Determinants of γ-herpesvirus shedding in saliva among Ugandan children and their mothers.

Robert Newton[1,2#], Nazzarena Labo[3], Katie Wakeham [1,4], Vickie Marshall[3], Romin Roshan[3], Angela Nalwoga[1,5], Ismail Sebina[6], Lawrence Muhangi[1,5], Emily L. Webb[5], Wendell Miley [3], Rosemary Rochford[7,] Alison M. Elliott[1, 5#], Denise Whitby[3].

1. MRC/UVRI and LSHTM Uganda Research Unit, Uganda
2. University of York, UK
3. Viral Oncology Section, AIDS and Cancer Virus Program, Leidos Biomedical Inc. Frederick National Laboratory for Cancer Research, USA
4. Sussex Cancer Center, Brighton and Sussex University Hospitals NHS Trust, UK
5. London School of Hygiene & Tropical Medicine, UK
6. Department of Immunology, University of Washington, USA.
7. Department of Immunology & Microbiology, University of Colorado, USA

AE is the Principal Investigator of the study in which this project is based; RN is a Senior Visiting Scientist at the International Agency for Research on Cancer, Lyon, France.

Abstract

Introduction

Epstein Barr Virus (EBV) and Kaposi's sarcoma associated herpesvirus (KSHV) are transmitted via saliva but factors associated with salivary shedding are unknown.

Methods

We measured the shedding of both viruses in the saliva of ~500 Ugandan mothers and their six-year old children, testing all participants for EBV and KSHV seropositive individuals for KSHV.

Results

EBV and KSHV were shed by 72% and 22% of the mothers, and by 85% and 40% of children, respectively; boys were more likely than girls to shed KSHV (48% versus 30%), but not EBV. Children shed more KSHV and EBV than mothers, however salivary of loads EBV and KSHV were similar. KSHV shedding increased with increasing anti-KSHV (K8.1) antibodies in mothers and with decreasing anti-malarial antibodies both in mothers and children. Among mothers, 40% of KSHV shedders also shed EBV, compared to 75% of KSHV non-shedders; for children, it was 65% versus 83%.

Conclusions

In summary, in this population, individuals were more likely to shed EBV than KSHV in saliva; we have identified several factors, including child's sex, that influence KSHV shedding, and an inverse relationship between EBV and KSHV shedding, suggesting a direct or indirect interaction between the two viruses.

KEY WORDS: EBV, KSHV, saliva, shedding, Uganda

Introduction

Kaposi's sarcoma associated herpesvirus (KSHV) is a necessary cause of Kaposi's sarcoma (KS) [1, 2]. Unlike other human herpesviruses, KSHV is not ubiquitous across human populations; rather, the prevalence of infection shows considerable geographical variation, largely mirroring the variations seen in the incidence of KS. KSHV prevalence is relatively high in sub-Saharan Africa, lower in some Mediterranean countries and lowest in most northern European and Asian populations [3], suggesting, in part, difference in transmission across regions. The factors that sustain higher rates of transmission in sub-Saharan Africa compared to most other parts of the world are unclear. In Africa, primary infection begins in childhood and prevalence increases with age. KSHV is shed in saliva [1, 4, 5], and this is the primary mode of transmission[1]. Conversely, infection by the related gammaherpesvirus, Epstein Barr virus (EBV), which is also causes a number of human malignancies, is highly prevalent in all human populations[6]. Like KSHV, EBV is transmitted via saliva, but, in low income settings, infection generally occurs much earlier in childhood, compared to high income settings [7].

To investigate possible explanations for the differing epidemiology of these viruses, we compare here the prevalence and determinants of shedding of KSHV and EBV in saliva of apparently healthy people from a population in Uganda with high KSHV seroprevalence, on the assumption that viral shedding is an essential step in transmission [8]. We examined sociodemographic, clinical and serological factors, including exposure to helminths and malaria, as, in previous work within this cohort, we have found these factors to be associated with KSHV seroprevalence[9, 10].

Methods

This was a cross sectional study carried out within the context of a clinical trial, the Entebbe Mother and Baby study (EMaBS) (ISRCTN32849447). EMaBS is an ongoing birth cohort that originated as a double blind randomised placebo-controlled trial designed to determine the impact of helminth infections and their treatment on vaccine responses and infectious diseases outcomes; the details have been reported elsewhere [11, 12][3, 4]. A total of 2507 pregnant women from Entebbe, Uganda, who consented, were enrolled into EMaBS and they and their children continue to be followed. In 2010, we systematically recruited into this sub-study consenting HIV negative mothers and their children, seen sequentially in the follow-up clinic. Additional plasma samples, together with a saliva sample, were collected and stored from both mother and child. Three mothers who had seroconverted for HIV after enrolment in the original study were excluded.

Stored plasma samples from mothers, and from their 6-year old children, were tested for the presence of KSHV antibodies using two Enzyme-linked Immunosorbent Assays (ELISA) employing KSHV recombinant proteins, a lytic structural glycoprotein, K8.1 and the main latent nuclear protein, the latency- associated nuclear antigen (LANA) encoded by ORF73. Results are reported as optical densities (ODs). Each plate contained three positive and three negative controls; each assay cut-off was calculated based on the performance of the negative controls. This procedure has been reported elsewhere [13][5]. Individuals positive in either assay were considered KSHV seropositive.

The same plasma samples were tested for malaria antibodies using two *P. falciparum* antigens: merozoite surface protein (MSP)-1 and apical membrane antigen (AMA)-1 [14][6]. A pool of malaria positive plasma samples from patients known to be infected with malaria

was used to make standard dilutions. This pool was diluted serially five times starting from 1:50 for MSP-1 and 1:100 for AMA-1 to make 6 standards with a four-fold dilution increment. Blank wells were used to subtract background absorbance from the standards and the samples. ODs obtained were then exported into Microsoft Excel and antibody titres for each sample and each antigen were derived from the standard curve of ODs. This procedure has been reported elsewhere [15][7].

Saliva was collected by having participants rinse their mouth with alcohol-based mouthwash and subsequently discharge this fluid into a 50-mL conical tube. DNA was extracted using Qiagen blood and body fluids kit according to the manufacturer's instructions.

KSHV viral load was measured using a quantitative real-time PCR assay targeting the K6 gene [16, 17][9, 10]. Similarly, EBV viral load was determined using a quantitative real-time PCR specific for the EBNA1 gene [18][11]. Another real-time assay for the human endogenous retrovirus 3 (ERV-3), present at two copies in each diploid cell, was used to quantify cellular DNA [19][12]. For each real-time PCR assay, each sample was assayed in triplicate and an average of the three individual reactions was used to estimate the number of copies of the target gene. DNA viral load was then calculated as viral copies (genome equivalents) per million cells. Samples were designated qualitative positive if they could not be reliably quantitated, i.e. if all three replicates amplified over threshold, but the average viral copy number was less than three in the KHSV assay, or less than 10 in the EBV assay, or if the sample failed to amplify in one or two of the three triplicate reactions. Qualitative positive samples were retested, and if the results were confirmed, they were assigned the arbitrary value of one viral copy in further analyses. All mothers and children were tested for EBV viral load in saliva, while KSHV seropositive individuals were also tested for KSHV viral load.

Data analysis preparations involved generating binary outcome variables for KSHV viral load shedding, creating binary and categorical variables for demographic, socioeconomic and illness information, as well as serological variables including malaria antibody titres and KSHV antibody levels. For quantitative analyses, viral loads were log10-transformed.

Variables considered to be possible risk factors / confounders for shedding in children were sex of child, maternal age (categorised as 14-19, 20-24, 25-29, 30-34 and 35+), maternal education (none or primary, secondary, tertiary), parity (1, 2-4 or 5+ pregnancies), household social economic status, whether mother was shedding or not, helminthiasis, anaemia, anti-KSHV antibody levels (anti-K8.1 antigen and ORF73 antigen, tertiles), anti-malaria antibody levels (tertiles). For shedding in mothers, possible risk factors considered were age, education, household socioeconomic status, HIV infection, anaemia, helminthiasis, anti-KSHV and anti-malaria antibody levels. Because analysis on KSHV shedding is subset on KSHV seropositive individuals, anti-KSHV antibody tertiles include only values predefined as positive, whilst anti-malaria antibody levels include the entire range encountered in the sample.

Initial analysis involved generating descriptive statistics by cross-tabulating viral load shedding outcome variables and demographic, socioeconomic, illness and immunologic variables that were considered to be possible risk factors for shedding.

Logistic and linear regression models were fitted to examine variables predictive of shedding and viral load, respectively; nested modelling was used when twin children were included. Both prior knowledge from previous studies and a $p<0.05$ level of significance in univariate analyses were used to select factors to be included in multivariable analyses. Likelihood ratio tests were used to determine adjusted p-values. Analyses were conducted using STATA v. 13.1 (StataCorp, College Station, Texas, USA).

Ethics Statement

This study was approved by the Science and Ethics Committee (SEC) of the Uganda Virus Research Institute (UVRI), Uganda National Council for Science and Technology (UNCST) and the London School of Hygiene & Tropical Medicine (LSHTM) Research Ethics Committee. Written and verbal information was provided in English and the vernacular; informed consent was obtained according to the Declaration of Helsinki, and was recorded by signature or, in case of participant's inability to provide a signature, by thumbprint, as approved by the study's IRBs. In Uganda, minors who are married, pregnant or have children are considered emancipated, and do not require parent/guardian consent to participate in research, therefore all participating mothers provided consent autonomously. Consents for the participating children was given by their mothers, fathers or guardians.

Results

Amongst participants in the Entebbe Mother and Baby Study[11], we accrued for the present investigation 560 HIV-negative mothers and their 567 (including twins) six-year old children with KSHV serological data. Socio-demographic and other characteristics of the mothers at enrolment are shown in Table 1. Compared to the entire EMaBS cohort, mothers participating in this study were more likely to have higher education and income, and less likely to have anaemia.

Mothers were generally young (median 23 years, range 14-40), half had no education above primary school, and about a quarter had enrolled at their first pregnancy. Mothers were roughly distributed between lower (42.6%) and higher (57.4%) household socioeconomic status (SES).

Of 560 mothers and 567 of their six-year old children, whose serological data were available, 299 (53%) and 102 (22%), respectively were KSHV seropositive. KSHV DNA was detected in saliva twice as frequently in seropositive children (40%, 40/99 tested) as in seropositive mothers (21.5%, 64/297 tested); however, while 27/56 (48%) of boys had detectable KSHV VL, only 13/43 (30%) of girls did (Figure 1). Amongst KSHV shedders, median KSHV viral load was 3.6 logs copies (IQR 2.3-4.6) in mothers, versus 4.4 (IQR 3.5-4.7) in children (p=0.03); there was no significant difference by sex of the child (median 4.5 logs, IQR 3.5-4.9 in boys versus 4.4 logs, IQR 3.1-4.6 in girls).

EBV DNA was prevalent in saliva of both mothers (72%, 402/559 tested) and their children (85% 474/560 tested); prevalence was similar in boys (84%, 232/277) and girls (86%, 238/277) (Figure 1). However, the median viral load amongst EBV shedders was

significantly higher in children than in their mothers (median 4.7 logs, IQR 3.6-5.4, versus 3.9 logs, IQR 2.4-4.7; P<0.001) and did not differ significantly by sex of the child (4.6 logs, IQR 3.6-5.4 in boys versus 4.8 logs, IQR 3.6-5.4 in girls).

In multivariate analysis, among mothers (Table 2), a detectable KSHV VL was not associated with any demographic or clinical variable. KSHV shedding was directly associated with increasing levels of antibodies against KSHV K8.1 (medium and high optical density [OD], vs. low OD, Odds Ratio [OR] 80, 95% confidence interval, [CI] 11-560) and inversely associated with antibodies against *P. falciparum* AMA-1 (medium and high titres vs. low titres, OR 0.25, CI 0.07-0.86), but there was no association with level of antibodies against KSHV ORF73, nor with anti-*P.f.*MSP-1 antibodies. Detection of EBV DNA in mothers was not associated with any sociodemographic, clinical or serological factor.

Amongst seropositive mothers shedding KSHV (Table 3), no factor was associated with KSHV viral load. Similarly, no factors were associated with EBV VL in EBV shedders.

The children of mothers shedding KSHV were not more likely to be KSHV seropositive (crude OR, 1.63, CI 0.9-2.93; OR adjusted for maternal age, education, SES and sex of the child, 1.63, CI 0.9-2.99). In multivariate analysis, among children (Table 4), the odds of shedding KSHV were higher in boys versus girls (OR 2.22, CI 0.97-5.10), and in children with helminthiasis compared to those without (OR 7.63, CI 0.63-83.45), although the differences did not reach statistical significance, and the latter finding was based on only four children. The odds of shedding KSHV were inversely associated with antibodies against *P. falciparum* AMA-1 (medium and high titres vs. low titres, OR 0.11, CI 0.01-0.77), and tended to increase with higher anti-KSHV antibody levels, although there was no statistically significant association.

The odds of shedding EBV tended to be lower in children born to mothers 20 years and older (OR 0.3, CI 0.09-1.05) but were not otherwise associated with any sociodemographic, clinical or serological factor.

Among children shedding KSHV (Table 5), VL was higher in those who had helminths, (+2.1 log copies, CI +0.37 to +3.83) but was not associated with any other factor, whether demographic, clinical or serological.

EBV viral load was significantly higher in children in those borne of mothers with higher EBV VL (+0.15 log copies, CI 0.04-0.26) Furthermore, EBV viral load was lower in children with medium or high *P.f.*MSP-1 antibody levels (-1.05 log copies, CI -1.77 to -0.33).

Among KSHV-seropositive participants tested for both viruses, 57% shed only EBV, 13% only KSHV, 13% shed both viruses and 17% did not shed either virus. Among mothers, 40% of KSHV shedders also shed EBV, compared to 75% of those who did not shed KSHV; for children, it was 65% versus 83% (Table 1S). When examining the relationship between KSHV VL and EBV VL, restricting to KSHV-seropositive individuals, we found a negative correlation (ρ=-0.22, $p<0.0001$). The correlation strengthened amongst KSHV shedders (ρ=-0.30, $p<0.001$). However, when restricting to EBV shedders, a modest positive correlation was observed (ρ=+0.15, $p=0.01$). Stratifying mothers and children yielded similar results (except that the variance was larger in the latter case, because of the smaller number of KSHV- seropositive children).

Discussion

To our knowledge, this is the first study of factors associated with shedding of KSHV and EBV in saliva – a mechanism for viral transmission – in a sample of apparently healthy, HIV uninfected people, from a population in which both viruses are very prevalent and in which the tumours they cause, Kaposi's sarcoma and Burkitt's lymphoma, are endemic[20].

The prevalence of KSHV shedding was similar between mothers and their female children, but it tended to be higher in male children. When examining KSHV viral load in individuals who did shed, it was higher in children than in mothers, but similar in boys and girls. The reasons for greater prevalence, and presumably frequency, of shedding among male children, when compared to their mothers and to female children are not clear. In two other studies of adults and children from Uganda examining prevalence of KSHV viral load in blood, Mbulaiteye and colleagues showed that males were twice as likely to have detectable virus in blood when compared to females [18, 21, 22]. One of these studies [21] found no difference in the prevalence of detectable virus in saliva between boys and girls. However, in combination with the fact that, among people without HIV infection, Kaposi's sarcoma shows a marked excess incidence among men compared to women [23, 24], this might suggest that males are less able to control KSHV replication than females. Our results further suggest that such differential control may be established at an early age, suggesting that non-reproductive factors might be at play, whether genetic, or environmental due to early sex- or gender-specific exposures/ behaviours.

That many more children than mothers shed also suggests that children may be an important source of KSHV transmission both within the family and in the wider community. Further studies of KSHV transmission between children are warranted.

In mothers, we detected strong associations between KSHV shedding and anti-KSHV K8.1 antibody levels; this is consistent with the hypothesis that antibodies against the K8.1 antigen reflect lytic viral replication [25]. Such association in children was not significant. While children shed more frequently, it is conceivable that the shorter time since KSHV acquisition did not yet result in increase of KSHV- specific antibodies compared to non-shedders; alternatively, or additionally, in the course of recent infection in children, the antibody response profile might be different than during established infection in adults.

Viral load in peripheral blood mononuclear cells is more likely to be relevant to KSHV pathogenesis than viral load in saliva, and the correlation between it and anti-KSHV antibody levels will need to be investigated directly, yet, we can now confirm a previously observed association between anti-K8.1 antibody levels and KSHV reactivation, resulting in salivary shedding [8]. This further validates our previous observations on the possible role of anti-K8.1 antibody levels as a prognostic marker in the natural history of infection [25].

In previous work within this cohort, we have identified associations between KSHV prevalence and malaria parasitaemia in both mothers and children [9, 10]. More recently we found that KSHV prevalence was also associated with antibodies against *P. falciparum* malaria (both *P.f.*AMA-1 and *P.f.*MSP-1) in both mothers and children [26]. In this study, we find an association between *P.f.AMA*-1 levels and KSHV shedding in mothers, and *P.f.*MSP-1 levels and KSHV shedding in children, but not between these anti-malaria antibodies and

VL in shedders, either mothers or children. Further prospective research on the role of malaria in the natural history of KSHV infection is justified. In keeping with our previous findings, we also observed a sizable association between faecal detection of helminths in children with both KSHV shedding and viral load, although, for the former finding, the small number of affected children did not allow the association to reach significance. This also deserves further study in larger cohorts, especially in light of the recent observations that murine gammaherpesvirus 68 undergoes reactivation in latently infected mice acutely infected with intestinal helminths [27].

Consistent with earlier reports, a large majority of participants (adults and children of both sexes) were shedding EBV[28]. Such a high proportion of shedders explains to some extent why, contrary to KSHV, EBV is ubiquitous in this population from a very early age. A child had presumably a higher likelihood of exposure to EBV than to KSHV at any given time, even though we detected similar VL in shedders: in children, median KSHV VL was 4.4 log copies (IQR 3.5-4.7), while EBV VL was 4.7 logs (IQR 3.6-5.4); in mothers, KSHV VL was 3.6 logs (IQR 2.3-4.6), and EBV VL 3.9 logs, (IQR 2.4-4.7). Susceptibility to acquisition of either viral infection might also differ in the same individuals, in part because of the different role of exposure cofactors.

In mothers, no factor was associated with EBV shedding nor with viral load. In children, however, some maternal factors were significant. Children of older mothers, tended to shed less, emphasizing the possible role of environmental exposures unmeasured in our study. Likewise, children of mothers with high EBV VL tended to shed more themselves, again suggesting a common exposure or perhaps a genetic factor, also worthy of further examination. We have recently performed a genome wide association study (GWAS) in a population-based rural Ugandan cohort, and we have identified association between 5 novel

loci and anti-EBV antibody levels [29]. Finally, we have identified negative associations between *P.f.*MSP-1 (but not anti- *P.f.*AMA-1) antibody levels and EBV viral load in shedding children (but not mothers). The relationship between EBV and malaria infections has been investigated since the discovery of the virus[30], and very recent data provide interesting insight on the role of malaria in EBV-associated lymphomagenesis[31]; yet, like for KSHV, the contribution of malaria to EBV pathogenesis and natural history, must be further investigated throughout the lifespan.

For the first time, we also present data on the relationship between KSHV and EBV shedding in saliva. It is notable that individuals shedding KSHV tend to shed less EBV, while individuals shedding EBV tend to shed more KSHV. This suggests that there may be direct or indirect interaction between EBV and KSHV oropharyngeal replication, but the mechanisms controlling replication and shedding in saliva have not yet been investigated. Sparse in vitro data is available on dually-infected primary effusion lymphoma lines, showing KSHV inhibition by EBV [32], mutual inhibition by the two viruses [33] or differential transactivation of the two viruses by host immune factors [34], and even synergistic in vivo tumorigenicity in animal models [35]. Further investigation on the interactions between the two human gammaherpesviruses and their host is warranted.

In conclusion, this study investigates salivary shedding of KSHV and EBV infection in apparently healthy mothers and their children and, identifies for the first time several factors that influence shedding or salivary viral load, in the course of either or both infections. Our findings contribute to knowledge of transmission, epidemiology and natural history of EBV and KSHV infection in an East African population, in which the two viruses and the associated malignancies are endemic.

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Conflict of interest

All authors declare to have no association that may pose conflicts of interest.

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Authors' summary

Among ~500 Ugandan mother-child pairs, EBV was more likely shed in saliva than KSHV; several factors, including child's sex and parasitic infections, influenced viral shedding. EBV and KSHV shedding were inversely related, suggesting an interaction between the two viruses.

Correspondence to: Denise Whitby, AIDS and Cancer Virus Program, Leidos Biomedical Research Inc., Frederick National Laboratory for Cancer Research, Frederick MD 21702. whitbyd@mail.nih.gov

Alternate: Robert Newton, University of York, UK. robert.newton@york.ac.uk

Figure Captions

Figure 1. Salivary shedding in mothers and children. Unadjusted proportion of mothers and children of either sex who are shedding KSHV are estimated on KSHV seropositive individuals only, whilst prevalence of EBV shedding is estimated on the entire sample, as all assumed to be EBV seropositive

Table 1: Characteristics of participating mothers (N= 560)

Characteristics	Number[a]	%
Age group		
14-19	112	20.0%
20-24	222	39.7%
25-29	136	24.3%
30-34	62	11.1%
35+	27	4.8%
Education		
None/primary	264	47.2%
Secondary	232	41.5%
Tertiary	63	11.3%
Marital status		
Single/divorced/separated/widower	70	12.5%
Married	488	87.5%
Mother's monthly income (UGS)[b]		
<30,000	428	80.6%
>= 30,000	103	19.4%
Household socioeconomic status		
Lower	234	42.6%
Higher	314	57.4%
Number of pregnancies		
1	139	24.9%
2-4	324	58.0%
5+	97	17.2%

[a] Numbers do not add to total because of missing data, percentages refer to total. [b] 30,000 Ugandan Shillings (UGS) corresponds to the median national income.

Table 2. Factors associated with shedding of KSHV and EBV in saliva among mothers

Factor	KSHV Shedding						EBV Shedding					
	N[a]	%	aOR[b]	95%	CI[b]	p[d]	N	%	aOR	95%	CI	p
Age												
14-19	14/73	19.2%	Ref.			n.s.[e]	80/112	71.4%	Ref.			n.s.
20-24	24/116	20.3%	1.09	0.52	2.29		162/221	73.3%	1.10	0.66	1.85	
25-29	16/65	24.6%	1.42	0.62	3.26		93/136	68.4%	0.89	0.50	1.57	
30-34	8/29	26.7%	1.47	0.53	4.06		45/62	72.6%	1.05	0.52	2.14	
35+	2/14	14.3%	0.72	0.14	3.65		21/27	77.8%	1.67	0.57	4.87	
Education												
Primary/none	36/147	24.2%	Ref.			n.s.	191/264	72.0%	Ref.	0.83		n.s.
secondary	22/123	17.7%	0.69	0.38	1.27		167/231	72.3%	1.00	0.66	1.49	
tertiary	6/27	22.2%	0.91	0.33	2.51		44/63	69.8%	0.89	0.47	1.69	
Household SES												
lower	29/154	18.8%	Ref.				168/234	71.8%	Ref.			
higher	35/140	24.5%	0.70	0.40	1.24	n.s.	228/314	72.6%	1.05	0.71	1.55	n.s.
Anaemia												

No	35/188	18.6%	Ref.				269/377	71.4%	Ref.			
Yes	29/109	25.9%	1.60	0.90	2.83	n.s.	132/181	72.9%	1.13	0.75	1.70	n.s.
Anti-KSHV antibodies OD												
ORF73												
Low	22/99	22.0%	Ref.			n.s.	34/99	34.3%				n.s.
Medium	16/99	15.0%	0.65	0.31	1.34		27/99	27.3%	1.00	0.53	1.90	
High	26/99	27.0%	1.20	0.61	2.34		33/99	33.3%	0.71	0.38	1.31	
K8.1												
Low	5/98	5.0%	Ref.			<0.001	29/99	29.6%				n.s.
Medium	25/98	25.0%	7.22	2.58	20.24		30/100	30.0%	1.36	0.73	2.53	
High	34/98	34.0%	10.61	3.87	29.12		35/99	35.4%	1.16	0.63	2.14	
Malaria antibody titres												
P.f.AMA-1												
Low	23/81	28.4%	Ref.			0.02	136/183	74.3%	Ref.			
Medium	21/104	20.2%	0.58	0.29	1.17		132/184	71.7%	0.87	0.54	1.39	n.s.
High	17/110	15.5%	0.39	0.19	0.82		129/186	69.7%	0.83	0.52	1.33	
P.f.MSP-1												
Low	20/79	25.3%	Ref.			n.s.	136/181	75.1%	Ref.			n.s.

Medium	20/105	19.0%	0.60	0.29	1.25	140/185	75.7%	1.04	0.64	1.70
High	21/111	18.9%	0.58	0.28	1.19	121/186	65.1%	0.60	0.38	0.96

[a]KSHV seropositive individuals only. [b]Adjusted odds ratio (aOR) are estimated from multivariate logistic regression models that include age, education, household socioeconomic status; the first three covariates are adjusted for the other two. [c] CI, Confidence Interval. [d]p values for ORs are from likelihood ratio tests. [e]$p<0.05$

Table 3. Factors associated with KSHV and EBV viral load (VL) in saliva of shedding mothers

	KSHV VL				EBV VL			
	[a]Coeff.	95%	CI[b]	p[c]	Coeff.	95%	CI	p
N	64				404			
Age								
14-19	Ref.			n.s.[e]	Ref.			n.s.
20-24	-0.36	-1.61	0.90		-0.17	-0.69	0.34	
25-29	0.14	-1.24	1.52		-0.39	-0.98	0.19	
30-34	-0.71	-2.36	0.94		-0.20	-0.90	0.50	
35+	0.71	-2.17	3.59		-0.13	-1.05	0.79	
Education								
Primary/none	Ref.			n.s.	Ref.			n.s.
secondary	0.78	-0.23	1.79		0.24	-0.17	0.64	
tertiary	1.35	-0.31	3.01		0.14	-0.51	0.79	
Household SES	-0.03	-0.98	0.93	n.s.	-0.22	-0.60	0.17	n.s.
Anaemia	-0.23	-1.22	0.76	n.s.	-0.01	-0.41	0.40	n.s.
anti-KSHV antibodies OD								
K8.1								
Low	Ref.			n.s.	-			
Medium	0.83	-1.05	2.71		-			
High	0.94	-0.89	2.77		-			
ORF73								
Low	Ref.			n.s.	-			
Medium	-0.81	-2.08	0.47		-			
High	0.46	-0.70	1.62		-			

Anti-malaria antibodies OD								
P.f.AMA-1								
Low	Ref.			n.s.	Ref.			n.s.
Medium	0.19	-0.98	1.36		0.26	-0.20	0.72	
High	0.19	-1.08	1.46		0.28	-0.18	0.73	
P.f.MSP-1								
Low	Ref.			n.s.	Ref.			n.s.
Medium	-0.09	-1.35	1.16		0.14	-0.31	0.59	
High	0.06	-1.17	1.29		0.36	-0.10	0.83	

[a]Coefficients expressing variation in log10 GE/million cells are estimated in shedding individuals only, from multivariate models that include age, education and household SES; first three covariates adjusted for the other two. [b]CI, Confidence Interval. [c]p values for coefficients are from likelihood ratio tests. [e]$p<0.05$

Table 4. Factors associated with shedding of KSHV and EBV in saliva among children

Factor	KSHV Shedding						EBV Shedding					
	N[a]	(%)	aOR[b]	95%	CI[b]	p[b]	N	(%)	aOR	95%	CI	p
Sex												
F	13/43	30.2%	Ref.				238/277	85.9%	Ref.			
M	27/56	48.2%	2.15	0.93	4.96	n.s.[e]	232/277	83.8%	0.72	0.30	1.73	n.s.
Maternal Age												
14-19	6/14	42.9%	Ref.			n.s.	100/110	90.9%	Ref.			0.05
20-24	15/36	41.7%	0.89	0.25	3.18		186/224	83.0%	0.28	0.07	1.09	
25-29	10/29	34.5%	0.72	0.19	2.73		111/131	84.7%	0.36	0.08	1.52	
30-34	6/11	54.5%	1.36	0.27	6.92		54/63	85.7%	0.42	0.07	2.34	
35+	3/9	33.3%	0.54	0.09	3.19		20/27	74.1%	0.10	0.01	0.98	
Parity												
1	12/22	54.5%	Ref.			n.s.	114/135	84.4%	Ref.			n.s.
2-4	16/55	29.6%	0.37	0.13	1.06		279/324	86.1%	1.27	0.45	3.62	
5+	12/23	52.2%	0.90	0.27	2.94		78/96	81.3%	0.66	0.17	2.52	
Maternal shedding												
No	29/68	42.6%	Ref.				124/150	82.7%				

Yes	4/17	23.5%	0.39	0.11	1.33	n.s.	338/396	85.4%	1.23	0.74	2.04	n.s.
Anaemia												
No	29/77	37.6%	Ref.				367/430	85.3%	Ref.			
Yes	11/22	50.0%	1.51	0.57	3.98	n.s.	107/130	82.3%	0.63	0.23	1.76	n.s.
Helminths												
No	15/41	36.5%	Ref.				251/287	87.6%	Ref.			
Yes	3/4	75.0%	7.23	0.63	83.45	n.s.	14/18	77.8%	0.18	0.01	5.52	n.s.
***anti*-KSHV antibodies OD**												
ORF73												
Low	11/33	33.3%	Ref.			n.s.	9/33	27.3%	Ref.			n.s.
Medium	12/33	36.4%	1.28	0.45	3.63		9/33	27.3%	0.86	0.28	2.65	
High	17/33	51.5%	2.60	0.92	7.38		6/33	18.2%	1.39	0.41	4.66	
anti-K8.1												
Low	12/33	36.4%	Ref.			n.s.	4/33	12.1%	Ref.			n.s.
Medium	9/33	27.3%	0.55	0.19	1.61		11/33	33.3%	0.33	0.09	1.23	
High	19/33	57.6%	2.06	0.75	5.70		9/33	27.3%	0.45	0.12	1.71	
***anti*-Malaria antibody titres**												
P.f.AMA-1												

Low	11/24	45.8%	Ref.			n.s.	157/184	85.3%	Ref.			
Medium	11/25	44.0%	1.02	0.32	3.23		152/186	81.7%	0.58	0.20	1.69	n.s.
High	17/48	35.4%	0.67	0.24	1.86		157/182	86.3%	1.06	0.37	3.05	
P.f.MSP-1												
Low	14/23	60.9%	Ref.			0.03	157/186	84.4%	Ref.			n.s.
Medium	12/36	33.3%	0.32	0.11	0.97		161/185	87.0%	1.46	0.50	4.27	
High	13/38	34.2%	0.33	0.11	1.00		148/181	81.8%	0.65	0.23	1.85	

[a]KSHV seropositive individuals only. [b]Sex -adjusted odds ratio (aOR), except when sex is the factor. [c] CI, Confidence Interval. [d]p values for ORs are from likelihood ratio tests. [e]$p<0.05$

Table 5. Factors associated with KSHV and EBV viral load in saliva of shedding children.

	KSHV VL				EBV VL			
	Coeff. [a]	95%	CI[b]	p[c]	Coeff.	95%	CI	p
N		40				477		
Male Sex	-0.10	-1.19	0.99	n.s.[e]	0.20	-0.14	0.56	n.s.
Maternal Age								
14-19	Ref.			n.s.	Ref.			n.s.
20-24	1.03	-0.53	2.60		-0.05	-0.52	0.42	
25-29	1.22	-0.46	2.89		-0.17	-0.69	0.35	
30-34	0.34	-1.52	2.21		-0.34	-0.98	0.30	
35+	2.05	-0.29	4.39		-0.23	-1.16	0.70	
Parity	Ref.			0.05	Ref.			n.s.
2-4	1.29	0.09	2.50		-0.25	-0.67	0.17	
5 or more	0.91	-0.36	2.19		-0.20	-0.76	0.36	
Maternal VL [d]	-3.83	-16.54	8.89	n.s.	0.15	0.04	0.25	0.007
Anaemia	0.09	-1.07	1.25	n.s.	-0.21	-0.62	0.21	n.s.
Helminths	2.10	0.37	3.83	0.02	-0.16	-1.26	0.93	n.s.
K8.1 OD								
Low	Ref.			n.s.	-			
Medium	-0.14	-1.60	1.32		-			
High	0.63	-0.67	1.93		-			
ORF73 OD								
Low	Ref.			n.s.	-			

Medium	0.54	-0.83	1.90		-			
High	0.09	-1.22	1.39		-			
pfAMA-1 OD								
Low	Ref.			n.s.	Ref.			n.s.
Medium	0.63	-0.79	2.05		-0.19	-0.62	0.25	
High	0.11	-1.22	1.45		-0.08	-0.51	0.35	
pfMSP-1 OD								
Low	Ref.			n.s.	Ref.			0.01
Medium	-0.35	-1.78	1.07		-0.37	-0.75	0.02	
High	-0.12	-1.47	1.24		-0.55	-0.97	-0.12	

[a]Coefficients expressing variation in log10 GE/million cells are estimated in shedding individuals only and sex adjusted, except when sex is the factor. [b]CI, Confidence Interval. [c]p values for coefficients are from likelihood ratio tests. [d] Corresponding maternal VL scaled in log10 copies increments. [e]$p<0.05$

Figure 1.