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Risk Factors for Kaposi's Sarcoma–Associated Herpesvirus DNA in Blood and in Saliva in Rural Uganda

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Background: Detectable Kaposi's sarcoma–associated herpesvirus (KSHV) DNA in blood and increased antibody titres may indicate KSHV reactivation, while the transmission of KSHV occurs via viral shedding in saliva.

Methods: We investigated the risk factors for KSHV DNA detection by real-time polymerase chain reaction in blood and by viral shedding in saliva, in 878 people aged 3 to 89 years of both sexes in a rural Ugandan population cohort. Helminths were detected using microscopy and the presence of malaria parasitaemia was identified using rapid diagnostic tests. Regression modelling was used for a statistical analysis.

Results: The KSHV viral load in blood did not correlate with the viral load in saliva, suggesting separate immunological controls within each compartment. The proportions of individuals with a detectable virus in blood were 23% among children aged 3–5 years and 22% among those 6–12 years, thereafter reducing with increasing age. The proportions of individuals with a detectable virus in saliva increased from 30% in children aged 3–5 years to 45% in those aged 6–12 years, and decreased subsequently with increasing age. Overall, 29% of males shed in saliva, compared to 19% of females ($P = .008$).

Conclusions: Together, these data suggest that young males may be responsible for much of the onward transmission of KSHV. Individuals with a current malaria infection had higher levels of viral DNA in their blood ($P = .031$), compared to uninfected individuals. This suggests that malaria may lead to KSHV reactivation, thereby increasing the transmission and pathogenicity of the virus.

Keywords. Kaposi's sarcoma herpesvirus DNA; risk factors; Uganda.

Kaposi's sarcoma-associated herpesvirus (KSHV) causes Kaposi's sarcoma (KS), multicentric Castleman disease, and primary effusion lymphoma [1–6]. The prevalence of KSHV and incidence of KS both vary geographically [7–9], and are endemic in sub-Saharan Africa [10, 11].

Salivary exchange is the main route of transmission of KSHV, normally occurring in early childhood and increasing with age [12–15]. In a rural population cohort in Uganda (the general population cohort [GPC]), we previously reported KSHV infections in children as young as 1 year [11, 16]. In addition to viral shedding in saliva, viral DNA detection in blood and increased antibody titres to lytic antigens are markers of frequent KSHV reactivation [13]. The KSHV-associated oncogenesis and progression of diseases, as well as virus transmission, are all thought to be related to virus reactivation [17].

Viral DNA detection in blood has been associated with KS disease risk and progression [18–21]. Additionally, treatment with combined antiretroviral therapy of KS patients living with acquired immunodeficiency syndrome has been shown to reduce the KSHV load in blood to undetectable levels [18, 22]. Determinants of KSHV DNA detection in blood among KSHV-seropositive people in the general population are not well understood. The presence of a viral load in plasma and peripheral blood mononuclear cells (PBMCs) has been reported mainly in high-risk groups, such as individuals living with human immunodeficiency virus (HIV) and patients with KSHV-related diseases [18, 23–27]. A few studies have reported KSHV viral loads in blood donors (adults) in nonendemic areas [28–30] and another study reported plasma viral loads in a population-based HIV survey in an endemic area [31].

Environmental factors may contribute to high KSHV transmission in endemic areas. We have previously shown that KSHV seroprevalence is associated with malaria parasitaemia [16], higher malaria antibody titres [32], and helminth infections [33, 34]. We and others have reported that KSHV shedding in saliva is more common in males, compared to females [35, 36], but no study has investigated KSHV viral loads in both blood and saliva in the same individuals within a population-based study in a KSHV-endemic area.

This study investigated KSHV viral DNA detection in PBMCs and saliva in KSHV-seropositive individuals aged 3 to 89 years

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from the GPC: a longstanding cohort in rural, southwestern Uganda. We also determined the risk factors associated with viral DNA detection and levels in PBMCs and in saliva, and the relationship between viral DNA detection in PBMCs and saliva and KSHV antibody levels in plasma.

METHODS

Study Population and Ethical Approvals

This work was carried out within the GPC. The GPC is a community-based cohort of 22 000 people in 25 adjacent villages in southwestern Uganda. It was established in 1989 to carry out HIV research; participants from the GPC have been followed ever since. Between July 2017 and November 2017, we nested a cross-sectional study within the GPC, enrolling 975 KSHV-seropositive (tested previously [32]) individuals who were living without HIV and aged 3 to 89 years. Participants were selected randomly after stratification for age, sex, and household. Blood, stool, and saliva samples were collected from these individuals. PBMCs and plasma were obtained from blood for immunological and virological analyses. Stool samples were used for helminth diagnoses, while saliva samples were used for KSHV viral DNA detection and quantification. Socio-demographic data were collected using standard questionnaires. This study was approved by the Uganda Virus Research Institute (UVRI) Research and Ethics Committee (reference number GC/127/16/09/566), the Uganda National Council for Science and Technology (reference number HS2123), and the London School of Hygiene & Tropical Medicine (LSHTM) Ethics Committee (reference number 11881). Written informed consent was obtained from all adults aged 18 years and above. Parents or guardians consented for children below 18 years; additionally, children aged 8–17 years provided written assent.

Laboratory Procedures

KSHV DNA was quantified in PBMCs and saliva from 878 KSHV-seropositive individuals. About 2 million PBMCs and saliva pellets were processed for DNA extraction. Study participants were instructed to rinse with 5 mL of Listerine mouthwash, emptying it, as well as saliva, in a falcon tube. Aliquots (of 1 mL each) of saliva were spun at 13 000 relative centrifugal force for 10 minutes to form saliva pellets. Thereafter, the supernatant was removed, and the saliva pellet was stored at -80°C . Genomic DNA was extracted from PBMCs and saliva pellets using a QIAamp blood kit (Qiagen, Valencia, CA), following the manufacturer's instructions. KSHV DNA was quantified using real-time polymerase chain reaction, following procedures previously reported [13, 37, 38]. KSHV DNA was detected using primers and a probe specific to the K6 gene region [39]. Additionally, the numbers of cellular equivalents in PBMCs were determined using a quantitative assay specific to human endogenous retrovirus 3 [39], which is present in 2 copies per genomic cell. Raw copies were reported for saliva KSHV DNA.

Samples were amplified in triplicate; the samples that were positive in 1 or 2 reactions in the KSHV K6 assay were designated as qualitative positives. The sensitivity of the K6 assay is 3 copies.

Using an in-house Luminex assay and enzyme-linked immunosorbent assay, as previously reported [40–42], plasma samples were tested for immunoglobulin G (IgG) antibody levels to the KSHV K8.1 (lytic) and open reading frame (ORF) 73 (latent) antigens. The enzyme-linked immunosorbent assay was used to confirm serostatus, while the Luminex assay was used to determine antibody levels, due to its wider dynamic range. The presence of malaria parasitaemia was diagnosed using rapid diagnostic tests (ONE STEP Malaria HRP-II [*Plasmodium falciparum*] and pLDH [*Plasmodium* species] Antigen Rapid Test). A single stool sample was provided by each participant. This was analyzed for helminths (*Schistosoma mansoni*, *Ascaris lumbricoides*, *Tichuris trichiura*, *Trichostrongylus* spp, and hookworm) using the Kato Katz microscopy method, following the manufacturer's instructions. Details of this procedure have been reported elsewhere [43, 44].

Statistical Analysis

A statistical analysis was carried out using STATA version 13 (Statacorp, College Station, TX). Graphs were drawn using STATA and GraphPad Prism version 6. Qualitative, positive samples were given a constant value of 0.04 for saliva and 0.5 for PBMCs, which were below the values of the lowest qualified samples for a quantitative analysis. Viral load levels were \log_{10} transformed. First, the risk factors associated with viral DNA detection (as a categorical outcome variable) in saliva and blood, separately, were obtained using logistic regression modelling. Thereafter, the risk factors associated with increasing levels of viral DNA (as a continuous outcome variable) in saliva and in blood were separately determined using linear regression modelling. Likelihood ratio tests were used to select the best fit models.

RESULTS

Study Participants' Characteristics

We tested 878 individuals for KSHV viral DNA; 49% (410/834) were males and 3% (27/840), 11% (95/840), 13% (110/840), 8% (67/840), 17% (139/840), 14% (121/840), 14% (118/840), 9% (74/840), and 11% (89/840) were aged 3–5, 6–12, 13–18, 19–25, 26–35, 36–45, 46–55, 56–65, and 66–89 years, respectively (Table 1). The proportions of individuals with malaria parasitaemia were 4% (34/834) overall and 11% (13/120) among children aged 3–12 years. Previously, we reported an annual malaria prevalence of 18% in the same population [16]. The lower prevalence of malaria infection in this study might be attributed to sample collection during the dry season. Hookworm was the most prevalent helminth, at 15% (104/686), followed by *Schistosoma mansoni* and *Ascaris lumbricoides* at 1% (8/686) each and *Trichuris trichiura* at 0.1% (1/686). We

Table 1. General Characteristics and Parasite Infection Status

Sex, males	49% (410/834)
Age, mean (range)	36 (3–89)
Age groups	
2–5	3% (27/840)
6–12	11% (95/840)
13–18	13% (110/840)
19–25	8% (67/840)
26–35	17% (139/840)
36–45	14% (121/840)
46–55	14% (118/840)
56–65	9% (74/840)
66–89	11% (89/840)
Malaria parasitaemia	
Overall	4% (34/834)
Children aged 3–12 years	11% (13/120)
<i>Schistosoma mansoni</i> infection status	1% (7/686)
Hookworm infection status	15% (104/686)
<i>Ascaris lumbricoides</i> infection status	1% (8/686)
<i>Trichuris trichiura</i> infection status	0.2% (1/685)

Data are among participants tested for Kaposi's sarcoma-associated herpesvirus viral DNA. The presence of malaria parasitaemia was determined using rapid diagnostic tests. Helminth status was determined from a single stool sample using the Kato Katz method.

may have slightly underestimated the true prevalence of helminths, because a single-sample test was used rather than a triple-sample test.

Blood and Saliva DNA Detection and Levels

We did not observe a correlation between KSHV DNA copy numbers in PBMCs and DNA copy numbers in saliva

(Figure 1). The proportion of individuals with detectable viral DNA in saliva was higher than the proportion of people with the detectable viral DNA in PBMCs (Figure 2). Children had the highest proportions of detectable viral DNA in PBMCs (Figure 2A) and in saliva (Figure 2B), decreasing with increasing age in adults. The trends were similar for females and males, with males having higher proportions of detectable viral DNA in saliva.

Associations Between Risk Factors and DNA

The proportion of individuals with detectable viral DNA in PBMCs decreased with increasing age; this trend was significant even after adjusting for sex and parasite infections (Table 2). Individuals infected with malaria parasites had higher levels of KSHV DNA in blood, compared to malaria-uninfected individuals (adjusted regression coefficient 0.79 [confidence interval [CI], .07–1.50]; $P = .031$; Table 3). We observed no statistically significant associations with other measured risk factors, including age, sex, hookworm infection, and *S. mansoni* infection (Table 3).

Associations Between Risk Factors and DNA in Saliva

Overall, males had a higher risk of shedding viral DNA, compared to females (adjusted odds ratio 1.63 [CI, 1.14–2.34]; $P = .008$; Table 4). Similar to PBMCs, the proportion of participants with shedding in saliva diminished with increasing age, even after adjusting for sex and parasite infections ($P = .0001$; Table 4). Additionally, compared to females, males had higher

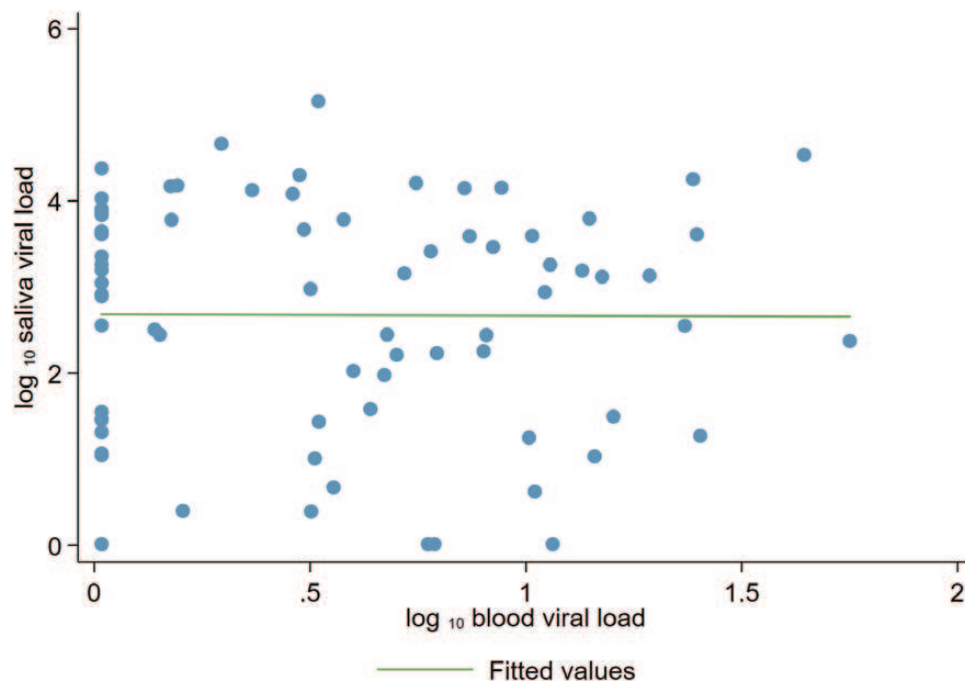


Figure 1. Kaposi's sarcoma-associated herpesvirus (KSHV) viral load in saliva and in peripheral blood mononuclear cells. KSHV viral loads were measured using real-time polymerase chain reaction.

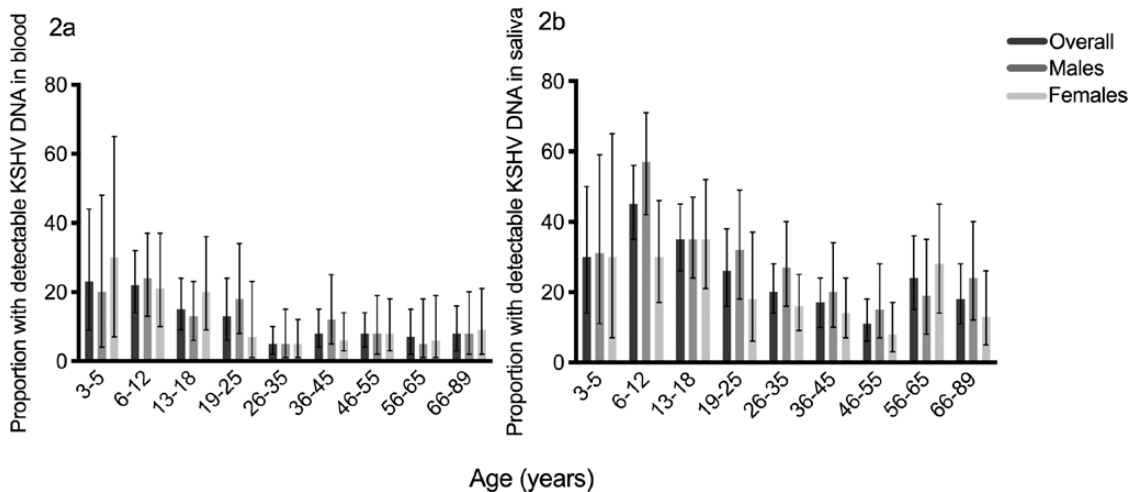


Figure 2. Proportion of individuals with detectable Kaposi's sarcoma-associated herpesvirus (KSHV) in (A) peripheral blood mononuclear cells and (B) saliva. KSHV viral loads were measured using real time polymerase chain reaction. Abbreviation: KSHV, Kaposi's sarcoma-associated herpesvirus.

levels of KSHV DNA in saliva (adjusted regression coefficient 0.46 [CI, .05–.87]; $P = .027$; Table 5).

DNA Detection and Antibody Levels

Individuals with detectable viral DNA in PBMCs (Supplementary Figure 1A) and in saliva (Supplementary Figure 1B) had higher IgG antibodies to the K8.1 antigen ($P < .0001$), as previously reported [13]. There were no differences in IgG antibody levels to the ORF73 antigen between individuals with or without detectable viral DNA in the blood (Supplementary Figure 1C) or in saliva (Supplementary Figure 1D).

DISCUSSION

This is the first population-based study to report on the presence and levels of KSHV viral DNA in the blood and saliva of apparently healthy people across the life course. The proportion of individuals with detectable viral DNA in saliva was higher than the proportion of individuals with detectable viral DNA in blood, consistent with previous reports [22, 45–47]. We previously reported KSHV and EBV DNA shedding in the saliva of children and their mothers in Uganda, and noted that Epstein-Barr Virus DNA was shed more frequently and at higher levels than KSHV [38].

Table 2. Risk Factors for the Presence of Detectable Kaposi's Sarcoma-Associated Herpesvirus DNA in Blood (Categorically)

	% Detectable Viral DNA in Blood	OR (95% CI)	PValue	Adjusted ^a OR (95% CI)	PValue
Age group					
3–12	23% (27/120)	1		1	
13–25	15% (26/177)	0.59 (.33–1.08)		0.63 (.33–1.17)	
26–50	7% (20/307)	0.24 (.13–.45)		0.29 (.15–.57)	
50+	8% (18/227)	0.30 (.16–.57)	<.0001	0.34 (.16–.72)	.0014
Sex					
Female	10% (41/419)	1		1	
Male	12% (49/406)	1.27 (.82–1.96)	.294	0.89 (.55–1.45)	.638
Malaria parasitaemia					
Negative	10% (83/791)	1		1	
Positive	21% (7/34)	2.21 (.93–5.24)	.071	1.59 (.64–3.95)	.321
Schistosoma mansoni					
Negative	11% (77/672)	1		1	
Positive	43% (3/7)	5.80 (1.27–26.38)	.023	11.04 (2.16–56.97)	.004
Hookworm					
Negative	13% (74/576)	1		1	
Positive	6% (6/103)	0.42 (.18–.99)	.048	0.41 (.16–1.04)	.061

Logistic regression was used for the statistical analysis. The presence of malaria parasitaemia was determined using rapid diagnostic tests. Helminth status was determined from a single stool sample using the Kato Katz method.

Abbreviations: CI, confidence interval; OR, odds ratio.

^aAdjusted for age, sex, malaria parasitaemia, *Schistosoma mansoni*, and hookworm infection status.

Table 3. Risk Factors Associated With Increasing Levels of Kaposi's Sarcoma–Associated Herpesvirus DNA in Blood (Continuously)

	Coef (95% CI)	PValue	Adjusted ^a Coef (95% CI)	PValue
Age group				
3–12	Ref		Ref	
13–25	–0.39 (–.86 to .07)		–0.35 (–.84 to .15)	
26–50	0.19 (–.31 to .69)		0.26 (–.30 to .83)	
50+	–0.32 (–.83 to .19)	.084	–0.06 (–.70 to .57)	.160
Sex				
Female	Ref		Ref	
Male	0.10 (–.27 to .47)	.594	0.21 (–.19 to .61)	.292
Malaria parasitaemia				
Negative	Ref		Ref	
Positive	0.71 (.05–1.38)	.036	0.79 (.07–1.50)	.031
Schistosoma mansoni				
Negative	Ref		Ref	
Positive	0.17 (–.87 to 1.21)	.750	–0.15 (–1.30 to 1.00)	.797
Hookworm				
Negative	Ref		Ref	
Positive	0.30 (–.45 to 1.05)	.79	0.37 (–.46 to 1.21)	.372

Linear regression modelling was performed on log₁₀ transformed KSHV DNA levels for statistical analysis. The presence of malaria parasitaemia was determined using rapid diagnostic tests. Helminth status was determined from a single stool sample using Kato Katz method.

Abbreviations: CI, confidence interval; Coef, linear regression coefficient; KSHV, Kaposi's sarcoma–associated herpesvirus; Ref, reference.

^aAdjusted for age, sex, malaria parasitaemia, *Schistosoma mansoni*, and hookworm infection status.

In this study, we observed no correlation between levels of KSHV DNA in blood and in saliva. The detection of KSHV DNA in blood and in saliva may reflect a reactivation of the virus or an initial infection that manifests with lytic replication. Viral shedding in saliva leads to transmission of the virus [13], while a viral load in blood has been implicated in disease risk and progression [18, 23]. The lack of a correlation between blood and saliva viral DNA suggests that the mechanisms for reactivation of the virus in blood and in saliva may be different. This may imply that distinct immune control

measures are required to prevent viral reactivation in the different compartments. For insistence, immunoglobulin A production in saliva may be important for viral control in oral fluids, while T and natural killer cell responses may play a more pivotal role in the control of viral reactivation in peripheral blood. Alternatively, environmental factors, such as plant derivatives that have been shown to reactivate KSHV in vitro [48], may play a role in viral reactivation in oral fluids if chewed. Therefore, studies of immune correlates of KSHV DNA detection in saliva and PBMC are warranted.

Table 4. Risk Factors for the Presence of Detectable Kaposi's Sarcoma–Associated Herpesvirus DNA in Saliva (Categorical)

	% Detectable Viral DNA in Saliva	OR (95% CI)	PValue	Adjusted ^a OR (95% CI)	PValue
Age group					
3–12	42% (50/120)	1			
13–25	31% (55/175)	0.64 (.40–1.40)		0.61 (.37–1.02)	
26–50	18% (56/310)	0.31 (.19–.49)		0.38 (.24–.63)	
50+	17% (39/231)	0.28 (.18–.47)	<.0001	0.30 (.18–.54)	.0001
Sex					
Female	19% (79/423)	1		1	
Male	29% (119/407)	1.80 (1.30–2.49)	<.0001	1.63 (1.14–2.34)	.008
Malaria parasitaemia					
Negative	24% (188/796)	1		1	
Positive	29% (10/34)	1.35 (.63–2.87)	.439	0.98 (.44–2.16)	.952
Schistosoma mansoni					
Negative	26% (174/675)	1		1	
Positive	43% (3/70)	2.16 (.48–9.74)	.317	2.43 (.51–11.52)	.265
Hookworm					
Negative	27% (158/578)	1		1	
Positive	18% (19/104)	0.59 (.35–1.009)	.054	0.66 (.38–1.14)	.136

Logistic regression was used for statistical analysis. The presence of malaria parasitaemia was determined using rapid diagnostic tests. Helminth status was determined from a single stool sample using the Kato Katz method.

Abbreviations: CI, confidence interval; Coef, linear regression coefficient.

^aAdjusted for age, sex, malaria parasitaemia, *Schistosoma mansoni*, and hookworm infection status.

Table 5. Risk Factors Associated With Levels of KSHV DNA in Saliva (Continuous)

	Coef (95% CI)	PValue	Adjusted ^a Coef (95% CI)	PValue
Age group				
3–12	Ref		Ref	
13–25	–0.50 (–1.02 to .21)		–0.61 (–.13 to –.09)	
26–50	–0.31 (–.82 to .21)		–0.26 (–.80 to .28)	
50+	–0.78 (–1.35 to –.21)	.049	–0.76 (–1.40 to –.11)	.048
Sex				
Female	Ref		Ref	
Male	0.51 (.12–.89)	.010	0.46 (.05–.87)	.027
Malaria parasitaemia				
Negative	Ref		Ref	
Positive	0.20 (–.67 to 1.07)	.651	0.05 (–.82 to .92)	.909
Schistosoma mansoni				
Negative	Ref		Ref	
Positive	–0.58 (–2.12 to .96)	.460	–0.38 (–1.94 to 1.18)	.635
Hookworm				
Negative	Ref		Ref	
Positive	–0.36 (–.96 to .32)	.326	–0.37 (–1.03 to .29)	.270

Linear regression modelling was performed on \log_{10} transformed KSHV DNA levels for statistical analysis. The presence of malaria parasitaemia was determined using rapid diagnostic tests. Helminth status was determined from a single stool sample using the Kato Katz method. Abbreviations: CI, confidence interval; Coef, linear regression coefficient; KSHV, Kaposi's sarcoma-associated herpesvirus; Ref, reference.

^aAdjusted for age, sex, malaria parasitaemia, *Schistosoma mansoni*, and hookworm infection status.

The proportion of KSHV DNA detected in blood and saliva was highest in children, compared to adults. Previous studies have reported a high risk of KSHV seropositivity in children born to KSHV-seropositive mothers [13, 35, 49]. However, seropositivity in children whose mothers were seronegative has also been reported [49, 50]. Our current study suggests that siblings or playmates may also be a major source of transmission to uninfected children.

High viral loads among children could be associated with coinfections that are very prevalent in childhood, such as coinfection with malaria; we also observed the highest prevalence of malaria parasitaemia in the same age group. We showed that participants with malaria parasitaemia have higher levels of KSHV DNA in blood, compared to those uninfected with malaria. This is the first study to relate malaria parasitaemia directly with KSHV load in blood. We have previously reported associations between malaria (parasitaemia and antibodies) and KSHV seroprevalence [16, 32–34]. Results from the current study support a potential role of malaria in KSHV pathogenesis. The mechanisms for the association between malaria and KSHV viral load could include immunomodulation and dysfunction associated with repeated malaria infections [51, 52].

In the present study, males (both men and boys) were more likely to shed KSHV DNA in saliva and had higher levels of viral DNA, compared to females (women and girls). This is consistent with previous studies by us and others [31, 36, 38]. Sex differences in the immune controls of KSHV infections might contribute to the higher risk of KS in men [53, 54]. These findings warrant further study.

We previously reported that high KSHV K8.1 antibody titres were associated with and predictive of KS risk [55]. In the current study, we have observed that people with detectable viral DNA in both blood and saliva have higher IgG antibody levels for K8.1, but not for ORF73, when compared to individuals without detectable viral DNA. This association confirms our previous hypothesis [55]: increased lytic antibody levels reflect more frequent KSHV reactivation.

In summary, our data are consistent with high rates of KSHV transmission in rural Uganda. This might be partly attributed to parasite coinfections, such as coinfection with malaria, which interferes with immune control or makes uninfected children susceptible to infections. Studies investigating the mechanism through which malaria affects KSHV are required. Additionally, the characterization of protective immune responses to KSHV is needed to inform vaccine development and to develop strategies to lower KSHV transmission in endemic areas.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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