Risk factors for Kaposi's sarcoma associated herpesvirus (KSHV) DNA in blood and in saliva in rural Uganda

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Summary: HHV8 shedding in saliva is associated with viral transmission while viral detection in blood is associated with disease pathogensis. Our data shows that children may be a major transmission source and malaria could play a role in susceptibility to infection.

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Abstract

Introduction

Detectable KSHV DNA in blood and increased antibody titres may indicate KSHV reactivation, while transmission of KSHV occurs via viral shedding in saliva.

Methods

We investigated risk factors for KSHV DNA detection by real-time PCR, in blood and 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 malaria parasitaemia was identified using rapid diagnostic tests. Regression modelling was used for statistical analysis.

Results and discussion

KSHV viral load in blood did not correlate with viral load in saliva, suggesting separate immunological control within each compartment. The proportion of individuals with detectable virus in blood was 23% among children aged 3-5 years , 22% among 6-12 years old, thereafter reducing with increasing age. The proportion of individuals with detectable virus in saliva increased from 30% in 3-5 year old children to 45% in those aged 6-12 and decreasing subsequently with increasing age. Overall, 29% of males shed in saliva compared to 19% of females (p = 0.008). 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 blood (p = 0.031) compared to malaria uninfected individuals. This suggests that malaria may lead to KSHV reactivation, thereby increasing transmission and pathogenicity of the virus.

Key words: Kaposi’s sarcoma herpesvirus DNA; Risk factors; Uganda

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Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV) causes Kaposi's sarcoma (KS), multicentric Castleman disease (MCD) and primary effusion lymphoma (PEL) (1-6). 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), for instance, we have reported KSHV infections in children as young as one 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). KSHV-associated oncogenesis and progression of dieases, 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 of AIDS-KS patients with cART has been shown to reduce KSHV load in blood to undetectable levels (18, 22). Determinants of KSHV DNA detection in blood among KSHV seropositive people in the general population is not well understood. Viral load in plasma and PBMCs has been reported mainly in high-risk groups such as HIV infected individuals and patients with KSHV related diseases (18, 23-27). A few studies have reported KSHV viral load in blood donors (adults) in non-endemic areas (28-30) and a study reported plasma viral load 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,

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36), but no study has investigated KSHV viral load 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 from the General Population Cohort (GPC) – a longstanding cohort in rural south-western 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 General Population Cohort (GPC). The GPC is a community-based cohort of 22,000 people in 25 adjacent villages in south-western 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)), HIV negative individuals aged three to eighty-nine years. Participants were selected randomly after stratification for age, sex and household. Blood, stool and saliva samples were collected from these individuals. Peripheral blood mononuclear cells (PBMCs) and plasma were obtained from blood for immunological and virological analyses. Stool samples were used for helminth diagnosis while saliva was used for KSHV viral DNA detection and quantification. Socio-demographic data were collected using standard questionnaires. This study was approved by the UVRI-Research and Ethics Committee (REC) (reference number: GC/127/16/09/566), the Uganda National Council for Science and Technology (UNCST) (reference number: HS2123) and 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.

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Laboratory procedures

KSHV DNA was quantified in PBMCs and saliva from 878 KSHV seropositive individuals. A pellet of about two million PBMCs and saliva pellets were processed for DNA extraction. Study participants were instructed to rinse with 5mL of Listerine mouthwash, empting it, as well as saliva in a falcon tube. Aliquots (of 1mL each) of saliva were spun at 13,000rcf for 10 minutes to form saliva pellets. Thereafter the supernatant was removed and the saliva pellet 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 PCR, 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 number of cellular equivalents in PBMCs were determined using a quantitative assay specific to human endogenous retrovirus 3 (ERV-3) (39), which is present in two copies per genomic cell. Raw copies were reported for saliva KSHV DNA. Samples were amplified in triplicate; the samples that were positive in one or two reactions in the KSHV K6 assay were designated as qualitative positives. The sensititivity of the K6 assay is 3 copies.

Using an in house Luminex assay and ELISA, previously reported (40-42), plasma samples were tested for IgG antibody levels to the KSHV K8.1 (lytic) and ORF73 (latent) antigens. The ELISA was used to confirm serostatus while the Luminex assay was used to determine antibody levels, due to its wider dynamic range. Malaria parasitemia was diagnosed using rapid diagnostic tests (RDT) (ONE STEP Malaria HRP-II (P.f) and pLDH (Pan) Antigen Rapid Test). A single stool sample was provided by each participant. This was analysed 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).

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Statistical analysis

Statistical analysis was carried out using STATA version 13 (Statacorp, College Station, Texas USA). 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 quantitative analysis. Viral load levels were $\log_{10}$ transformed. First, risk factors associated with viral DNA detection (as a categorical outcome variable) in saliva and blood, separately, were obtained using logistic regression modelling. Thereafter, risk factors associated with increasing levels of viral DNA (as a continuous outcome variable) in saliva and in blood, separately, were 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, while 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 proportion of individuals with malaria parasitaemia was 4% (34/834) overall and 11% (13/120) among children 3-12 years. Previously we have 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. The prevalence of helminths was as follows: Hookworm was the most prevalent 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 may have slightly underestimated the true prevalence of helminths, because a single, rather than triple sample test, was used.

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Blood and saliva KSHV DNA detection and levels of viral DNA

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 2a and 2b). Children had the highest proportion of detectable viral DNA in PBMCs (Figure 2a) and in saliva (Figure 2b), decreasing with increasing age in adults. The trend was similar for females and males, with males having higher proportions of detectable viral DNA in saliva.

Associations between risk factors and KSHV DNA in PBMCs

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 (0.07,1.50), p=0.031) (Table 3). We observe no statistically significant associations with other measured risk factors including age, sex, hookworm and *S. mansoni* infections (Table 3).

Associations between risk factors and KSHV DNA in saliva

Overall, males had a higher risk of shedding viral DNA compared to females; adjusted Odds Ratio 1.63 (1.14, 2.34), p=0.008 (Table 4). Similar to PBMCs, the proportion of shedders in saliva diminished with increasing age, even after adjusting for sex and parasite infections p=0.0001 (Table 4). Additionally, compared to females, males had higher levels of KSHV DNA in saliva (adjusted regression coefficient 0.46 (0.05,0.87), p=0.027) (Table 5).

KSHV DNA detection and antibody levels

Individuals with detectable viral DNA in PBMCs (Supplementary Figure 1a) and in saliva (Figure 1b) had higher IgG antibodies to the K8.1 antigen (p<0.0001), as previously reported

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(13). There was no difference in IgG antibodies to 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 presence and levels of KSHV viral DNA in blood and saliva in apparently healthy people across the lifecourse. 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 have previously reported KSHV and EBV DNA shedding in saliva of children and their mothers in Uganda and noted that EBV DNA was shed more frequently and at higher levels than KSHV (38).

In this study, we observed no correlation between levels of KSHV DNA in blood and in saliva. Detection of KSHV DNA in blood and in saliva may reflect 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 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, IgA production in saliva may be important for viral control in oral fluids, while T and NK cell responses may play a more pivotal role in the control of viral reactivation in peripheral blood. Alternatively environmental factors such as plant derrivates that have been shown to reactivate KSHV *in vitro* (48) may play a role in viral reactivation in oral fluids if chewed. Studies of immune correlates of KSHV DNA detection in saliva and PBMC are therefore warranted.

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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 load among children could be associated with co-infections that are very prevalent in childhood such as 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. This is consistent with previous studies by us and others (31, 36, 38). Sex differences in immune control of KSHV infection might contribute to the higher risk of KS in men (53, 54). These findings warrant further study.

We have previously reported that high KSHV K8.1 antibody titres are 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 ORF73, compared to individuals without the detectable viral DNA. This association confirms our

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previous hypothesis (55) that 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 co-infections such as malaria which interfere with immune control, or make uninfected children susceptible to infections. Studies investigating the mechanism through which malaria affects KSHV are required. Additionally, characterisation of protective immune responses to KSHV is needed to inform vaccine development and to develop strategies to lower KSHV transmission in endemic areas.

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Disclaimer

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Figure legends

Figure 1: KSHV viral load in saliva and in peripheral blood mononuclear cells (PBMCs). KSHV viral loads were measured using real time PCR.

Figure 2: Proportion of individuals with detectable KSHV in peripheral blood mononuclear cells (PBMCs) (A) and saliva (B). KSHV viral loads were measured using real time PCR.

Supplementary Figure 1: IgG antibody levels to K8.1 (A & B) and ORF73 (C& D) protein among individuals with and without detectable KSHV in blood (A & C) and in saliva (B & D). Antibodies were measured using Luminex assay. These antibodies were $\log_{10}$ transformed. P values were obtained from a ttest after $\log_{10}$ transformation of the data.

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Table 1: General characteristics and parasite infections status among participants tested for KSHV viral DNA

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)
Schistosoma mansoni infection status	1% (7/686)
Hookworm infection status	15% (104/686)
Ascaria lumbricoides infection status	1% (8/686)
Trichuris trichiura infection status	0.2% (1/685)

Malaria parasitaemia was determined using rapid diagnostic tests (RDTs). Helminth status was determined from a single stool sample using Kato Katz method.

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Table 2: Risk factors for the presence of detectable KSHV DNA in blood (categorically)

	% detectable viral DNA in blood	OR[a] (95% CI)	P value	Adjusted[b] OR (95% CI)	P value
Age group					
3-12	23% (27/120)	1		1	
13-25	15% (26/177)	0.59 (0.33, 1.08)		0.63 (0.33, 1.17)	
26-50	7% (20/307)	0.24 (0.13, 0.45)		0.29 (0.15, 0.57)	
50+	8% (18/227)	0.30 (0.16, 0.57)	<0.0001	0.34 (0.16, 0.72)	0.0014
Sex					
Female	10% (41/419)	1		1	
Male	12% (49/406)	1.27 (0.82, 1.96)	0.294	0.89 (0.55, 1.45)	0.638
Malaria parasiatemia					
Negative	10% (83/791)	1		1	
Positive	21% (7/34)	2.21 (0.93, 5.24)	0.071	1.59 (0.64, 3.95)	0.321
S. mansoni					
Negative	11% (77/672)	1		1	
Positive	43% (3/7)	5.80 (1.27, 26.38)	0.023	11.04 (2.16, 56.97)	0.004
Hookworm					
Negative	13% (74/576)	1		1	
Positive	6% (6/103)	0.42 (0.18, 0.99)	0.048	0.41 (0.16, 1.04)	0.061

[a] OR: odds ratio, [b] adjusted for age, sex, malaria parasitaemia, *S. mansoni* and hookworm infection status. Logistic regression was used for statistical analysis. Malaria parasitaemia was determined using rapid diagnostic tests (RDTs). Helminth status was determined from a single stool sample using Kato Katz method.

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Table 3: Risk factors associated with increasing levels of KSHV DNA in blood (continuously)

	Coef[a]. (95% CI)	P value	Adjusted[b] Coef. (95% CI)	P value
Age group				
3-12	Ref		Ref	
13-25	-0.39 (-0.86, 0.07)		-0.35 (-0.84, 0.15)	
26-50	0.19 (-0.31, 0.69)		0.26 (-0.30, 0.83)	
50+	-0.32 (-0.83, 0.19)	0.084	-0.06 (-0.70, 0.57)	0.160
Sex				
Female	Ref		ref	
Male	0.10 (-0.27, 0.47)	0.594	0.21 (-0.19, 0.61)	0.292
Malaria parasitaemia				
Negative	Ref		Ref	
Positive	0.71 (0.05, 1.38)	0.036	0.79 (0.07, 1.50)	0.031
S. mansoni				
Negative	Ref		Ref	
Positive	0.17 (-0.87, 1.21)	0.750	-0.15 (-1.30, 1.00)	0.797
Hookworm				
Negative	Ref		Ref	
Positive	0.30 (-0.45, 1.05)	0.79	0.37 (-0.46, 1.21)	0.372

[a] Coef: linear regression coefficient, [b] adjusted for age, sex, malaria parasitaemia, *S. mansoni* and hookworm infection status. Linear regression modelling was performed on $\log_{10}$ transformed KSHV DNA levels for statistical analysis. Malaria parasitaemia was determined using rapid diagnostic tests (RDTs). Helminth status was determined from a single stool sample using Kato Katz method.

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Table 4: Risk factors for the presence of detectable KSHV DNA in saliva (categorically)

	% detectable viral DNA in saliva	OR[a] (95% CI)	P value	Adjusted[b] OR (95% CI)	P value
Age group					
3-12	42% (50/120)	1			
13-25	31% (55/175)	0.64 (0.40, 1.40)		0.61 (0.37, 1.02)	
26-50	18% (56/310)	0.31 (0.19, 0.49)		0.38 (0.24, 0.63)	
50+	17% (39/231)	0.28 (0.18, 0.47)	<0.0001	0.30 (0.18, 0.54)	0.0001
Sex					
Female	19% (79/423)	1		1	
Male	29% (119/407)	1.80 (1.30, 2.49)	<0.0001	1.63 (1.14, 2.34)	0.008
Malaria parasitaemia					
Negative	24% (188/796)	1		1	
Positive	29% (10/34)	1.35 (0.63, 2.87)	0.439	0.98 (0.44, 2.16)	0.952
S. mansoni					
Negative	26% (174/675)	1		1	
positive	43% (3/70)	2.16 (0.48, 9.74)	0.317	2.43 (0.51, 11.52)	0.265
Hookworm					
Negative	27% (158/578)	1		1	
Positive	18% (19/104)	0.59 (0.35, 1.009)	0.054	0.66 (0.38, 1.14)	0.136

[a] OR: odds ratio, [b] adjusted for age, sex, malaria parasitaemia, *S. mansoni* and hookworm infection status. Logistic regression was used for statistical analysis. Malaria parasitaemia was determined using rapid diagnostic tests (RDTs). Helminth status was determined from a single stool sample using Kato Katz method.

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Table 5: Risk factors associated with levels of KSHV DNA in saliva (continuously)

	Coef.[a] (95% CI)	P value	Adjusted[b] Coef. (95% CI)	P value
Age group				
3-12	Ref		Ref	
13-25	-0.50 (-1.02, 0.21)		-0.61 (-0.13, -0.09)	
26-50	-0.31 (-0.82, 0.21)		-0.26 (-0.80, 0.28)	
50+	-0.78 (-1.35, -0.21)	0.049	-0.76 (-1.40, -0.11)	0.048
Sex				
Female	Ref		Ref	
Male	0.51 (0.12, 0.89)	0.010	0.46 (0.05, 0.87)	0.027
Malaria parasitaemia				
Negative	Ref		Ref	
Positive	0.20 (-0.67, 1.07)	0.651	0.05 (-0.82, 0.92)	0.909
S. mansoni				
Negative	Ref		Ref	
positive	-0.58 (-2.12, 0.96)	0.460	-0.38 (-1.94, 1.18)	0.635
Hookworm				
Negative	Ref		Ref	
Positive	-0.36 (-0.96, 0.32)	0.326	-0.37 (-1.03, 0.29)	0.270

[a] Coef: linear regression coefficient, [b] adjusted for age, sex, malaria parasitaemia, *S. mansoni* and hookworm infection status. Linear regression modelling was performed on log_{10} transformed KSHV DNA levels for statistical analysis. Malaria parasitaemia was determined using rapid diagnostic tests (RDTs). Helminth status was determined from a single stool sample using Kato Katz method.

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Figure 1

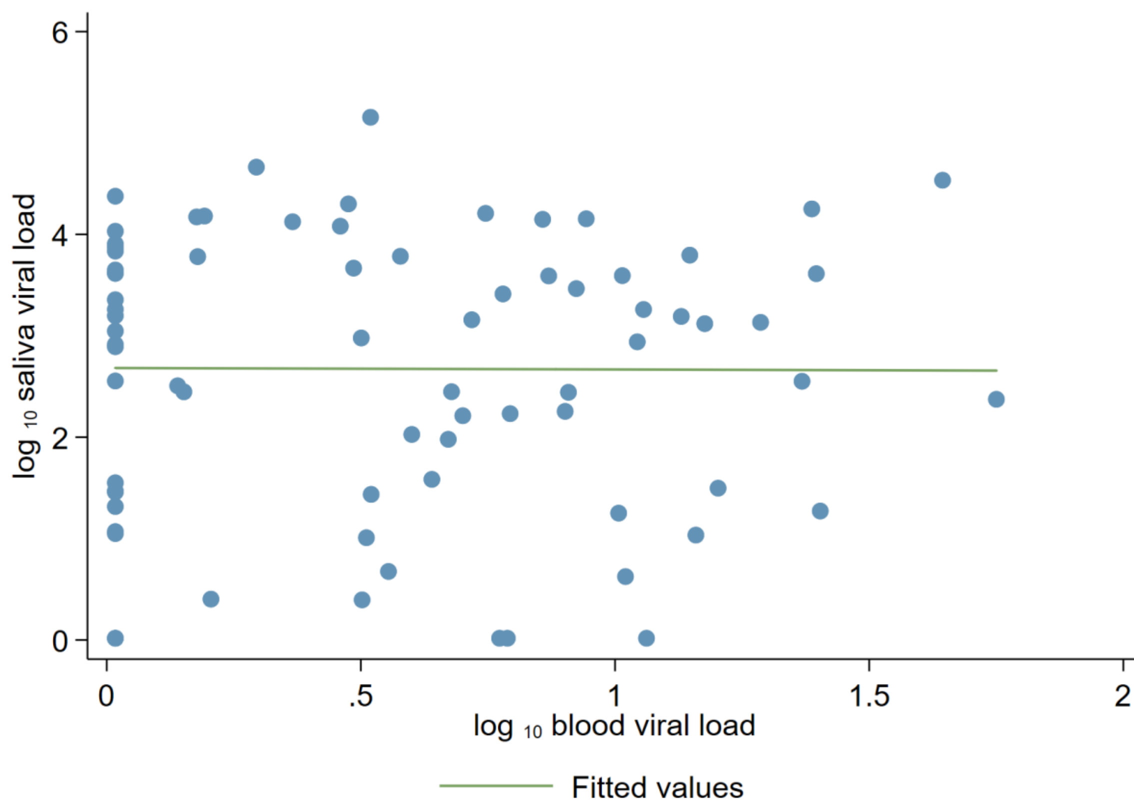

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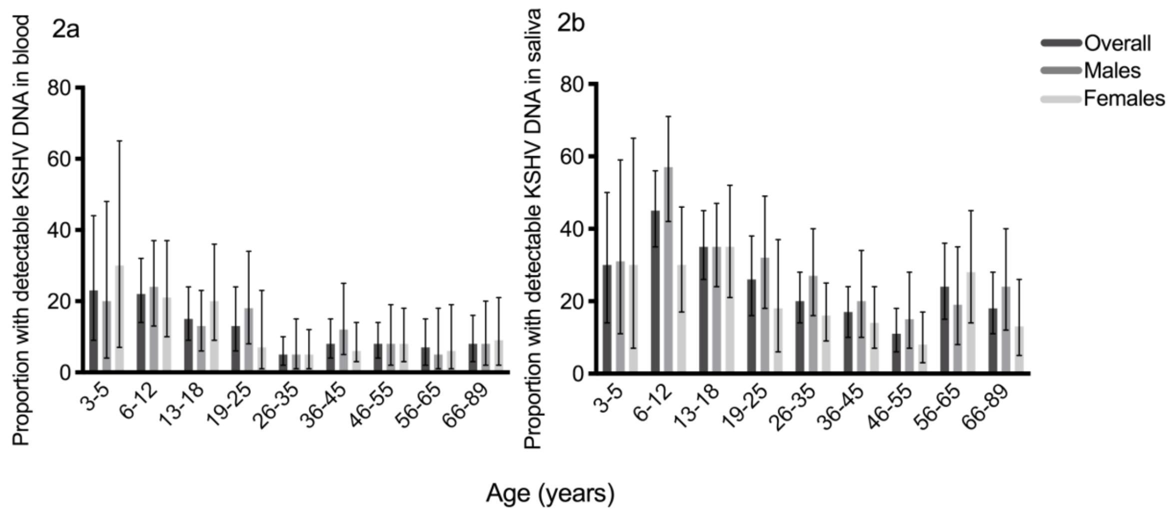
Figure 2



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