Relationship between anaemia, malaria co-infection and Kaposi Sarcoma-associated Herpesvirus (KSHV) seropositivity in a population-based study in rural Uganda

Running title: Anaemia, malaria co-infection and KSHV seropositivity

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Summary

KSHV seroprevalence is very high in rural Uganda. Malaria infection and anaemia are the risk factors associated with KSHV seroprevalence and antibody levels in this study. These factors might cause reactivation of the virus and hence lead to increased transmission.

Abstract

We examined anaemia and malaria as risk factors for KSHV seropositivity and antibody levels in a long-standing rural Ugandan cohort, in which KSHV is prevalent. Samples from 4134 children, aged 1-17 years, with a sex ratio of 1:1 and 3149 adults aged 18-103 years, 41% of whom were males, were analysed. Among children, malaria infection was associated with higher KSHV prevalence (61% versus 41% prevalence among malaria infected and uninfected respectively); malaria was not assessed in adults. Additionally, lower haemoglobin level was associated with an increased prevalence of KSHV seropositivity, both in children and in adults.

Keywords: Kaposi's sarcoma herpesvirus antibodies, rural population, anaemia, malaria

Introduction

We recently reported KSHV prevalence of 95% among adults in a rural population cohort (the General Population Cohort (GPC) in Uganda [1], the highest prevalence of KSHV ever reported, in addition to high KSHV antibody levels. We propose that the very high prevalence in this population may be driven by frequent KSHV reactivation, viral shedding and transmission rates and that the high antibody levels also reflect frequent reactivation. It is important therefore to study potential co-factors for reactivation in relation to KSHV prevalence and antibody levels. We have previously reported an association between malaria and hookworm infections and KSHV seropositivity in an urban population in Uganda [2, 3]. Since both malaria and hookworm are associated with anaemia, we hypothesized that anaemia may have a role in KSHV transmission via viral reactivation. This hypothesis is supported by data from *in vitro* experiments showing reactivation of KSHV in conditions of hypoxia [4]. In this study we aimed to confirm the high prevalence of KSHV in the GPC in recent years, with higher ART coverage, and determine the role of anaemia and malaria co-infection as risk factors for KSHV prevalence and antibody levels in a highly endemic population.

Methods

Study population and socio-demographic data collection

The General Population Cohort (GPC) is located in south-western Uganda in Kyamulibwa sub-community of Kalungu district with an altitude of approximately 1200m above sea level. It is community-based open cohort of about 22,000 people in 25 adjacent villages [5]. This cross sectional study analysed plasma samples collected from two surveys, the adult survey and the children survey. The adults were surveyed in 2014/2015 and the children in 2016. Adults without haematological parameter data and children without either haematological parameter data or

malaria parasitaemia status data were excluded in the laboratory analysis. Children less than 1 year of age were excluded from the statistical analysis and children less than 2 years were not tested for HIV serostatus, due to the potential for maternal IgG to be present, which could affect antibody measurement. Socio-economic scores were generated for adults using Principal Component Analysis of various household indicators during the previous survey.

Ethical approval

The study was approved by the Research and Ethics Committee Uganda Virus Research Institute and the Uganda National Council for Science and Technology.

Haematological and serological analysis

During these two surveys, blood was collected from study participants and tested immediately after collection for HIV; a smaller proportion of samples were also tested for malaria parasitaemia and haemoglobin levels, using point-of care assays and rapid tests. HIV serostatus was determined using rapid diagnostic tests. Malaria parasitaemia was measured in children only, using malaria Rapid Diagnostic Tests (ONE STEP Malaria HRP-II (P.f) and pLDH (Pan) Antigen Rapid Test). Haemoglobin levels in g/dL were obtained from the Haemocue 201 analyzer.

Stored plasma samples for both children and adults were retrieved and tested for KSHV antibodies using an in-house ELISA as previously described [6]. Samples from the two surveys were tested separately after simple randomisation onto ELISA plates. Antibodies to both K8.1 and ORF73 proteins were measured as optical density. Each ELISA plate contained three negative and positive control wells; negative controls were used to calculate a cut-off value for every plate as previously described [2, 7]. Seropositivity was defined as reactivity to either K8.1 or ORF73 proteins.

Statistical analysis

Statistical analysis was carried out using STATA13 (Statacorp, College Station, Texas USA). Children's and adults' results were analysed separately. Haemoglobin levels were mean centred for easier interpretation. Anaemia was defined using haemoglobin levels in g/dL after altitude adjustment following WHO guidelines [8]. A constant value 0.5 was subtracted from haemoglobin levels for altitude adjustment, the results were then categorised into normal and anaemic using the following cut-off values: 11.0 for pregnant females and children below 5 years, 11.5 for children 5 to 11 years, 12.0 for children 12 to 14 years and other females 15 years and above, 13.0 for males 15 years and above. These WHO haemoglobin reference ranges used to define anaemia may not be representative of African populations, as previously reported [9, 10], because they are based on western population data. We therefore analysed haemoglobin both as a continuous variable and as categorised into normal and anaemic using separate regression models.

Linear regression with bootstrapped confidence intervals was used for antibody levels analysis, because they were severely skewed. Logistic regression was used for seroprevalence analysis, furthermore, we adjusted for clustering at the village level using survey commands. We assessed interaction between age and haemoglobin levels, as well as between age and anaemia in relation to anti-K8.1 antibody levels, anti-ORF73 antibody levels and KSHV prevalence based on *a priori* suspicions of interaction, using likelihood ratio tests.

Results

The characteristics of the individuals analysed are shown in supplementary Table 1. We analysed results from 3149 adults and 4134 children. This analysis included children aged 1 to 17 years and adults aged 18 to 103 years (supplementary Table 1).

Risk factors for KSHV prevalence and antibody levels among adults

KSHV prevalence was 91% in all adults (2871/3149) (supplementary Figure 1). Every 1g/dL decrease in haemoglobin values was associated with increased odds of being KSHV seropositive (OR=0.86 (0.77, 0.96), P=0.006 and anaemic individuals were more likely to be KSHV seropositive compared to people with normal haemoglobin values, but this association was not statistically significant OR=1.25 (0.87, 1.79), P=0.229 (Table 1A).

We then analysed antibody levels to K8.1 and ORF73 proteins as continuous variables without categorising participants as seropositive or seronegative. Anaemic adults had higher antibodies to ORF73 protein compared to individuals with normal haemoglobin values (coef. 0.28 (0.16, 0.39), P<0.0001. Similarly, every 1g/dL decrease in haemoglobin was associated with an increase in ORF73 antibody ODs (Table 1B). The association between haemoglobin and antibodies to ORF73 protein was strongest among older people (Table 1C). Conversely, anti-K8.1 antibody levels were not significantly associated with either haemoglobin levels or anaemia (Table 1B). This might be attributed to the relative abundance of LANA compared to late lytic proteins such as K8.1, even during KS disease [11].

Compared to HIV negative adults, HIV positive adults had lower antibodies to KSHV, especially those with CD4 counts of 500cell/μL or less (Table 1B). This may be due to B cell dysfunction caused by HIV infection, and consequent decreased antibody responses [12, 13]. On the other hand, HIV positive adults with CD4 counts above 500cells/μL, compared to HIV negative adults were more likely to be KSHV seropositive (Table 1A), which may be due to antiretroviral treatment.

Risk factors for KSHV prevalence and antibody levels among children

We then investigated associations between KSHV prevalence and antibody levels and risk factors among children. Overall, KSHV prevalence was 51% (2117/4134) in the children, the prevalence

increased with age, rising from 31% among 1-5 year olds, to 53% among 6-12 year olds, to 73% among 13-17 year olds (supplementary Figure 1). We first adjusted for HIV status, age and sex, then malaria parasitaemia and anaemia/haemoglobin levels were added in the full models. In the first analysis, haemoglobin levels, malaria parasitaemia and age were strongly associated with KSHV prevalence (Table 2A). Every 1 g/dL decrease in haemoglobin levels increased the odds of being KSHV seropositive by 11% (P<0.0001) and the odds of being KSHV positive if anaemic compared to with normal haemoglobin levels was 1.42 (1.18, 1.71) (p<0.0001). The odds of being KSHV seropositive, if malaria infected, compared to the uninfected were 2.26 (1.85, 2.77) (P<0.0001) and every annual increase in age was associated with a 17% increased risk of being KSHV seropositive (Table 2A).

After adjusting for malaria parasitaemia, the risk of being KSHV seropositive for every 1 g/dL decrease in haemoglobin reduced to 7% (p=0.005). Similarly, the odds of being KSHV seropositive in comparing anaemic individuals to people with normal haemoglobin levels reduced to 1.23 (1.01, 1.49) p=0.037. After adjusting for haemoglobin levels, the odds of being KSHV seropositive comparing people with and without malaria parasitaemia changed little (OR=2.12 (1.75, 2.57), p<0.0001) (Table 2A). Every annual increase in age remained strongly associated with increased KSHV prevalence risk, OR=1.18 (P<0.0001) even after adjusting for malaria parasitaemia and haemoglobin (Table 2A).

We then finally investigated associations between the same risk factors and KSHV antibody levels (OD) as continuous variables without categorising participants as seropositive or seronegative. Only malaria parasitaemia was associated with both anti-K8.1 and anti-ORF73 antibody levels in the fully multivariate analysis (Table 2B).

Discussion

We observed a significant association between haemoglobin levels and KSHV prevalence among children and adults, where people with low levels of haemoglobin were more likely to be KSHV seropositive. As a categorical variable, anaemia was associated with KSHV prevalence among children. Reduction in haemoglobin has been shown to cause hypoxia/low tissue oxygen, while hypoxia has been shown to reactivate KSHV *in-vitro* [4]. We therefore hypothesise that low haemoglobin levels leads to reactivation of KSHV through hypoxia. Increased reactivation may help spread the virus during initial infection. Alternately, hypoxia may enhance initial infection of cells, possibly through upregulation of the replication and transcription activator [14]. In this cross sectional study, we did not directly measure KSHV reactivation or KSHV viral load in blood or plasma, although antibody levels may be viewed as a surrogate marker for frequent reactivation. The connection between KSHV reactivation, hypoxia and anaemia requires further investigation.

We showed that children infected with malaria are more likely to be KSHV seropositive. Additionally, the effect of anaemia and/or haemoglobin levels on KSHV prevalence and antibody levels reduced to about 50% after adjusting for malaria infection. Malaria causes anaemia, and in part the anaemia effect in children could be explained (confounded) by malaria infection. The consistent association between malaria infection and KSHV prevalence suggests malaria may be driving KSHV transmission in malaria endemic areas. This might imply that exposure to malaria significantly impacts on KSHV reactivation, which might also have long lasting effects. The mechanism through which malaria may reactivate KSHV requires further investigation.

Conclusion

Findings from this study suggest malaria infection as a risk factor for KSHV prevalence. Malaria associated anaemia is one mechanism that likely contributes to this association but cannot entirely

explain it. In KSHV and malaria endemic areas, a number of other parasite co-infections such as helminths, which cause anaemia and/or immunomodulation are common. The role of multiple parasitic infections and KSHV transmission and pathogenesis warrants further careful study.

Footnote page

All authors have declared that they have no association which may cause any conflict of interest.

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Table 1: Risk factors for KSHV prevalence and antibody levels among adults 18 to 103 years.

1A: Risk factors for KSHV prevalence

Risk factor	KSHV prevalence	Adjusted [a] Odds Ratio (95% CI)	P value
Age		1.01 (1.003, 1.02)	0.010
Sex			
Males	94% (1218/1297)	1	
Females	89% (1653/1852)	0.61 (0.46, 0.80)	0.001
HIV and CD4 count status			
(-) CD4 count unknown	92% (2615/2841)	1	
(+) CD4 count > 500	70% (32/46)	0.16 (0.10, 0.26)	<0.0001
(+) CD4 count ≤ 500	90% (76/84)	0.61 (0.16, 2.36)	0.451
(+) CD4 count unknown	83% (146/175)	0.53 (0.29, 0.96)	0.039
Haemoglobin levels [b]		0.86 (0.77, 0.96)	0.006
Anaemia [c]			
Normal	91% (2153/2370)	1	
Anaemic	92% (718/779)	1.25 (0.87, 1.79)	0.229

1B: Risk factors for anti-K8.1 and anti-ORF73 antibody levels

	K8.1		ORF73	
Risk factor	Adjusted [a] Coef. [d] (95% CI)	P value	Adjusted [a] Coef. [d] (95% CI)	P value
Age	0.004 (0.001, 0.007)	0.015	0.01 (0.007, 0.013)	<0.0001
Sex				

Males	Ref			
Females	-0.25 (-0.35, -0.14)	<0.0001	-0.24 (-0.34, -0.14)	<0.0001
HIV and CD4 count status				
(-) CD4 count unknown	Ref		Ref	
(+) CD4 count > 500	-0.77 (-1.21, -0.33)	0.001	-0.71 (-1.15, -0.29)	0.001
(+) CD4 count ≤ 500	-0.33 (-0.64, -0.02)	0.036	-0.48 (-0.79, -0.17)	0.003
(+) CD4 count unknown	-0.28 (-0.51, -0.06)	0.014	-0.38 (-0.59, -0.16)	0.001
Haemoglobin levels[b]	-0.03 (-0.06, 0.01)	0.119		
Anaemia[c]				
Normal	Ref			
Anaemic	0.007 (-0.11, 0.13)	0.910	0.28 (0.16, 0.39)	<0.0001

1C: Age group specific association between anti-ORF73 antibody levels and haemoglobin levels

	Age group	Adjusted[a] Coef. (95% CI)	P value	Interaction P value
Haemoglobin levels[b]	18-24	-0.09 (-0.16, -0.02)	0.009	
	25-44	-0.08 (-0.12, -0.03)	0.001	
	45-103	-0.17 (-0.21, -0.12)	<0.0001	0.007

[a] adjusted for age, sex, HIV status and household socio-economic status, CI: confidence interval. Anti-K8.1 and anti-ORF73 antibody levels (measured as optical density) were obtained from ELISA. KSHV seropositivity was defined as reactivity to either K8.1 or ORF73 antigens. [b]haemoglobin levels were mean (13.7 g/dL) centred. [c]haemoglobin levels were altitude adjusted and categorised into normal and anaemic following WHO guidelines. [d]Coef.: regression coefficient, CI: confidence interval. Logistic regression, allowing for the survey design was used for statistical analysis of risk factors for KSHV prevalence. Linear regression with bootstrapped confidence intervals was used for statistical analysis of risk factors for antibody levels. Table 1C: Age group specific associations between ORF73 antibody levels and haemoglobin levels were reported due to interaction between age and Haemoglobin levels. CD4 counts were measured in cells/μL.

Table 2: Risk factors for KSHV prevalence and antibody levels among children aged 1 to 17 years.

2A: Risk factors for KSHV prevalence

		Adjusted[a]		Adjusted[b]	
Risk factor (n)	KSHV prevalence	OR (95% CI)	P value	OR (95% CI)	P value
Haemoglobin levels (3199)[c]		0.89 (0.84, 0.93)	<0.0001	0.93 (0.88, 0.98)	0.005
Anaemia[d]					
Normal	44% (1143/2584)	1			
Anaemic	48% (294/615)	1.42 (1.18, 1.71)	<0.0001	1.23 (1.01, 1.49)	0.037
Malaria					
Negative	41% (1088/2630)	1		1	
Positive	61% (348/569)	2.22 (1.84, 2.69)	<0.0001	2.13 (1.75, 2.58)	<0.0001
Age (4134)		1.17 (1.15, 1.18)	<0.0001	1.18 (1.15, 1.21)	<0.0001

Sex					
Boys	52% (1076/2061)	1		1	
Girls	50% (1041/2073)	0.93 (0.82, 1.07)	0.27	0.94 (0.82, 1.07)	0.33
HIV					
Negative	53% (2030/3812)	1		1	
Positive	40% (19/48)	0.49 (0.25, 0.96)	0.04	0.55 (0.30, 1.03)	0.06

2B: Risk factors for antibody levels

	K8.1				ORF73			
	Adjusted[a]		Adjusted[b]		Adjusted[a]		Adjusted[b]	
Risk factor (n)	Coef[e]. (95% CI)	P value	Coef. (95% CI)	P value	Coef. (95% CI)	P value	Coef. (95% CI)	P value
Malaria								
Negative (2630)	ref		ref		ref		ref	
Positive (569)	0.27 (0.18, 0.38)	<0.0001	0.26 (0.16, 0.37)	<0.0001	0.30 (0.21, 0.38)	<0.0001	0.26 (0.18, 0.34)	<0.0001
Age (4134)	0.08 (0.07, 0.09)	<0.0001	0.1 (0.08, 0.11)	<0.0001	0.06 (0.05, 0.07)	<0.0001	0.05 (0.04, 0.06)	<0.0001

Haemoglobin levels (3199)[c]	-0.04 (-0.06, -0.01)	0.003	-0.02 (-0.05, 0.01)	0.2	-0.03 (-0.06, -0.01)	0.001	-0.02 (-0.04, 0.005)	0.13
Anaemia[d]								
Normal	ref		ref		ref		ref	
Anaemic	0.04 (-0.05, 0.13)	0.414	-0.02 (-0.12, 0.07)	0.649	0.12 (0.05, 0.19)	0.001	0.07 (-0.01, 0.14)	0.073
Sex								
Boys (2061)	ref		ref		ref		ref	
Girls (2073)	-0.05 (-0.11, 0.02)	0.17	-0.01 (-0.09, 0.06)	0.75	0.01 (-0.04, 0.07)	0.63	-0.01 (-0.07, 0.05)	0.66
HIV								
Negative (3808)	ref		ref		ref		ref	
Positive (270)	-0.24 (-0.56, 0.07)	0.13	-0.12 (-0.48, 0.24)	0.5	0.20 (-0.17, 0.57)	0.29	0.29 (-0.13, 0.72)	0.18

[a]adjusted for age, sex and HIV status and [b]adjusted for age, sex, HIV status, haemoglobin and malaria parasitaemia. Logistic regression, allowing for survey design was used for statistical analysis of risk factors and KSHV prevalence. OR: Odds Ratio, [e]Coef.: regression coefficient CI: confidence Interval. ORF73 and K8.1 antibody levels (measured as optical density) were obtained from ELISA. KSHV prevalence defined as antibody reactivity to either K8.1 or ORF73 antigens. HIV status obtained using rapid diagnostic tests. Malaria parasitaemia determined using rapid diagnostic tests. [c]haemoglobin levels were mean (13.0 g/dL) centred. [d]haemoglobin levels were altitude adjusted and categorised into normal and anaemic following WHO guidelines. Linear regression with bootstrapped confidence interval used in the analysis of risk factors for KSHV antibody levels.