7 buffy-coat samples from children. Among children, when the KSHV genome could be quantified (30 saliva and 29 buffy-coat samples), the median KSHV-genome copy numbers were 10,100 copies/mL of saliva (interquartile range [IQR], 320–29,333 copies/mL of saliva) and 133 copies/ 10^6 PBCs in buffy-coat samples (IQR, 34–275 copies/ 10^6 PBCs). The levels of KSHV DNA detected in saliva and in peripheral blood were similar for girls and boys. For mothers, KSHV DNA could be quantified in 19 of 22 saliva and in 6 of 11 buffy-coat samples. Their median KSHV-genome copy numbers were 7200 copies/mL of saliva (IQR, 220–32,000 copies/mL of saliva) and 42 copies/ 10^6 PBCs in buffy-coat samples (IQR, 38–88 copies/ 10^6 PBCs), but the log-transformed levels were not significantly different in mothers and children.

Among children, detection of KSHV DNA in saliva and buffy-coat samples was associated with household density but was not associated with sex, tribe, source of drinking water, or location of residence, controlling for age and serostatus (table 1). KSHV DNA was detected 2.9-fold (95% CI, 1.03–7.91-fold) more frequently in saliva samples from children reporting recent sickle cell dactylitis and 0.37-fold (95% CI, 0.14–0.98-fold) more frequently in saliva samples from children taking folic acid daily to prevent severe anemia. Recent fever, cough, malaria, and sore throat were unrelated to detection of KSHV DNA in either saliva or buffy-coat samples.

DISCUSSION

Our results have broadened our understanding of the frequency of KSHV salivary shedding and have emphasized the potential role that saliva might play as a vehicle for KSHV transmission in the spread of KSHV in regions where the virus is endemic. High rates of KSHV detection have been previously reported in Africans [5, 7, 8]. The present study has corroborated these earlier findings, using a larger study population, providing quantitative data, and simultaneously evaluating the detection of KSHV DNA in saliva and buffy-coat samples from children and their mothers.

We found that KSHV DNA was detected more frequently and at higher levels in saliva than in peripheral blood and in children than in mothers. Detection of KSHV DNA was associated with the presence and level of KSHV antibody (as measured by the K8.1 and, to a lesser extent, the orf73 EIAs), younger age, and high household density. High antibody reactivity, particularly against K8.1 lytic antigens, appears to be associated with detection of KSHV DNA in saliva, peripheral blood, or both, indicating that K8.1 antibody is an immune response to KSHV structural proteins released during lytic vi-

Table 1. Frequency of detection of Kaposi sarcoma-associated herpesvirus (KSHV) DNA in saliva and buffy-coat samples from Ugandan children with sickle cell disease, by demographic variables.

Category, variable	KSHV DNA detected in saliva sample (n = 216) ^a			KSHV DNA detected in buffy-coat sample (n = 233)		
	No. (%)	OR ^b (95% CI)	P ^c	No. (%)	OR ^b (95% CI)	P ^c
Sex			.84			.09
Female	18 (18)	Ref.	...	23 (21)	Ref.	...
Male	19 (17)	0.93 (0.43–2.00)	...	17 (14)	0.53 (0.25–1.11)	...
Age in years, quartiles			.74			.02
1.0–3.8	5 (19)	Ref.	...	8 (22)	Ref.	...
3.9–6.8	6 (12)	0.32 (0.07–1.40)	...	10 (19)	0.68 (0.22–2.12)	...
6.9–10.9	11 (15)	0.33 (0.08–1.29)	...	13 (18)	0.47 (0.16–1.40)	...
11.0–16.0	15 (22)	0.48 (0.12–1.81)	...	9 (13)	0.28 (0.09–0.89)	...
Tribe			.89			.03
Ganda	28 (18)	Ref.	...	32 (19)	Ref.	...
Luo	2 (15)	0.76 (0.14–4.20)	...	2 (14)	0.85 (0.16–4.51)	...
Soga	2 (18)	1.59 (0.26–9.58)	...	4 (15)	2.80 (0.66–11.90)	...
Other	5 (14)	0.77 (0.26–2.31)	...	2 (5)	0.19 (0.04–0.88)	...
Source of drinking water			.90			.05
Private tap	0	0
Communal standpipe	21 (17)	Ref.	...	18 (13)	Ref.	...
Surface water	16 (20)	0.95 (0.44–2.07)	...	22 (24)	2.14 (1.01–4.56)	...
Location of residence			.23			.28
Urban	15 (21)	Ref.	...	16 (21)	Ref.	...
Periurban	9 (13)	0.43 (0.16–1.17)	...	11 (14)	0.52 (0.21–1.29)	...
Rural	13 (18)	0.78 (0.31–2.00)	...	12 (16)	0.53 (0.21–1.35)	...
Household density ^d			.03			.06
Low	4 (9)	Ref.	...	5 (11)	Ref.	...
Medium	8 (13)	1.84 (0.48–7.10)	...	8 (12)	1.24 (0.36–4.33)	...
High	7 (23)	3.84 (0.89–16.60)	...	6 (18)	1.51 (0.39–5.92)	...
Very high	18 (23)	3.69 (1.05–13.10)	...	21 (24)	2.50 (0.82–7.64)	...

NOTE. CI, confidence interval; OR, odds ratio; Ref., reference.

^a Excludes 17 children without saliva samples.

^b Adjusted for age group and KSHV-antibody serostatus (seronegative for both K8.1 and orf73, seropositive for orf73 only, seropositive for K8.1 only, and seropositive for both K8.1 and orf73), except for age group, which is adjusted only for KSHV-antibody serostatus.

^c P values for age and household density are for trend.

^d Household density is calculated as the no. of persons per the no. of rooms in the house (low, ≥ 1.50 ; medium, 1.51–2.24; high, 2.25–2.99; very high, ≥ 3.00).

rus replication. Detection of KSHV DNA in both saliva and buffy-coat samples was frequent among the study subjects. This finding suggests that KSHV infection in the oral cavity, as manifested by salivary shedding, may be in a dynamic relationship with infection in circulating infected B lymphocytes.

Detection of KSHV DNA was inversely related with age in positive subjects. It is possible that some recently infected children were still in the lytic phase of their primary infection. Because we had no follow-up samples, we could not examine this hypothesis. Alternatively, because the children in our study had sickle cell anemia, humoral immune deficits associated with sickle cell disease could account for the higher prevalence of KSHV DNA in children. Anemia could also be a factor, as suggested by an in vitro study that reported KSHV replication in cells subjected to hypoxia [15]. We found that children who took folic acid daily to prevent severe anemia were less likely

to shed virus, whereas those who had sickle cell dactylitis, a condition resulting from hypoxia-induced infarction of bone marrow and cortical bone of the hands and feet, were more likely to shed virus.

Mothers who were positive for KSHV DNA in saliva, buffy-coat samples, or both were more likely to have children with detectable KSHV DNA in their saliva or peripheral blood, but the association was only statistically significant for detection of KSHV in buffy-coat samples. The findings for detection in saliva samples might reflect transmission between mother-child pairs, whereas the findings for detection in buffy-coat samples could indicate shared host responses to KSHV infection due to the subjects' being genetically related or to common environmental exposures. Environmental factors were also suggested by the association between detection of KSHV DNA in saliva and buffy-coat samples from children and high house-

hold density, implying person-to-person transmission of KSHV and persistence of viremia in crowded households.

In the present study, 6 children and 1 mother were seronegative but were positive for KSHV DNA in either saliva or buffy-coat samples. Although they may have been misclassified as seronegative or virus positive because of errors in sample labeling or testing, the preponderance of KSHV detection in children and the unique K1 sequence obtained from the 1 child for whom sufficient viral DNA allowed for KSHV genotyping argue against such misclassification. Perhaps these subjects had pre-seroconversion infections. Alternatively, sickle cell disease-associated humoral immune deficits may have resulted in a less robust immune response, resulting in these children being falsely classified as seronegative.

The strengths of the present study include its size and our evaluation of paired saliva and peripheral-blood samples from children and women in a region where KS is endemic. Although all of the children in our study had sickle cell disease, which may limit generalization of our findings, this disease is not known to be associated with risk for acquiring KSHV or for developing KS. A further limitation is that the cross-sectional design may have prevented us from observing transient shedding or viremia and from finding their associations.

To summarize, detection of KSHV DNA was significantly associated with lytic anti-KSHV antibodies, younger age, and high household density. The frequent detection and high levels of KSHV DNA in the saliva of our subjects support the hypothesis that KSHV may be transmitted via saliva and suggest that person-to-person spread among children as well as among mothers contributes to the endemicity of KSHV in Uganda.

Acknowledgments

We thank the study subjects and the staff at the Mulago Sickle Cell Clinic (Kampala, Uganda), for their participation; Amali Amarasinghe (Research Triangle Institute, Washington, DC), for coordinating the fieldwork; Benon Biryahwaho, for storing and shipping samples; and Christine Gamache and

Georgina Mbisa, for performing the serologic assays. We also thank Mulago Hospital, Makerere University Medical School, and the Uganda Ministry of Health, for allowing the study to be conducted.

References

1. Martin JN. Diagnosis and epidemiology of human herpesvirus 8 infection. *Semin Hematol* **2003**;40:133–42.
2. Corey L, Brodie S, Huang ML, Koelle DM, Wald A. HHV-8 infection: a model for reactivation and transmission. *Rev Med Virol* **2002**;12:47–63.
3. Vieira J, Huang ML, Koelle DM, Corey L. Transmissible Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in saliva of men with a history of Kaposi's sarcoma. *J Virol* **1997**;71:7083–7.
4. Pauk J, Huang ML, Brodie SJ, et al. Mucosal shedding of human herpesvirus 8 in men. *N Engl J Med* **2000**;343:1369–77.
5. Andreoni M, Sarmati L, Nicastrì E, et al. Primary human herpesvirus 8 infection in immunocompetent children. *JAMA* **2002**;287:1295–300.
6. Gessain A, Mauclore P, van Beveren M, et al. Human herpesvirus 8 primary infection occurs during childhood in Cameroon, Central Africa. *Int J Cancer* **1999**;81:189–92.
7. Enbom M, Urassa W, Massambu C, Thorstensson R, Mhalu F, Linde A. Detection of human herpesvirus 8 DNA in serum from blood donors with HHV-8 antibodies indicates possible bloodborne virus transmission. *J Med Virol* **2002**;68:264–7.
8. Mantina H, Kankasa C, Klaskala W, et al. Vertical transmission of Kaposi's sarcoma-associated herpesvirus. *Int J Cancer* **2001**;94:749–52.
9. Mbulaiteye SM, Biggar RJ, Bakaki PM, et al. Human herpesvirus 8 infection and transfusion history in children with sickle-cell disease in Uganda. *J Natl Cancer Inst* **2003**;95:1330–5.
10. Biggar RJ, Whitby D, Marshall V, Linhares AC, Black F. Human herpesvirus 8 in Brazilian Amerindians: a hyperendemic population with a new subtype. *J Infect Dis* **2000**;181:1562–8.
11. de Sanjose S, Marshall V, Sola J, et al. Prevalence of Kaposi's sarcoma-associated herpesvirus infection in sex workers and women from the general population in Spain. *Int J Cancer* **2002**;98:155–8.
12. Yuan CC, Miley W, Waters D. A quantification of human cells using an ERV-3 real time PCR assay. *J Virol Methods* **2001**;91:109–17.
13. Whitby D, Marshall VA, Bagni RK, et al. Genotypic characterization of Kaposi's sarcoma-associated herpesvirus in asymptomatic infected subjects from isolated populations. *J Gen Virol* **2004**;85:155–63.
14. Zeger SL, Liang KY. Longitudinal data analysis for discrete and continuous outcomes. *Biometrics* **1986**;42:121–30.
15. Davis DA, Rinderknecht AS, Zoetevej J, et al. Hypoxia induces lytic replication of Kaposi sarcoma-associated herpesvirus. *Blood* **2001**;97:3244–50.