Entebbe Mother and Baby Study

PROTOCOL AMENDMENT

Amendment title: Testing for KSHV, EBV and malaria among five year old children and mothers in the Entebbe Mother and Baby Study stored samples.

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1. Summary

Kaposi sarcoma-associated herpes virus (KSHV) infection is necessary but not sufficient for the development of Kaposi's sarcoma (KS). Sub-Saharan Africa has the highest KSHV prevalence and KS incidence. The occurrence of KS among individuals without evident immunosuppression in sub-Saharan Africa has a distinct geographical distribution. This suggests co-factors for KSHV pathogenesis. Malaria is still a burden in sub-Saharan Africa. The co-occurrence of malaria and KSHV in sub-Saharan Africa has raised questions as to the effect of malaria on KSHV pathogenesis. In a cross sectional survey conducted within the Entebbe Mother and Baby Study (EMaBS) we have shown that in both mothers and their children, asymptomatic malaria parasitaemia is significantly associated with antibodies to KSHV [1]. This novel finding raises important questions as to the impact of malaria on control of KSHV replication. The impact of *P. falciparum* malaria on EBV, another gamma herpes virus closely related to KSHV is established [2]. During malaria infection T-cell immunity is impaired. We hypothesise that impaired T cell immunity during malaria infection leads to (1) viral replication leading to increased transmission of KSHV; (2) increased susceptibility of KSHV seronegative children to the virus; and (3) endemic KS disease. KSHV and EBV reactivation is characterised by increased viral load both in saliva and in peripheral blood and raised KSHV and EBV specific antibody titres. We propose that malaria is associated with increased KSHV and EBV viral load in blood; this is a sign of KSHV and EBV reactivation. We shall investigate this hypothesis by testing peripheral blood for KSHV and EBV viral load in the 5 year old children and mothers, who are KSHV and EBV seropositive respectively. We shall also test for malaria parasitaemia and antibodies in the same individuals using PCR and ELISA respectively.

2. Background

Kaposi's sarcoma associated Herpes Virus (KSHV)

Kaposi's Sarcoma Herpes virus (KSHV) is the aetiological agent responsible for Kaposi's sarcoma (KS) [3]. The prevalence of KSHV infection shows considerable variation geographically, largely reflecting the variations seen in the incidence of KS [4]. KSHV prevalence is high in sub-Saharan Africa and some Mediterranean countries and low in most northern European and Asian populations [5]. Studies from sub-Saharan Africa report high KSHV prevalence, with primary infection beginning in childhood; seroprevalence increases with age. There is some evidence to suggest that, in Africa, transmission occurs via saliva [4].

KSHV latent and lytic life cycles

KSHV, like other herpes viruses, displays two phases. During latency only a few viral genes are expressed. During latent infection, the KSHV genome persists as a circular double-stranded DNA molecule in the nucleus with most viral genes being silenced except a few viral latent genes located in the latency locus. As a result, there is no production of virus. Latent infection allows KSHV to evade the host immune surveillance and facilitate the establishment of a lifelong persistent infection. KSHV latent cells constitute a reservoir of chronic viral infection tightly controlled by the host immune system [6]. In the lytic phase KSHV is reactivated with initiation of extensive viral DNA replication and gene expression, resulting in production of new viral particles [7]. Viral reactivation is crucial for KSHV pathogenesis and contributes to the progression of KS [8] [9]. In KS tumors, a small subset of cells also undergoes spontaneous lytic replication and inhibition of KSHV lytic replication with anti-herpes viral drugs that block lytic replication causes KS tumor regression [10].

Laboratory markers of KSHV reactivation

Clinical markers of KSHV reactivation are increasing KSHV lytic antibodies titres [11] [12] and increasing KSHV viral load in blood and saliva. Serology to KSHV lytic antigen has been adopted by the majority of researchers to study reactivation.

Factors driving KSHV reactivation

Factors implicated in driving KSHV reactivation can be broadly divided into two groups: (1) Immunosuppression, and (2) environmental agents.

Immunosupression

KSHV reactivation and aggressive forms of KS are seen in individuals with immunosuppression. These sub-types of KS are HIV–associated KS and iatrogenic or transplant associated KS [13] [14].

HIV can drive KSHV reactivation directly via the HIV-1 transactivator Tat [15]. The HIV-1 Tat protein can independently promote lytic cycle induction and replication of KSHV in culture [13]. HIV also acts indirectly through suppression of KSHV-specific T cell responses [16] and induction of inflammatory cytokines [17]. Anti-retroviral therapy is associated with regression of KS and decreasing KSHV detection, and this is accompanied by restoration of numbers and functions of KSHV-specific CD4 positive [18] [19] cells and CD8 positive cells [20].

KS development in KSHV infected individuals after solid organ transplant is linked to calcineurin inhibitors. This class of drugs prevents organ rejection by immunosuppression largely through T cell inhibition. Loss of KSHV-specific T cell repertoire has been demonstrated with calcineurin inhibitors, with recovery and remission of KS on stopping medication [21]. The majority of post-transplant KS patients are KSHV seropositive prior to transplantation, suggesting that reactivation of latent virus is responsible for the disease [22] [23].

Environmental agents

A number of environmental co-factors for KSHV reactivation and disease have been reported. Studies in this area are sparse and less credence is given to these factors compared to immunosuppression. Environmental factors fall into three groups: parasites, soil, plants.

I. Parasites

Parasites have been hypothesized as potential co-factors to KSHV transmission and infection. KSHV is an immune-sensitive virus [24] and the survival of parasites is dependent upon their ability to interfere with host immune function [25]. Epidemiological studies from Italy report substantial declines in KSHV seroprevalence and KS incidence in association with eradication of mosquitoes [26]. It is notable that malaria is known to affect immune control of another human gamma herpes virus, the Epstein Barr Virus (EBV). In addition, malaria interacts with the EBV thereby increasing the risk of Burkitt's lymphoma, the most common cancer reported in children in East Africa [27] [28]. Malaria infection may cause KSHV reactivation increasing viral replication and shedding in saliva. Repeated malaria infections have been shown to suppress T cell immunity [20], and this may have a detrimental effect in the control of KSHV infection. We have previously reported an association between KSHV seropositivity and malaria in women in Uganda [29].

II. Soil

Areas of high incidence of endemic KS are characterized by a common geologic substrate, composed of fertile reddish-brown volcanic clay soils. Volcanic mountains fringe the western branch of the Great Rift Valley in eastern Congo, in western Uganda and Tanzania, and further south in Malawi [29]. The unusually high prevalence of endemic forms of KS in regions of intercontinental rifts and volcanism may point to prolonged exposure to indigenous iron oxide rich volcanic soils as a common etiological risk factor [30].

III. Plants

The ''oncoweed'' hypothesis is that certain extracts of natural products collected from African countries known to have a high prevalence of KSHV infection and endemic KS can cause reactivation of KSHV in latently infected PEL cell lines. It was proposed that this may provide a biological mechanism to explain geographic variations in KSHV reactivation rates. Higher reactivation rates may explain higher prevalence, viral loads and transmission frequency in KS endemic African regions [31].

Epstein Barr Virus (EBV)

EBV is another gamma herpes virus closely related to KSHV. Like KSHV, EBV is highly prevalent in sub-Saharan Africa. Transmission of EBV is through saliva. EBV and malaria co-infection have been implicated in the cause of endemic Burkitt's lymphoma (eBl). Two theories have been suggested to explain the relationship between EBV and *P. falciparum* Malaria in the cause of eBl. One suggests that *P. falciparum* induces polyclonal B-cell expansion and consequently lytic EBV reactivation, thus leading to the expansion of latently infected B-cells and the likelihood of c-myc translocation, which is a hallmark of all B-cell tumours. The other theory suggests that EBV-specific T-cell immunity is selectively impaired during *P.falciparum* malaria co-infection, either as a cause or consequence of enhanced EBV replication, leading to loss of viral control. Anti-EBV antibody titres and EBV viral load are some of the markers of EBV reactivation [32].

Malaria

Malaria is a vector borne disease transmitted by female anopheles mosquitoes. In 2010, WHO estimated 219 million malaria cases worldwide with 79% from the African region. Of the 219 million cases, 660,000 people are estimated to have died from malaria [33]. Malaria is caused by a protozoan parasite of the genus *Plasmodium*. There are four plasmodium species which infect humans, *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae*. *P. falciparum* is the most prevalent species and the cause of most malaria deaths worldwide.

The human host elicits a complex immune response against various stages of *P. falciparum*. The course of the immune response is determined by a number of factors including genetics, age, exposure history of the host, and the species and strain of parasite [34]. During the course of infection with *P. falciparum* immunosuppression to malaria and other unrelated antigens has been observed. This partial immunosupression may lead to (1) failure to fight malaria infection; (2) could make the infected individual vulnerable to secondary infections, and (3) reduced immune response to certain vaccines. A

number of mechanism through which malaria causes immunosuppression have been hypothesised. Hemozoin, a malaria pigment produced from the breakdown of haemoglobin by the parasite in infected red blood cells, is believed to affect the function of myeloid lineage cells. Myelod derived dendritic cells have been shown to be affected by hemozoin. A number of studies have shown a great reduction and loss of function of dendritic cells during the course of malaria infection. This state is normally short lived once the parasites have been removed. Since dendritic cells play a major role in antigen presentation, the initiation of the adaptive immune response is greatly affected during malaria infection [35].

3. Rationale for the study

KSHV is the aetiological agent responsible for Kaposi's sarcoma (KS). In southern Uganda, malaria is ubiquitous and the prevalence of KSHV is up to 80% in some areas. Transmission of KSHV in Africa has been proposed to occur via saliva and throughout life, including during childhood, as opposed to sexual transmission among adults seen in affluent countries. Reactivation of KSHV is a key step in KS development and progression and possibly also for transmission to uninfected individuals.

KSHV, like all other herpes virus, has two stages: the lytic stage and the latent stage. After infection with KSHV the virus goes into the latent stage where it expresses minimal antigens to avoid recognition by the immune system. CD8-T cells play a key role in eliminating/destroying virus infected cells and controlling viral replication. During immunosuppression, for example in HIV infected, elderly, and organ transplant recipients, an individual's viral control is lost, leading to virus replication and KS disease. As discussed above, some parasitic infections such as malaria infection also impair T-cell immunity. This might explain high incidences of KS and KSHV in sub-Saharan Africa. We propose to investigate the association between KSHV and malaria co-infection using more sensitive laboratory techniques.

The EMaBS is a cohort study which enrolled 2500 pregnant mothers. These mothers and their children have been followed with samples taken every 12 months for the past 10 years, currently the children are 9 years old. This cohort has collected extensive data on helminth burden, anaemia, HIV infection, malaria infection and other demographic factors. The highly organised data collected provides the opportunity to carry out a well organised study that can be adjusted for confounders and explore other potential co-factors.

4. Hypothesis

Malaria is associated with increased KSHV and EBV viral loads.

5. Objectives

- To examine the association between malaria and KSHV viral load in Ugandan KSHV seropositive five year old children and **mothers**
- To examine the association between malaria and EBV viral load in Ugandan EBV seropositive five year old children and **mothers**

5.1. Specific objectives

We will address these aims by comparing in Ugandan women and/or 5 year old children:

1) Malaria parasitaemia by PCR and KSHV and EBV viral load in whole blood

2) Malaria antibody responses and KSHV and EBV viral load in whole blood

6. Methodology

6.1. Study design and study population

This cross sectional study will use stored samples from the Entebbe Mother and Baby Study (EMaBS) - a large double blind randomised placebo controlled trial designed to determine the impact of helminth infections and their treatment on vaccine responses and infectious diseases outcomes.

Through EMaBS peripheral blood samples were collected from mothers and their children (now aged 9 years) from Entebbe Wakiso district of Uganda. Whole blood pellets were stored for DNA extraction: serum and plasma samples were stored for further serological assays. Data on demographic variables such as socioeconomic status were collected. Through EMaBS, we screened mothers for KSHV antibodies as part of Dr Katie Wakeham's PhD project. We also screened these mothers and children at 1-5 years time points for malaria antibodies as part of Dr Juliet Ndibazza's PhD project.

The plasma samples from 5 year old children will be used to identify KSHV and EBV seropositive children using an ELISA assay. The whole blood pellets of KSHV and EBV seropositive mothers and 5 year old children will be used to test for malaria parasitemia, KSHV and EBV viral load using real time quantitative PCR assays. The inclusion criteria will be, mothers and 5 year old children who are KSHV and/or EBV seropositive **who agreed for their samples to be stored for future assays.**

6.2. Outcome and exposure measures

The main outcomes of the study will be KSHV and EBV viral load. The exposures or risk factors are malaria parasitaemia and malaria antibodies. Already collected demographic data, socio economic status, HIV status, helminths and anemia will be used to adjust for possible confounders and look for other associations.

6.3. Laboratory investigations

Immunological and molecular biology assays will be performed on these samples such as: (1) a multiplex quantitative real time PCR assay testing for KSHV, EBV and malaria parasitemia; (2) serological assays testing for KSHV and EBV specific antibodies. Some of the assays which will be used are explained in detail below.

KSHV Serology

Plasma/serum samples from 5 year old children will be screened for the presence of KSHV antibodies using an Enzyme linked immunosobent assay (ELISA) to a lytic structural glycoprotein, K8.1 and a latent protein ORF73. Each plate contains three positive and three negative controls. Each assay cut-off is calculated based upon the performance of the negative control wells. Samples with optical densities above the cut-off for seropositivity will be re-tested at doubling dilutions to determine titre. Protocols for this assay are currently held in the Co-infections studies Programme (CiSP) laboratory.

KSHV quantitative real-time PCR

DNA will be extracted from whole blood with the QIAamp DNA blood mini kit protocol (Qiagen, Valencia, CA) following the manufacturer's directions. Quality and concentration of DNA will be assessed by optical density using a Nanodrop spectrophotometer. KSHV DNA will be quantified using real time PCR. Negative control wells will be run in triplicate as a separate quality control check on every assay plate. The KSHV DNA will be detected using primers and a probe previously reported to be specific to the K6 gene region [36]. Additionally, the number of cellular equivalents will be determined using a quantitative assay specific to human endogenous retrovirus 3 (ERV-3), which is present in two copies per genomic cell [37]. All DNA samples will be tested in triplicate reactions for both assays and the estimated copy number for each individual reaction will be averaged. The KSHV viral load will be determined by calculating the viral DNA copies per million cells. Any sample that will be quantifiable by the ERV-3 assay but not positive in all three reactions in the KSHV K6 assay will be designated as positive

but not quantifiable. Non-quantifiable low level KSHV PCR positive samples will be repeated in a second assay for confirmation. The lower limit of detection for the ERV-3 assay has been previously determined as 10 copies per million cells while the KSHV K6 assay has a lower limit of sensitivity of 1 copy per million cells [33].

Malaria PCR

Genomic DNA will be extracted from whole blood pellets using the QIAamp DNA blood mini kit protocol (Qiagen, Valencia, CA), following the manufacturer's directions. DNA quantification will be carried out using a Nanodrop spectrophotometer. Malaria DNA will be determined using Taqman real time PCR. Negative samples will be tested twice to confirm if they are true negatives. Phocine herpes virus will be added to every sample at the point of extraction to act as an internal control. Using a standard curve generated from malaria positive controls whose parasitaemia was quantified using fluorescence microscopy, we will derive parasite concentrations for each patient.

6.4. Sample size determination

From previous studies we have conducted in the EMaBS, KSHV seroprevalence among five year old children and mothers was approximately 20% and 60% respectively. Asymptomatic malaria parasiteamia prevalence in the same cohort is 11% among mothers assessed using blood smears. PCR, which is a more sensitive technique, will be used to determine asymptomatic malaria parsitaemia in this study. Approximately 2500 mothers were enrolled into the EMaBS. The available samples are approximately 1000 at each time point; therefore approximately 200 children and 600 mothers are KSHV seropositive. From previous studies elsewhere we assume a geometric mean KSHV viral load of 6 (a mean viral load on a log scale of 1.8 with a standard deviation of 0.8). The tables 1 and 2 below show the power to detect an increase in viral load of a given relative magnitude as being significant at the 5% level, corresponding to a number of possible breakdowns of the sample into malaria positive and malaria negatives.

Table 1: power values at different % increase in viral load, if a sample size of 600 mothers is used

	Relative percentage increase of geometric mean KSHV viral load		
Number of malaria positives	**40%**	**30%**	**25%**
75	**92% power**	**71.6% power**	**52.6% power**
100	**96% power**	**81% power**	**62.6% power**
125	**98.4% power**	**87.5% power**	**70.1% power**

Table 2: power values at difference % increase in viral load, if a samples size of 200 children is used

	Relative percentage increase of geometric mean KSHV viral load		
Number of malaria positives	**40%**	**30%**	**25%**
50	**71% power**	**48.2% power**	**33.4% power**
75	**81% power**	**57% power**	**40.2% power**
100	**83% power**	**60% power**	**42.4% power**

6.5. Ethical considerations

The Entebbe Mother and Baby Study (EMaBS) has ethical approval from the Science and Ethics Committee (SEC) of Uganda Virus Research Institute (UVRI), and also from the Uganda National Council for Science and Technology (UNCST). This project will use stored EMaBS samples and we will seek a protocol amendment approval from the UVRI SEC and UNCST. This project will investigate further, the idea developed by Dr Robert Newton and PhD student Dr Katie Wakeham through the research they conducted within the EMaBS. They investigated the association between malaria parasitemia (tested using blood slides) and KSHV antibody responses in EMaBS's mothers and children. These results are published [1] and the work was approved by UVRI SEC and UNCST ("The effect of HIV infection on the prevalence and transmission of oncogenic infections with amendment 25th July 2010")

6.6. Data management

Clinical and socio-demographic data has been collected and it is managed by the Co-infection studies programme (CiSP). The new data generated from the lab assays will be held securely by MRC/UVRI.

6.6.1. Analysis plan

Data generated from KSHV/EBV viral load assays and malaria parasitemia will be compared, first using simple descriptive analysis, then latter on regression models will be designed mainly to adjust for possible confounders and look for other associations.

7. Quality assurance and audit

To ensure quality and reliability of the data of this study Standard operating Procedures (SOPs) will be developed for laboratory procedures. The study will be regular monitored and conducted in accordance to the principles of Good Clinical laboratory Practice (GCLP).

8. Potential limitations

- The major limitation is in un-measured or un-identified confounders.
- Failure to find KSHV in circulating blood is a possible limitation.

9. Significance of the proposed work

This would be the first study to investigate KSHV specific immune modulation by malaria co-infection in Ugandan children, a population most susceptible to KSHV infection. This study could potentially be a major step forward in understanding the reasons for high KSHV prevalence in Uganda.

10. Plan for dissemination

Results will be disseminated to the community, MRC, UVRI and UNCST. International dissemination of results will take place through conferences and publications.

10.1. Timeframe

Activity	August 2013	September 2013	October 2013	November 2013	December 2013	January 2014	February 2014	March 2014	April 2014	June 2014	July 2014	August 2014	September 2014
Proposal writing and ethical clearance													
Performing laboratory assays													
Data cleaning and analysis													
Results publication													

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