Kaposi’s Sarcoma-Associated Herpesvirus Transmission and Infection among Young Zambian Children

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KAPOSI’S SARCOMA-ASSOCIATED HERPESVIRUS TRANSMISSION AND INFECTION

AMONG YOUNG ZAMBIAN CHILDREN

by

Landon N. Olp

A DISSERTATION

Presented to the Faculty of
The Graduate College at the University of Nebraska
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Kaposi’s sarcoma-associated herpesvirus (KSHV) is the etiologic agent for all forms of Kaposi’s sarcoma (KS)—one of the most common pediatric cancers in sub-Saharan Africa during the AIDS epidemic. KS was endemic in sub-Saharan Africa prior to the HIV/AIDS epidemic, but KS cases drastically increased thereafter. Our laboratory previously observed that KSHV infection is common among Zambian children and saliva is the major route of transmission. However, additional factors associated with the transmission of KSHV to children are poorly understood. Since a vaccine against KSHV is not currently available, it is paramount to understand factors associated with transmission so that alternative strategies can be developed to prevent KSHV acquisition during early childhood.

The studies reported here reveal that early childhood transmission of KSHV is multifactorial. We show that household members served as primary KSHV transmission sources to children, but transmission from outside the household also occurred. We also describe a prospective cohort study—that followed HIV-exposed, KSHV-negative children—to assess the impact of HIV and antiretroviral therapy (ART) on risk of KSHV acquisition. Data from this cohort suggest that early ART and prevention of immune suppression significantly reduce the risk of KSHV acquisition among HIV-infected
children. After primary KSHV infection, however, KSHV antibody titer was highly variable and did not correlate with available clinical information, HIV/ART status, or KSHV DNA detection.

Additionally, next-generation deep sequencing was used to examine KSHV genomic diversity in an endemic setting as the first step to investigate the possible impact of genetic variations on pathogenesis and transmission. We detected distinct phylogenetic clustering between KSHV isolates from Zambia and Western countries, and identified four genes with unprecedented levels of polymorphisms.

The results described herein present a deeper understanding of epidemiological, immunological, and viral factors that may be related to KSHV transmission among young children in Zambia—a region where KSHV is endemic and HIV is epidemic. The findings from this study will be important for developing public health strategies to reduce KSHV spread among young children. This, in turn, will help reduce the burden of KS among children and adults in endemic settings.
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CHAPTER 1
REVIEW OF LITERATURE

KSHV CLINICAL MANIFESTATIONS

Kaposi’s sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus-8 (HHV-8), was first identified in Kaposi’s sarcoma (KS) lesions of AIDS patients in 1994 [1]. Since then, it has been etiologically linked to all clinical manifestations of KS, as well as B cell lymphomas—primary effusion lymphoma (PEL) and multicentric Castleman’s disease (MCD)—and KSHV inflammatory cytokine syndrome (KICS) [2-4].

Kaposi’s sarcoma (KS)

KS was first described in 1872 by Moritz Kaposi [5]. KS manifests as multifocal, pigmented endothelial lesions primarily on the skin but can also involve mucosal membranes and visceral organs [6]. Globally, KS is rare; but prevalence is significantly increased in certain geographical regions and populations where associated cofactors such as immune suppression or HIV infection exist. Because all cases of KS are indistinguishable by histology, the aforementioned epidemiological features have been used to classify KS into four forms: classic, endemic, iatrogenic, and HIV-associated.

Classic KS

Classic KS was the epidemiological form first described by Moritz Kaposi as a rare disease that typically presents on the lower extremities [5]. It primarily occurs among
men older than 65 years of age and of Mediterranean and Jewish descent [7-9]. As such, the prevalence of classic KS is much higher among men with a male to female ratio estimated between 4:1 and 15:1 [8, 10]. Classic KS is the least aggressive form of all KS types and remains slow- or non-progressing even if left untreated [8, 11]. For example, the mean survival of patients with classic KS is 10 to 15 years, although the cause of death is commonly attributed to unrelated conditions associated with age [11].

**Endemic KS**

KS was prevalent in many countries of sub-Saharan Africa even before the AIDS epidemic, and was first recognized as endemic within the region in 1962 [12]. Subsequently, a broad strip across equatorial Africa—termed the ‘KS belt’—was documented to have high incidence of endemic KS. Countries within the ‘KS belt’ include Cameroon, the former Zaire, Uganda, Tanzania, as well as the southern countries of Malawi, Zambia, Zimbabwe, and South Africa [13]. Several clinical forms of endemic KS exist in sub-Saharan Africa, but can be differentiated as those found in adults (benign, florid, and aggressive) or children (lymphadenopathic) [14]. Among the adult forms, the male to female ratio is similar to that of classic KS; however, the median age of disease onset is lower, at 40 years of age. Moreover, aggressive and florid KS have a mortality rate of 36%, which is much higher than benign and classic KS [15]. On the other hand, lymphadenopathic KS a is less frequent and very unique variant of KS. This form presents almost exclusively in prepubescent children (mean age of 3 year old) with virtually no skew between sexes [14]. Lymphadenopathic KS rapidly disseminates, in the
absence of cutaneous lesions, and does not respond to treatment regimens—resulting in 100% mortality within three years [15, 16].

**Iatrogenic KS**

As solid-organ transplants became more common in the 1970s due to the advancements in immunosuppressant regimens to prevent graft-versus-host disease, a rise in KS was also detected [14, 17]. Hence, a third form of KS was recognized and called iatrogenic, or transplant-associated, KS. Despite the rarity of this form, an epidemiological study based in the United States observed that transplant recipients have a 54-fold higher risk of developing KS compared to the general population [18]. Of importance, iatrogenic KS often resolves without further treatment once the immunosuppressant regimen has been stopped [17]. This was the first indication highlighting a role of immune dysfunction in the development of KS.

**AIDS-associated KS**

In the early 1980s large numbers of young, homosexual men began presenting to hospitals in New York and California with generalized KS [19-21]. These men had no previously known risk factors for KS, but all had an immune deficiency that would later come to be known as AIDS. Subsequently, KS became the most common malignancy in AIDS patients and is most well known as a prominent AIDS-defining illness [21, 22]. Due to the close association with the AIDS epidemic, this recent and unique form of KS is aptly referred to as epidemic or AIDS-associated KS.

AIDS-associated KS is the most aggressive form of KS. The red/purple pigmented lesions are present on the skin but rapid dissemination to visceral organs such as the
lungs, gastrointestinal tract, spleen, and liver is common [22]. Development of KS among AIDS patients was common, with approximately 40% of AIDS patients presenting with KS at the beginning of the epidemic [23]. A subsequent longitudinal analysis of HIV and KSHV co-infected homosexual men revealed that the 10-year probability of developing KS in this population was nearly 50% [24]. This study also points out that KS can develop in HIV-infected individuals before the onset of AIDS and severe immunodeficiency. HIV infection is hypothesized to contribute to KS development through immune dysregulation, and this will be discussed in a subsequent section.

Primary effusion lymphoma (PEL)

KSHV DNA sequences were identified in PELs shortly after the virus was discovered [2]. PELs comprise a rare and unusual subset of AIDS-associated non-Hodgkin’s lymphoma (NHL) that are present in pleural, pericardial, and abdominal cavities; hence it was initially referred to as body cavity based lymphomas (BCBL) [25]. These lymphomatous effusions normally occur without an identifiable tumor mass. The tumor cells lack almost all B-cell, T-cell, or myeloid cell markers, but genetic analysis identified the cells to be of B-cell origin [26]. PELs only account for 3% of AIDS-related lymphomas and 0.4% of all AIDS unrelated NHLs [27]. Nevertheless, prognosis is very poor with a median survival of approximately six months [28].

Despite the low frequency of PEL cases, this disease has been important to KSHV research as they were used to develop the first cell lines harboring KSHV (e.g. BCBL-1, BC-1, and BC-3). These cell lines harbor large numbers of KSHV genomes, ranging from
40 to 80 copies per cell, and have consequently been used as tools in serological assays, virus purification, analysis of virus reactivation, and were even used to determine the first genomic sequence of KSHV [29].

**Multicentric Castleman’s disease (MCD)**

In 1995, KSHV sequences were also detected in MCD and a viral association with the disease was described [3]. MCD is a rare variant of Castleman’s disease—a polyclonal nonneoplastic disorder—that is associated with multiple lymphadenopathies. Hence, the name “multicentric” Castleman’s disease. Conversely, the most common clinical variant of Castleman’s disease is a hyaline vascular type, which presents as a solitary mass and is not associated with KSHV [6]. Patients with MCD frequently develop secondary malignancies, of which KS and non-Hodgkin’s lymphoma are the most common [3]. Although MCD is poorly understood, KSHV is present in almost all AIDS-associated cases and disease it is thought to be related to immune dysregulation, particularly excessive IL-6 production [29]. This again, highlights a potential role of immune dysregulation in the progression of KSHV-related disease.

**KSHV inflammatory cytokine syndrome (KICS)**

A recent study described a novel inflammatory syndrome associated with KSHV infection that resembled MCD even though there was no pathological evidence of MCD [4]. The KSHV inflammatory cytokine syndrome (KICS), is characterized by high KSHV viral load and substantial elevation of both human IL-6 and a virally-encoded homologue
of IL-6 compared to patients with KS [4, 30]. Little is known regarding what may trigger
the excessive lytic activation of KSHV in KICS, but a recent phylogenetic study revealed
an unusually high rate of polymorphisms in the microRNA sequences from MCD and
KICS patients [31]. This suggests that microRNA polymorphisms may be associated with
risk of disease.

**KAPOSI’S SARCOMA-ASSOCIATED HERPESVIRUS**

**Discovery and classification**

KSHV was initially discovered from a KS lesion of an AIDS patient by Chang and
Moore in 1994 using representational difference analysis [1]. This technique utilizes
DNA libraries generated from two separate samples to differentially amplify the DNA
unique to one library but not present in the other. KSHV sequences were therefore
amplified as a result of presence in KS tissue but not normal tissue from the same AIDS-
patient—suggesting KSHV may be the etiological agent for KS. Concordantly, PCR
analysis revealed that KSHV DNA was present in 100% of confirmed AIDS-KS tissues with
amplifiable DNA, while KSHV DNA was only detected 15% of non-KS tissues from
patients with AIDS and 0% of samples from non-AIDS patients [1]. Sequence analysis of
the differentially amplified DNA revealed homology to capsid and tegument proteins of
Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS), making KSHV the eighth and
most recently discovered human herpesvirus. Additional sequence analysis confirmed
that KSHV is a gamma-herpesvirus in the rhadinovirus genus (Figure 1.1) [32]; hence the
alternative designation of HHV-8.
Among the herpesviruses there are three subfamilies—alpha, beta and gamma—classified primarily on morphology, genome structure, and more recently sequence homology [33]. Herpesviruses consist of an electron dense core containing a linear double stranded DNA, an icosahedral capsid, a proteinaceous layer surrounding the capsid termed the tegument, and an outer membrane that contains viral glycoproteins. A unique feature of all herpesviruses is the ability to establish a persistent, latent infection in the host with periodic lytic reactivation. Latency is utilized to maintain an immunologically silent state where only a small subset of genes are expressed and infectious virus is not produced. Herpesviruses of different subfamilies establish latency in specific cell types. For example, the alpha-herpesviruses establish latency in neurons while the gamma-herpesviruses establish latency in lymphocytes [33].

KSHV genomic structure and subtyping

The KSHV genome is a largely conserved double stranded DNA comprised of a 140kb long unique region flanked by terminal repeats, resulting in a final genome size of approximately 165kb [34]. The initial KSHV genome sequence, generated from the PEL cell line BC-1, was published in 1996. This study conservatively assigned 81 open reading frames (ORFs) to the genomic sequence based on homology to herpesvirus saimiri (HVS) or a canonical AUG initiation site with a predicted amino acid sequence of >100 residues [34]. ORFs with homology to HVS were named consecutively from left to right (ORF4 – ORF75), whereas KSHV unique genes were given a “K” designation (Figure 1.2) [34]. Subsequently, six additional small ORFs have been identified for a total of 87, in addition
to small noncoding RNAs, microRNAs, and a polyadenylated nuclear (PAN) RNA [29].

Some of the KSHV genes encode multiple proteins through alternative splicing and alternative translation initiation sites. Recently, Arias et al. [35], completed a comprehensive annotation of KSHV using next-generation RNA-sequencing. This study confirmed previous KSHV annotations but also identified numerous new genomic features including alternative splicing, non-coding RNAs, and viral mRNA editing—substantially expanding the current understanding of KSHV genomic structure and coding capacity.

**KSHV genomic subtyping**

Despite the high level of conservation throughout the KSHV genome, the extreme 5’ and 3’ termini are highly variable and have been used to subtype the virus [36-38]. The 5’ terminus contains an 870-nucleotide, KSHV unique gene termed K1 [34]. K1 encodes a signal transducing, cell surface glycoprotein important in transformation of KSHV-infected and surrounding cells as well as induction of inflammatory proteins and downregulation of the B-cell receptor [39]. Sequence analysis revealed that K1 can vary by up to 30% at the amino acid level [36]. At the nucleotide level, 85% of polymorphisms within K1 are nonsynonymous, suggesting that strong selective pressure acts on the gene. The majority of K1 sequence variation is concentrated within two regions of the gene’s extracellular domain, variable region (VR) 1 and 2, which are 40-and 38-nucleotides respectively (Figure 1.3A) [36, 39]. Due to this high variability, K1 is routinely used to classify KSHV into at least six different genotypes (A, B, C, D, E, and F), and various sub-genotypes (Figure 1.3B) [36, 38, 40].
The 3’ terminus of the KSHV genome encodes a membrane associated signaling protein called K15, also known as latency-associated membrane protein (LAMP). The K15 protein has 12 transmembrane domains and is encoded by an alternatively spliced mRNA with eight exons [37, 41, 42]. Functional studies of K15 have shown that Src kinases as well as TRAF1, 2, and 3 interact with the cytoplasmic tail to induce multiple inflammatory cytokines and chemokines [43, 44]. K15 has also been implicated to inhibit B-cell receptor signal transduction [42]. Sequence analysis of K15 revealed three major allelic subtypes (P, M, and N) that have diverged by up to 70% at the amino acid level [37, 41]. The P allele is the predominant subtype, while the M allele is found in approximately 20% of samples and the N allele is very rare [45]. It is interesting to note, however, that despite the high divergence between alleles, the cytoplasmic tail is highly conserved and all K15 alleles contain SH2 and TRAF binding motifs (Figure 1.3C and D).

Within the central region of the KSHV genome, between the highly variable termini, sequences of nine discrete loci have been extensively analyzed [37, 45, 46]. These loci encompass approximately 5.6% of the KSHV genome and include the viral RNA T0.7 locus as well as portions of the following genes: K2, K3, ORF18/19, ORF26, K8, ORF73, and two loci within ORF75. Together, twelve KSHV genotypes have been proposed based on these nine discrete loci along with K1 and K15 [45]. However, the remaining KSHV genes, representing more than 90% of the genome, have not been used to fully characterize KSHV genetic structure and diversity.
KSHV infection and life cycle

KSHV has a typical herpesvirus particle consisting of a core, capsid, tegument, and envelope. The envelope contains conserved herpesvirus glycoproteins—gB, gH/gL, and gM/gN—as well as the less conserved ORF4 and KSHV unique K8.1 glycoproteins that are utilized in binding and entry (Figure 1.4) [47]. Immediately following infection, KSHV concurrently expresses latent and a select set of lytic genes involved in immune modulation. However, expression of lytic genes is quickly reduced, suggesting that the default program for KSHV infection is latency [48]. During latency, only a small subset of genes are expressed and the KSHV genome exists in the nucleus as an episome maintained by the latency-associated nuclear antigen (LANA/ORF73) and cellular histones [49-51]. During lytic infection, however, the entire KSHV genome is expressed in a temporally regulated fashion similar to other herpesviruses [52]. Although physiological stimuli for KSHV lytic reactivation are not fully understood, the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) and histone deacetylase inhibitor sodium butyrate are routinely used to reactivate virus replication in vitro [49, 53, 54].

Acute manifestations of KSHV infection

It is not clear whether primary infection of KSHV elicits any acute clinical symptoms. Very few studies have investigated primary infection of KSHV, as the timing of infection is difficult to ascertain in many populations. One study of immunocompetent children in Egypt reported that a febrile, maculopapular rash may be associated with primary infection [55]. However, only children with febrile syndromes of
undetermined origin were admitted into the study, potentially biasing the findings. A study from our laboratory reported that children who had rashes were also more likely to have recently undergone KSHV seroconversion [56]. However, this association was weak. Additional prospective studies of large cohorts are needed to further delineate the possibility of acute clinical symptoms associated with primary KSHV infection.

**HOST IMMUNE RESPONSE TO KSHV INFECTION**

KSHV infection elicits both humoral and cellular immune responses within infected individuals. The humoral response, however, is utilized as a marker for infection and therefore has been studied more in depth.

**Humoral immune response to KSHV**

Several KSHV proteins, expressed during both lytic and latent phases, have been shown to elicit antibody responses. In patients who have developed KS, antibody responses are nearly ubiquitous [57, 58]. However, among KSHV infected but asymptomatic individuals, antibody responses may be undetectable [59]. The predominant viral protein produced during latency is LANA [60]. Antibodies specific to LANA are regularly detected in as many as 82% of individuals who have developed KS [60-62]. Several proteins produced during lytic replication were initially reported to react with KS patient antibodies [63, 64], but the most immunodominant lytic-associated antigens identified were K8.1, a virion-associated glycoprotein, and ORF65, the minor capsid protein [58, 65-67].
In a recent study, Labo et al [68] individually expressed nearly 85% of all KSHV proteins and systematically analyzed this KSHV proteome for reactivity with antibodies in plasma of adults with KSHV-associated malignancies. The pattern of seroreactivity observed among these patients was diverse and highly variable. However, a set of antigens was identified that showed significant reactivity to antibodies in nearly all analyzed patients but not healthy controls. K8.1 produced the strongest and most frequent reactions with patient antibodies, followed by ORF65, ORF73/LANA, ORF38, ORF61, ORF59, and K5; therefore not only confirming but also expanding previous KSHV serological data and generating a powerful new serological tool [68]. Despite this in-depth characterization of antibody responses in adults, the spectrum of antibody responses has not been investigated during the early stages of infection in children or asymptomatic adults.

Whether the KSHV immunodominant antigens also elicit a neutralizing antibody response in KSHV infected individuals is not known. In fact, the clinical relevance of neutralizing antibodies during KSHV infection is unclear. As neutralizing antibodies are an important component of the humoral immune response to control progression of EBV-associated disease [69], it is important to establish the role of neutralizing antibodies against KSHV. To date, only three studies have investigated the role of neutralizing antibodies, each with conflicting results. The first two studies were conducted using a small number of KS patients from the United States. One reported that neutralizing antibodies were lower in KS patients compared to KSHV-infected asymptomatic patients [70], whereas the other study found no difference [71]. The
most recent study was conducted in a cohort of patients from Zambia, where KS is endemic, and found that neutralizing antibodies were significantly higher in KS patients compared to KSHV-infected asymptomatic patients [72]. Hence, the role of KSHV neutralizing antibodies during infection is yet to be fully elucidated.

**Cellular immune response to KSHV**

CD8⁺ cytotoxic T lymphocytes (CTLs) may also play a role in the KSHV immune response. Numerous reports have demonstrated that CTLs produce IFN-γ in response to epitopes within KSHV proteins; such as lytic proteins gB, gH, ORF25, ORF26, ORF57, ORF70, K3, K5, and K8.1, in addition to latent proteins ORF73/LANA and K12 [73-77]. Furthermore, several epitopes in gB, K8.1, ORF73/LANA, and K12 can induce both monofunctional and polyfunctional CTL responses in healthy KSHV-seropositive individuals [77]. However, very limited and somewhat conflicting data exists as to whether these CTL responses directly contribute to virus control or prevent disease progression in vivo. Guihot et al. [78] demonstrated that KSHV-specific CTL responses against LANA, K12, and K15 were weaker and detected less frequently among both AIDS-KS and classic KS patients compared to KSHV-infected asymptomatic patients. In partial contrast, a recent small study showed that CTL responses against LANA were associated with classic KS, whereas responses against K8.1 were inversely correlated with presence of KS [79]. Detailed longitudinal studies are required in order to adequately assess the direct role of KSHV-specific CTL responses in virus control and disease progression.
KSHV DETECTION METHODS

Polymerase chain reaction was utilized in the discovery of KSHV sequences from KS biopsies and therefore had the potential for use as a detection method. However, PCR only detects KSHV DNA in about 95% of KS biopsies and 52% of PBMC samples from patients clinically diagnosed with KS [60]. Low sensitivity and high expense of PCR therefore limits the routine clinical application of KSHV DNA detection for population screening. Consequently, immunofluorescence assays (IFAs) were developed using PEL-derived cells lines as a more sensitive and cost effective method to screen populations for antibodies against KSHV expressed proteins and determine global KSHV seroprevalence rates [61, 80]. As proteins that elicited strong antibody responses to KSHV infection were identified, enzyme-linked immunosorbent assays (ELISAs) were developed using recombinant KSHV peptides to measure antibodies against ORF65, K8.1 and ORF73/LANA [67, 81, 82]. ELISAs are preferable to IFA primarily because the latter is labor intensive and not easily scalable or automated. Nevertheless, the most frequently used ELISA design only utilizes two KSHV peptides whereas PEL-based IFAs are able to detect antibodies against all expressed KSHV antigens. Furthermore, ELISAs are not as sensitive as IFAs [83] and are unable to detect very low titer antibodies—thereby underestimating KSHV prevalence. In the absence of a “gold standard”, therefore, the choice of KSHV detection method is partially dependent on the population to be screened. Thus, our lab developed a monoclonal-enhanced IFA based on the BC-3 cell line with which we have obtained high sensitivity and specificity even among low antibody titer samples ([84] and described in Chapter 3).
**KSHV EPIDEMIOLOGY**

The global distribution of KSHV seroprevalence is uneven, but mirrors prevalence of KS, such that it is high in sub-Saharan Africa, moderate in the Mediterranean, and low in the United States and Western Europe (Figure 1.5, [85]). Seroprevalence rates higher than 80% have been reported in certain regions of sub-Saharan Africa. Numerous studies have detailed high seroprevalence in countries including Botswana (76-87%), Cameroon (28-62%), The Gambia (29-84%), South Africa (35%), Uganda (14-86%), and Zambia (47% - 58%) [80, 86-94]. The high variability within some sub-Saharan African countries is likely due to cohort sampling, but may also be due to lack of a standard serological assay [95]. Regardless, KSHV seroprevalence is significantly higher in sub-Saharan Africa compared to other regions. Around the Mediterranean Sea, where classic KS is prevalent, seroprevalence rates range from approximately 15 - 25% [96-98]. In the United States, Europe, and South America general KSHV seroprevalence rates are low, at approximately 5% [61, 67, 99-101]. Interestingly, certain ethnic groups show drastically higher seroprevalence rates compared to the general population. Among Amerindians in South America there is nearly 80% KSHV seroprevalence, and similar to patterns in sub-Saharan Africa, familial dependence was observed [102, 103]. Likewise, KSHV seroprevalence rates are 65% and 48% in adults and children, respectively, of the Kazak and Ughur ethnic groups in Xinjiang, China [104].

As described earlier, the KSHV glycoprotein K1 is highly variable and therefore routinely used to classify KSHV into at least six different genotypes (A, B, C, D, E, and F), and various sub-genotypes. Molecular epidemiology studies have revealed that these K1
genotypes have a distinct global distribution [36, 38, 45]. Genotypes A and C are found predominantly in the United States, Europe and the Mediterranean. The B genotype is found almost exclusively in sub-Saharan Africa, as is the A sub-genotype A5 and the rare F genotype [40, 105]. Genotype D is found commonly in KSHV-infected individuals of the Pacific Islands, including New Caledonia and the Solomon Islands [106], while the E genotype is found in the Amerindian populations of South America [102, 107].

The exact risk factors that predispose individuals, particularly children, to acquisition of KSHV are not completely understood. Uneven global distribution of KSHV seroprevalence and the increased seroprevalence in certain ethnic groups suggest that risk factors may include host genetic or environmental factors. Along these lines, Whitby et al. [108] demonstrated that 184 natural plant extracts from KSHV endemic countries are capable of reactivating KSHV replication. However, whether these extracts increase viral shedding in vivo or have any association with higher KSHV prevalence is unknown. Alternatively, malaria—a parasitic infection that is endemic in sub-Saharan Africa—was recently found to be associated with KSHV serostatus in Ugandan children [109, 110]. HIV infection is also a major risk factor for KSHV acquisition. A prospective longitudinal cohort study from our laboratory demonstrated that HIV infected children had a five-fold higher risk of acquiring KSHV compared to HIV uninfected children [59]. As described subsequently, HIV-induced immune dysregulation is hypothesized to be the major contributing factor for increased risk of KSHV infection.
KSHV TRANSMISSION

The modes of KSHV transmission appear to vary among different populations depending on seroprevalence and endemicity. In regions of low seroprevalence, sexual acts may be the primary route of transmission [24, 111, 112]. However, prepubescent children are also infected at high rates in regions of high KSHV seroprevalence, strongly suggesting a non-sexual mode of transmission [59, 113].

Sexual transmission

Among homosexual men in the United States, KSHV prevalence is associated with high number of sexual partners [24]. Moreover, sexual activity and sexually transmitted infections such as syphilis, hepatitis A, hepatitis B, and herpes simplex virus-2 are associated with KSHV infection [24, 57, 114, 115]. KSHV has been detected in semen, prostate glands, cervico-vaginal secretion, as well as saliva [57, 116-119]. These observations led to the initial hypothesis that KSHV was primarily a sexually transmitted disease in both KSHV endemic and non-endemic regions. However, a large cohort study in South Africa showed no association between KSHV infection and sexual behavior [120], suggesting that sexual transmission may only be a significant route of transmission in regions of low KSHV seroprevalence. Although transmission of KSHV among homosexual men is clearly related to sexual behavior, whether KSHV is indeed transmitted sexually or via salivary exchange in this population is less clear as the virus is infrequently detected in genital secretions but high viral burden can be detected in saliva [118, 119, 121].
Non-sexual transmission

High rates of KSHV infection observed among young children in sub-Saharan African countries led researchers to hypothesize that KSHV is transmitted via non-sexual routes. Both vertical and horizontal modes of transmission have been implicated. KSHV is frequently detected in cervical and vaginal secretions of HIV/KSHV co-infected women, suggesting that KSHV viral load in the female genital tract might influence vertical transmission of KSHV [119, 122, 123]. Rare cases of KS in newborn children further indicate that vertical transmission is possible [124, 125]. Our laboratory reported that KSHV DNA was detected in PBMCs of 2 out of 89 (2%) children born to KSHV infected mothers within 24 hours after birth—confirming that in utero or intrapartum KSHV infection can occur, albeit rarely [126].

Horizontal transmission, therefore, is most likely the predominant route of non-sexual KSHV transmission. Bloodborne transmission of KSHV has been suggested to occur at a very low rate [127, 128]. The most conclusive evidence was presented from a prospective cohort study of blood transfusion recipients in Uganda. In this study the excess risk of KSHV seroconversion after transfusion with KSHV seropositive blood was 2.8% compared to transfusion with KSHV seronegative blood—suggesting that an estimated 12 of the 425 patients were infected due to receiving KSHV contaminated blood [129]. Moreover, the risk of infection through blood transfusion decreased as the length of blood storage increased. Several case studies have also documented KSHV infection during organ transplantation, with molecular analysis demonstrating that the virus originated from the donor organ in some cases [130]. Although bloodborne
transmission of KSHV is possible, these studies also indicate that transmission via this route is rare. This is most likely due to uncommon viremia and the cell associated nature of the predominantly latent virus. Additional evidence that KSHV transmission is not primarily bloodborne includes the observation that KSHV seroprevalence is not correlated with intravenous drug use or infection by hepatitis C virus—a known bloodborne pathogen [131].

Breastmilk is a route of transmission for CMV and EBV, suggesting that KSHV might also be spread via this route [132, 133]. Indeed, an initial study showed that KSHV could be detected in breast milk samples [134]. However, a subsequent study from our laboratory did not detect any KSHV in breastmilk [135]. No additional studies have been conducted to detect KSHV DNA in breastmilk, suggesting that this route of transmission may be rare. In fact, a recent study showed that breastfeeding may even protect from KSHV infection [136].

KSHV transmission mainly occurs from mother to child or between siblings during childhood in endemic settings [137]. Accordingly, a recent study from our laboratory demonstrated that children who live in households with higher numbers of KSHV seropositive individuals have a higher risk of infection [136]. These observations suggest a route of horizontal transmission that involves close contact and familial interactions, for which salivary exchange is feasible. Soon after the discovery of KSHV, studies reported the detection of KSHV DNA in saliva of HIV infected individuals [138, 139]. KSHV found in saliva is infectious [140], and can replicate in primary oral epithelial cells [141]. Several additional studies have demonstrated that KSHV can be detected in
saliva of adults and children, regardless of HIV status, and that high KSHV burden can be detected [118, 135, 142-144]. These studies also demonstrated that KSHV shedding in adults is variable, with some individuals shedding occasionally, while others on a daily basis. Finally, specific child feeding behaviors were recently shown to be associated with early childhood KSHV infection [136]. Collectively, these studies provide strong evidence that salivary exchange is the primary route of KSHV transmission in areas of high seroprevalence.

**HIV AND KS/KSHV IN ZAMBIA**

Zambia is one of the poorest countries in Africa and also has one of the highest HIV prevalence rates in the world—with 12.5% of adults being HIV positive [145]. The overall HIV prevalence among Zambian women of childbearing age is 16%, but has been reported up to 30% from ages 25 – 35 [146]. Furthermore, mother-to-child transmission of HIV has been estimated to be as high 36% in mother-infant pairs not taking antiretroviral therapy (ART) [147]. Zambia is also one of the countries within the ‘KS belt’ [13]. Therefore, prior to the AIDS epidemic, endemic KS was prevalent in Zambia. After HIV infection became widespread, though, the number of KS cases drastically increased [148-150]. For example, by 1992, KS accounted for approximately 25% of all pediatric malignancies, with a peak incidence between one and two years of age, making it the most common childhood cancer [151]. Our laboratory also documented that KSHV infection is common among Zambian children as approximately 40% of children acquired KSHV by four years of age [59]. The large number of children infected
with KSHV in Zambia provides an opportunity to explore KSHV transmission, decipher the role of HIV and immune dysregulation, and use knowledge about KSHV transmission to aid in developing strategies to prevent infection.

**ROLE OF IMMUNE DYSREGULATION AND ANTIRETROVIRAL THERAPY ON KS/KSHV**

Numerous factors could result in the previously described associations between HIV and KS/KSHV; however, several lines of evidence suggest that immune dysregulation caused by HIV infection is a primary factor. Most significantly is the strong association between low CD4+ T-cell count and increased risk of developing KS. Biggar et al. [152] demonstrated that the risk of KS increased for every decline of 50 CD4+ cells per microliter of blood. Patients who have developed KS may also have lower CTL IFN-γ responses to KSHV epitopes compared to asymptomatic KSHV positive patients [78]. In addition to cellular immunity, HIV also disrupts normal humoral immune responses and low CD19+ cell count is associated with KS development [153]. KS in HIV infected individuals is associated with high total KSHV antibody titer and high KSHV viral load [154, 155]. Recent data from our laboratory also shows that prevalence and titer of KSHV neutralizing antibodies are higher in KS patients [72]. Together, these data indicate a loss or delay of an immune response that is able to control KSHV replication during the course of infection in HIV-positive individuals, which can lead to KS development.

Further substantiating the role of the immune system in KS development, antiretroviral therapy (ART) is closely associated with reductions in KS. The introduction
of highly active ART in 1996 coincides with decreases of KS incidence in HIV-infected adults by 80% and 85% in the United States and Europe, respectively [152, 156]. Moreover, patients who developed KS while on ART had significantly lower CD4+ T-cell counts and higher HIV viral loads at ART initiation [156]. Furthermore, a meta-analysis of patients with low risk KS tumors (T0) indicated that 81% of patients treated by ART without KS-specific chemotherapy exhibited regression of KS [157], suggesting that immune reconstitution plays an important role in preventing KS progression.

Despite data demonstrating the role of HIV-induced immune dysregulation and ART on KS, very little is known about the effect of ART on KSHV infection prior to KS development, or whether an effective immune response during early infection can limit KSHV replication and subsequently the development of KS. High KSHV viral load in PBMC and high titer lytic antibodies are two strong predictors of disease progression to KS [155, 158, 159], but the effect of ART on these predictors, particularly during acute KSHV infection, is yet to be fully elucidated. In a small cohort of HIV-infected homosexual men, KSHV viral loads were lower in patients undergoing ART and KSHV viral load decreased after ART initiation [160]. Other studies demonstrated less frequent detection of KSHV DNA in PBMC, plasma, and the oral cavity after ART initiation, but did not show a reduction in KSHV load [161, 162]. Current reports on the effect of ART on KSHV antibodies indicate increases in both anti-lytic and –latent antibodies after ART initiation [161, 163], but whether these antibodies are neutralizing and effective in reducing viral replication was not investigated. Taken together, the effects of ART on
KSHV infection are not well understood, and no data exists regarding the effects of ART on the immune response against KSHV during the early stages of infection.

RESEARCH AIMS

The overall objective of this dissertation research was to determine the epidemiological, immunological, and viral factors that may affect KSHV transmission to young children in a region where KSHV is endemic and HIV is epidemic. Important factors that may influence KSHV transmission include those associated with 1) the source of transmission (donor), 2) the newly infected individual (recipient), and 3) the virus itself. Donor-associated factors include viral burden in the oral cavity and the amount of contact between the donor and recipient, since saliva is implicated as the primary route of KSHV transmission [134, 135]. As HIV infection is associated with increased risk of KSHV infection [59], recipient-associated factors include immune function and concurrent infections by other pathogens. Conversely, virus-associated factors would primarily be related to genetic variations that lead to functional modifications in viral products such as microRNAs, entry machinery, or immunomodulatory proteins [31]. KSHV utilizes a complex entry process [47] and encodes multiple immunomodulatory genes [164]; consequently, changes in these proteins may alter pathogenesis and transmission. This project, therefore, employed a multifaceted design—utilizing three separate Zambian cohorts while employing molecular and epidemiological techniques—to investigate the range of factors that affect KSHV transmission to children. Our hypotheses were as follows:
Individuals other than a child’s primary caregiver serve as KSHV transmission donors.

Prevention of HIV-induced immune suppression by early ART reduces KSHV infection rates among HIV-infected children to a rate similar among HIV-uninfected children.

HIV-infected children on ART have a more robust humoral immune response to KSHV and therefore lower levels of KSHV replication compared to HIV-infected children not taking ART. The KSHV specific humoral response among HIV-infected children on ART is similar to HIV-uninfected children.

Distinct KSHV variants are present in sub-Saharan Africa compared to Western countries.

To test these hypotheses the following specific aims were pursued:

1) Elucidate the role of household members as transmission sources for early childhood infection of KSHV in an endemic setting

2) Evaluate the impact of ART on KSHV acquisition in a prospective cohort of young Zambian children.

3) Delineate the role of ART on the KSHV-specific humoral immune response and the effect it has on KSHV lytic replication during the early stages of infection in HIV-infected children.

4) Characterize KSHV genome-wide diversity from a geographical region where KSHV and KS are endemic.
Since a vaccine against KSHV is not currently available, and efforts to develop one are limited, it is paramount to develop alternative strategies to prevent KSHV acquisition during early childhood. The findings from this study will be important for developing and establishing such public health strategies to help reduce KSHV spread among young children. This, in turn, will help reduce the burden of KS among young children and adults in endemic settings.
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FIGURE LEGENDS

Figure 1.1. Figure from Chang et al. [34]. Radial, unrooted phylogenetic tree of amino acid sequences from 12 herpesvirus major capsid proteins using neighbor joining method with 100 bootstrap replicates. Phylogenetic tree shows the relationships between members of the alpha-, beta-, and gammaherpesvirus subfamilies.

Figure 1.2. KSHV genomic map from Mesri et al. [85]. Open reading frames (ORFs) with homology to herpesvirus saimiri were named consecutively, ORF4 – ORF75, whereas KSHV unique genes were given a “K” designation. KSHV encodes at least 87 ORFs and 17 microRNAs (purple boxes). Sense and antisense ORFs are designated in blue and red, respectively. The predominant genes expressed during latency are represented in green, and the multiple ORFs that encode cellular orthologues are indicated by yellow boxes.

Figure 1.3. KSHV variable genes K1 and K15. (A) Structure of the K1 protein. K1 consists of an N-terminal extracellular domain, a transmembrane domain, and a cytoplasmic C-terminal domain with an immunoreceptor tyrosine-based activation motif (ITAM). Locations of the variable regions (VR) 1 and 2 are noted on the extracellular domain. Figure from Brinkman and Schulz [41]. (B) Radial phylogenetic tree for selected KSHV K1 amino acid sequences. The diagram shows the relationships among the primary KSHV genotypes indicated by arcs. Figure from Hayward and Whitby [42]. (C) Structure of the K15 protein. K15 is composed of 12 transmembrane domains and a C-terminal cytoplasmic domain. While the transmembrane domains of K15 are highly variable, the
cytoplasmic domain is highly conserved and all alleles contain SH2 and TRAF binding motifs. Figure from Brinkman and Schulz [41]. (D) Radial phylogenetic tree for representative amino acid sequences of the K15 alleles illustrating the relationships among the highly diverged K15 alleles.

**Figure 1.4.** KSHV infection and life cycle. (1) KSHV utilizes several virion associated glycoproteins for attachment and entry; including gB, gH/gL, gM/gN, ORF4, and K8.1. (2) KSHV enters the cell and is shuttled to the nuclear membrane [49]. (3) Upon nuclear delivery of the linear genome, latent and a restricted set of lytic genes involved in immune modulation are concurrently expressed [50]. (4) Lytic gene is quickly reduced and latency is established soon after infection, suggesting that latency is the default program [50]. During latency, only a small subset of genes are expressed and the KSHV genome exists as an episome, attached to cellular chromatin, maintained by KSHV LANA/ORF73 and cellular histones [51-53]. (5) Physiological (unknown) or chemical (e.g. TPA, sodium butyrate) stimuli can induce lytic reactivation of KSHV [55]. (6) During lytic reactivation, the entire KSHV genome is expressed in a temporally regulated fashion similar to other herpesviruses [54] and the genome is replicated, encapsulated, and transported out of the nucleus. (7) The KSHV capsid then acquires the virion envelope and is transported within vesicles to the cell surface. (8) Virions are released into the extracellular space [29].
Figure 1.5. Figure from Mesri et al. [85]. (A) Global distribution of KS in males. (B) Global distribution of KSHV seroprevalence. White indicates data not available.
Figure 1.1
Figure 1.2
Figure 1.3
Figure 1.4

1) Virus attachment
2) Entry
3) Latent and restricted lytic gene expression
4) Latency established
5) Stimuli
6) Lytic reactivation
7) Envelopment
8) Virus release
Figure 1.5
CHAPTER 2

EARLY CHILDHOOD INFECTION OF KAPOSI’S SARCOMA-ASSOCIATED HERPESVIRUS IN ZAMBIAN HOUSEHOLDS: A MOLECULAR ANALYSIS

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ABSTRACT

Sub-Saharan Africa is endemic for Kaposi’s sarcoma-associated herpesvirus (KSHV) and there is a high rate of early childhood infection; however, the transmission sources are not well characterized. We examined household members as potential KSHV transmission sources to young children in the KSHV-endemic country of Zambia. To this end, we enrolled and followed Zambian households with at least one KSHV-seropositive child and collected longitudinal buccal swab samples. KSHV burden was evaluated and K1 sequences from the children were determined and analyzed for differences to K1 sequences from household members. The K1 sequences were also analyzed for evolution over time. We generated K1 sequences from 31 individuals across 16 households. Nine households contained multiple KSHV-positive members, including at least one child. In 6 of 9 households, the child had 100% sequence identity to all household members. However, in two households the child and mother had distinct K1 sequences. In the remaining household, the children were the only KSHV-infected individuals. Furthermore, we report that 1 of 18 individuals had K1 sequence variation within the timespan analyzed. In the present study, we provide evidence that (1) early childhood KSHV transmission occurs from both within and outside the household, (2) intra-household transmission can occur via non-maternal sources, (3) viral shedding in the buccal cavity is highly variable, and (4) the dominant K1 sequence within an individual did not rapidly evolve over time. These results are important for developing KSHV intervention strategies.
INTRODUCTION

Kaposi’s sarcoma-associated herpesvirus (KSHV), or human herpesvirus-8 (HHV-8), is the etiological agent of all forms of Kaposi’s sarcoma (KS), along with primary effusion lymphoma and multicentric Castleman’s disease [1-5]. Global seroprevalence of KSHV is uneven; it is low in the United States and Western Europe, moderate in the Mediterranean, and as high as 80% in sub-Saharan Africa [6-9]. Zambia is one such country in sub-Saharan Africa stricken by high KSHV and KS prevalence, and with the onset of the HIV-1 epidemic, a drastic increase in KS cases was seen in both adults and children [10, 11]. By 1992, KS accounted for approximately 25% of all childhood cancers diagnosed in Lusaka, the capital of Zambia, making KS one of the most frequently diagnosed cancers [12].

Since a KSHV vaccine is not likely to be available in the near future, elucidating the route and people involved as sources of transmission is imperative for the development of strategies to reduce KSHV spread and infection rates in endemic areas such as Zambia. Our previous study established that early childhood infection by KSHV is common in Zambia: approximately 40% of children were infected by four years of age [13]. We and others have also shown that KSHV DNA is frequently detectable in saliva, indicating salivary contact as the major route of horizontal transmission to children in endemic regions [7, 14, 15]. Nevertheless, the individuals involved as sources of KSHV transmission to children have yet to be identified. Previous studies of KSHV transmission have primarily focused on mother-to-child transmission, but salivary contact with children is not limited to the child’s mother. Moreover, we have shown that KSHV
incidence is similar in children born to KSHV-positive and -negative mothers, suggesting that KSHV transmission can come from sources other than the mother [13]. Therefore, to develop adequate KSHV intervention strategies in endemic areas, it is essential to evaluate the role of all personal contacts, including household members, as potential viral transmission sources during early childhood.

Despite a high level of genomic conservation, KSHV contains a gene on the extreme left-hand end of the genome, K1, that is highly variable [16]. K1 is an 870-nucleotide gene that encodes a signal transducing, cell surface glycoprotein important in transformation of KSHV-infected and surrounding cells as well as induction of inflammatory proteins and downregulation of the B-cell receptor [17]. The majority of K1 sequence variation is concentrated within two regions of the gene’s extracellular domain, variable region (VR) 1 and 2, which are 40- and 38-nucleotides respectively [16]. Due to its high variability, K1 is routinely used to classify KSHV into at least five different genotypes (A, B, C, D, and E), and various sub-genotypes [16, 18].

Using molecular analysis of K1, we examined a longitudinal cohort of KSHV-seropositive children and all members of their households in the KSHV endemic country of Zambia—making this study the first of its kind. Here, we provide evidence that transmission of KSHV to children can occur from both within and outside the household and intra-household transmission may occur via non-maternal sources. Furthermore, we report that superinfection was not detected in any individuals, and the dominant K1 sequence in the buccal cavity of an individual did not rapidly change over time. Additionally, we analyzed KSHV burden in the buccal cavity longitudinally and report
that viral shedding is highly variable within an individual over time. Our findings have important implications for the development of strategies to prevent KSHV transmission to young children in endemic regions.

**METHODS**

**Study Population**

In the present study, participants were recruited from various compounds within Lusaka District, Zambia. We enrolled and followed all willing and eligible complete households (n = 134,455 individuals) during September 2004 to November 2009. Eligible complete households were defined as family units in which (1) all related individuals who resided in the same dwelling agreed to participate in the study and (2) there was at least one KSHV-seropositive child under the age of four years (index child). Family units described in this study will herein be denoted as “households.” Venous blood and buccal swab samples were collected from each index child and mother every four months and from all other household members once a year at the University Teaching Hospital (UTH) in Lusaka for up to four years. KSHV serostatus was determined for each household member by monoclonal-enhanced immunofluorescence assay, as previously described [19]. Additionally, presence of HIV-1 antibodies was determined for each individual as described previously [20]. Trained nurses from the UTH provided information about the study and obtained written informed consent from participants or their guardians. This study was approved by the Institutional Review Board at the University of Nebraska-Lincoln and the Ethics Committee of the University of Zambia.
**DNA extraction and polymerase chain reaction for KSHV detection**

DNA was extracted from buccal swab samples using the Puregene Genomic DNA Purification Kit (Qiagen) according to manufacturer’s protocol. Extracted DNA was subjected to polymerase chain reaction (PCR) using primers for human β-actin (Actin1 [5’-TTCTACAATGAGCTGCGTGT-3’] and Actin2 [5’-GCCAGACAGCACTGTGTTGG-3’]) or GAPDH (GAPDH1 [5’-CCATGGAGAAGGCTGGGG-3’] and GAPDH2 [5’- CAAAGTTGTCATGGATGACC-3’]). Subsequently, PCR-positive samples were analyzed by nested PCR for presence of KSHV DNA using previously described primers for the ORF26 gene [21]. Each PCR reaction was performed in a total volume of 25 μl using 0.4 μM primers and TaKaRa Ex Taq DNA polymerase kit (TaKaRa Biotechnology) according to manufacturer’s protocol, with the exception of 2.5 units enzyme. For β-actin and GAPDH reactions, 1 μl genomic DNA was used. For first- and second-round ORF26 PCR, 2 μl genomic DNA and 2 μl PCR product were used, respectively. All reactions were performed using the following conditions: 95°C for 5 min, 35 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec, and one cycle of 72°C for 7 min.

**K1 sequencing**

Nested primers were used to PCR amplify the K1 gene from KSHV DNA-positive buccal swab samples: outer primers K1-R2 (5’-AGTACCAATCCACTGGTTGCG-3’) and K1-1Wh (5’-TGTCCTTCAGACCTTTGTTGG-3’); and inner primers K1-F1 (5’- ATGTTCCTGTATGTTGCTGC-3’) and K1-4Wh (5’-TGGTTGCGTATAGTCTTCCG-3’). K1 PCR reaction conditions were similar to those described above, except the elongation
temperature was 68°C. K1 PCR products were gel purified using the QIAQuick Gel
Extraction Kit (Qiagen) according to manufacturer’s instructions. Purified K1 PCR
products were sequenced with primers K1-F1 and OLK1R2 (5’-GCACGTGTGGTTTGAGTCAC-3’) using the BigDye Terminator v3.1 Cycle Sequencing Kit
and ABI Prism 3100-Avant DNA Sequencer (Applied Biosystems) according to
manufacturer’s protocols.

**Sequence analysis**

A 624-nucleotide sequence (positions 100-723) that encompasses VR1 and VR2
of the K1 gene was examined. All sequences were analyzed using BLAST (National
Center for Biotechnology Information) to ensure the amplicon was K1. Subsequently, K1
sequences from KSHV-infected individuals were aligned to sequences from members of
the same household and inspected for differences using Vector NTI software (v11.0,
Invitrogen). A maximum likelihood tree was constructed for all households using MEGA
(v5.04). Additionally, longitudinal K1 sequences from each individual were aligned and
inspected for differences using Vector NTI software. KSHV genotype of each individual
was determined by K1 sequence alignment to reference strains from GenBank and
construction of a maximum likelihood tree using MEGA (v5.04). K1 reference sequences
used for analysis were AF133038 (A1), AF130305 (A2), U86667 (A3), AF133039 (A4),
AF178823 (A5), AF133040 (B), AF133041 (C1), AF133042 (C3), AF133043 (D1), AF133044
(D2), and AF220292 (E). The K1 sequences generated in this study are available from
GenBank under accession numbers JQ422520 to JQ422550.
**Quantitative real-time PCR**

Longitudinal samples with K1 sequence data were also analyzed by a duplex quantitative real-time PCR (qPCR) assay developed to simultaneously quantitate the number of KSHV genomic copies and cellular equivalents in each sample. All qPCR template samples were subjected to phenol-chloroform purification, according to standard methods, to ensure maximum purity for accurate qPCR analysis. Briefly, at least three rounds of phenol-chloroform and one chloroform purification, followed by ethanol precipitation, were performed on each DNA sample. qPCR reactions were performed using Taqman chemistry (Applied Biosystems). Each 25 μl reaction mixture contained the following: 100 ng genomic DNA template, 300 nM and 200 nM ORF26 forward and reverse nested primers respectively, 200 nM ORF26 dual-labeled probe RT26p (5'-FAM-CCATGGTCTGCGGCACGCA-BHQ1-3'), 30 nM each β-globin primer described elsewhere [22], 200 nM β-globin dual-labeled probe BGX1 (5'-HEX-CTCCTGAGGAGAAGTCTGCCGTTACTGCC-BHQ1-3'), 1x Taqman buffer, 5 mM MgCl₂, 0.4 mM each dNTP, 0.0125 units Amplitaq Gold, and 0.01 units uracil-DNA glycosylase (UNG). All reactions were performed with the BioRad iCyclerIQ using the following conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec, 55°C for 1 min. The standard for KSHV was generated using a sequence of the ORF26 gene cloned into the pCR2.1 vector (pCR2.1.ORF26) according to the manufacturer’s protocol (Invitrogen). pCR2.1.ORF26 copy number was calculated based on molecular mass. The β-globin standard was generated using genomic DNA extracted from the 8E5 cell line.
and copy number was calculated by qPCR to a known laboratory standard. The duplex standard was generated by mixing calculated copy numbers of pCR2.1.ORF26 and 8E5 genomic DNA in Tris-EDTA, followed by five serial 10-fold dilutions. The cycle threshold values from PCR amplification of ORF26 and β-globin in the duplex standard were used to generate a curve for each amplicon. The duplex standard curve was run in parallel with buccal swab samples for each reaction. KSHV copy number was calculated for each well and normalized to the β-globin equivalent. All samples were run in triplicate and the mean KSHV copy number per 10,000 cellular equivalents was calculated.

RESULTS

**KSHV screening and genotype analysis**

In the present study, we examined buccal swab samples from individuals \((n = 455)\) of 137 complete households. Among these, 94 individuals from 43 households had KSHV DNA detectable by PCR in the buccal cavity and we attempted to amplify K1 for sequence analysis from these samples. Of the 94 KSHV DNA-positive individuals, the K1 gene was successfully amplified and sequenced in 31 individuals (12 index children and 19 household members) across 16 households (Figure 2.1). A maximum likelihood tree with 11 K1 reference sequences was generated for KSHV genotype analysis of the 31 K1 sequences (Figure 2.2). As expected, sequences from all 31 individuals clustered with genotypes A5 \((n = 17)\) or B \((n = 14)\), which are the most prevalent KSHV genotypes in Africa [23].
**KSHV transmission in complete households**

Of the 16 households containing individuals in which K1 was sequenced, nine were examined for intra-household transmission of KSHV to children because K1 sequence data from the index child and at least one other household member was obtained. Table 2.1 summarizes demographic information collected from all members of these nine households (12 index children and 30 household members). Of note, all 12 children had detectable levels of KSHV antibodies in sera and 11 had detectable KSHV DNA shedding in the buccal cavity. Twenty-five of the 30 household members were KSHV-seropositive, of which 15 had detectable levels of KSHV DNA in the buccal cavity for at least one time point during the study. We were then able to obtain K1 sequence from all 11 KSHV DNA-positive index children and 13 of the 15 household members for at least one time point. Household members from whom K1 sequences were obtained were mothers and older siblings as KSHV DNA was not frequently detected in the buccal cavities of other household members.

K1 sequences from the 11 index children and 13 household members of these nine households were aligned with A5 and B reference sequences and differences represented phylogenetically (Figures 2.3a and 2.3b). In six households (310, 402, 565, 602, 611, and 638), the index child had 100% K1 sequence identity to all analyzed household members (Figure 2.3a). Additionally, members from each household clustered to a distinct phylogenetic group with no overlap between households, providing evidence that cross-contamination between samples did not occur.
Interestingly, in three other households (163, 488, and 519) the index child had 100% K1 sequence identity to a sibling but not the mother. For household 163, the K1 sequence of the mother was distinct from her two children, with three amino acid substitutions. For household 488, the mother’s K1 sequence differed from her children’s by 56 amino acids, consequently belonging to a different KSHV genotype: A5 versus B (Figures 2.3b and 2.3c). For the remaining household (519), the two children had identical KSHV K1 sequence, despite the fact that the only other household member was KSHV-negative as determined by both antibody and DNA detection (Table 2.1 and Figure 2.3b).

**Longitudinal quantification of KSHV in the buccal cavity**

To further explore factors that may affect KSHV transmission within the household, we used qPCR to quantify KSHV burden in 25 of the 31 KSHV genotyped individuals, each of whom had KSHV-positive buccal swab samples at more than one time point (Figure 2.1). Lack of sufficient quantities of sample or absence of KSHV DNA in previous detection assays prevented us from quantifying KSHV copy numbers for all time points. Figure 2.4a illustrates the KSHV shedding levels in the buccal cavity for each of the 25 individuals analyzed. Of note, 76% of individuals analyzed had levels of KSHV shedding that varied substantially. Furthermore, six individuals had undetectable KSHV shedding for at least one time point, which was preceded or succeeded by detectable levels of KSHV.
To examine whether HIV-1 infection increases KSHV shedding in the buccal cavity, the mean number of KSHV copies/10^4 cells for all time points of each individual was compared between HIV-1 infected (n = 10) and uninfected (n = 15) individuals (Figure 2.4b). Individuals who were HIV-1 positive had a marginally higher mean and median level of KSHV buccal shedding compared to uninfected individuals. However, there was no significant difference (p = 0.57) due to high variation and a small number of samples.

**Lack of K1 genetic diversity over time within KSHV-infected individuals**

We also sought to determine whether the dominant KSHV genotype in an individual changes over time, whether these changes correlate with transmission of variant genotypes within the same household and whether superinfection by other KSHV variants can occur. For each individual that we obtained K1 sequences from at least two samples over a minimum of 12 months (n = 18), we inspected sequences for evolution over time. Table 2.2 summarizes the number of samples analyzed, timespan of samples, and whether K1 sequence varies over time within each individual. Surprisingly, 17 of the 18 individuals had no variation in the dominant K1 sequence over the period analyzed, even up to 40 months. Only one individual (163-I01) had detectable K1 sequence variation: a single adenosine/guanosine mixed nucleotide that resulted in a cysteine/tyrosine mixture at the 100th amino acid position. However, the sequence variation was only observed at the sixth of seven follow up time points over the course
of a 28-month period. At the subsequent time point, the variant reverted to the original sequence resulting in no net change in the dominant K1 sequence.

**DISCUSSION**

The principal objective of the present study was to elucidate the role of household members as transmission sources for early childhood infection of KSHV in an endemic setting. To this end, we used molecular analysis of viral sequences to link donor and recipient pairs. Our analysis of household K1 sequences revealed that in 6 of 9 households the index child had 100% K1 sequence identity to all other household members examined. This suggests that intra-household transmission is frequent and household practices common to all members within the household are the means by which KSHV contaminated saliva is transmitted. Indeed, Butler et al. [24] showed that sharing food and/or sauce plates with other household members is associated with a child being KSHV seropositive. Therefore, if an infected household member is shedding KSHV in saliva, sharing household food/sauce plates may provide a route for transmission to all other household members.

Additionally, three households provided evidence of extra-household KSHV transmission. For two households (488 and 519), we are confident that the index child and sibling did not acquire KSHV infection from the mother as there was high K1 sequence divergence within household 488 and no maternal KSHV infection for household 519. For household 163, the K1 sequences obtained from the children and mother varied by <1.5%. Differences in K1 sequences could be a result of viral evolution.
in the children as suggested by Mbulaiteye et al. [25] or a minor KSHV variant, undetectable by direct sequencing, may have been transmitted. Nevertheless, the observation that both children in each household had identical K1 sequences distinct from their mothers’ suggests that these children did not acquire KSHV infection from their mothers. Due to dense living conditions within compounds of Lusaka District, it is likely that these children acquired distinct KSHV variants from personal contact with community members outside of the household, which were not analyzed in the present study. Thus, our findings provide molecular evidence to support what previous reports have suggested: household members other than the mother, as well as personal contacts from outside the household may play an important role in KSHV transmission to children [24, 26, 27].

We also report that viral burden in the buccal cavity is highly variable over time (e.g. from undetectable to high levels, and vice versa) for the majority of household members, highlighting the importance of longitudinal observation in KSHV transmission studies. This is consistent with reports in longitudinal cohorts of men who have sex with men in low infection prevalence settings [28, 29]. Within our cohort, adults had periodic and robust lytic KSHV infection in the buccal cavity, which could increase the opportunity for transmission to children. Additionally, the high variability of viral shedding may partially explain a lack and/or inconsistency of correlation between KSHV infection and specific behaviors in which children are exposed to saliva, making it more difficult to identify the common practices that may increase the spread of KSHV in endemic areas.
Given the sequence diversity observed within K1, it is unclear whether viral quasispecies can develop in individuals during the course of KSHV infection. In the present study, we found that the dominant sequence of K1 in the buccal cavity of an individual did not change rapidly within the time analyzed. We observed sequence variation in only 1 of 18 individuals, suggesting that K1 evolution can occur within an individual; however, this variation was not sustained over time. Forward and reverse sequences were of excellent quality, indicating that the variation detected was genuine. However, it is possible that the mutation detected was a PCR artifact. In support of our finding that K1 evolution is possible within an individual, two recent studies have demonstrated that multiple KSHV variants and even closely related genotypes can exist within the same individual at a given time point [25, 30]. In contrast to our study, these groups sequenced individual K1 clones. This method is advantageous for detecting minor sequence variants; nevertheless, a small number of clones can misrepresent the proportion of variants present in the individual. Hence, high quality direct sequencing of PCR product is ideally suited to determine the dominant sequences present within a sample. Our findings suggest that although ongoing evolution of the K1 gene can occur in an individual, the level of variants is minor in the scope of the individual’s entire viral population and that K1 evolution does not induce a shift in the dominant viral genotype within an individual over the analyzed time.

One limitation of our study is the small final sample size. In our cohort, we detected KSHV DNA via PCR in 94 out of 455 individuals. This is not unexpected as not all household members were KSHV-seropositive. Moreover, a number of previous reports
demonstrated that KSHV DNA is not always detectable in the buccal cavity of KSHV-seropositive individuals [14, 15, 25]. Further limiting the number of households available for analysis, the K1 gene has high sequence variability resulting in PCR detection and sequencing that is not as sensitive as for the conserved ORF26 gene. Another limitation of our study is the use of households where children were already KSHV-seropositive, preventing us from directly correlating levels of household member KSHV shedding in the buccal cavity to the time of childhood infection.

Despite these limitations, our study is significant as we were able to analyze samples from complete households in a longitudinal cohort within an endemic area. The cohort analyzed was representative of urban families in Zambia, and to our knowledge child care practices do not differ significantly in the rural setting. However, to further substantiate our findings in other endemic areas, it will be important to correlate KSHV infection in household/community members with viral transmission to young children in those areas. The findings of our study are important for developing strategies—such as behavior modification—to prevent KSHV transmission during early childhood and therefore reduce KS incidence in endemic areas.
ACKNOWLEDGMENTS

We thank all study members from Lusaka for their participation in the study. We thank Veenu Minhas, Kay Crabtree, and all community health workers and laboratory staff at UTH for their contributions to recruitment, data management, and sample processing. We also thank Sandra Gonzalez for technical assistance and Jesse Thompson for helpful discussion. This work was supported by the National Institutes of Health (NIH; RO1 CA75903, T32 AIO60547, and P30 GM103509 to C.W.) and the Fogarty International Center (D43 TW01492 to C.W.). L.N.O. is supported by the NIH under a Ruth L. Kirschstein National Research Service Award from the National Institute of Allergy and Infectious Diseases, and C.G. is a Fogarty Fellow.
REFERENCES


FIGURE LEGENDS

Figure 2.1. Flowchart summarizing sample analysis from a longitudinal cohort of Zambian households with at least one KSHV-positive child. Inset boxes indicate number of households comprised of analyzed individuals. N/A, not applicable.

Figure 2.2. Phylogenetic analysis of K1 amino acid sequence to determine KSHV genotype of each individual. A maximum likelihood tree was generated with K1 sequences generated in this study and 11 prototypic reference sequences obtained from GenBank. Sequences generated in this study are boxed and reference sequences are labeled A1, A2, A3, A4, A5, B, C1, C3, D1, D2, and E.

Figure 2.3. Alignment of K1 sequences from members of nine households where sequence data was obtained from the index child and at least one other household member. Maximum likelihood phylogenetic tree of K1 nucleotide sequence from individuals indicating intra-household (a) and extra-household (b) transmission of KSHV to children. (c) K1 amino acid alignment for individuals of households 163 and 488. H02 designates the mother in each household.

Figure 2.4. Longitudinal quantification of KSHV burden by quantitative real-time PCR analysis of buccal swab samples. Buccal swab samples were collected from each index child and mother every four months and from all other household members once a year at UTH in Lusaka for up to four years. (a) Twenty-five individuals who had KSHV-positive
buccal swab samples by PCR at two or more time points were analyzed. Index children are denoted with the letter “I”, and household members are denoted with the letter “H”. KSHV burden is reported as the number of KSHV genomes per $10^4$ cellular genomes with a detection limit of 10 KSHV genomes. (b) Box and scatter plots of mean KSHV burden in buccal swab samples from 25 HIV-1 negative and positive individuals. Mean (X) and median (—) values are shown. $p = 0.57$, two-sided Student’s t-test.
<table>
<thead>
<tr>
<th>Study ID</th>
<th>Relation to Index Child</th>
<th>Sex</th>
<th>Age at Baseline</th>
<th>HIV-1 Status</th>
<th>KSHV Serostatus</th>
<th>KSHV Buccal Shedding</th>
<th>KSHV K1 Sequence</th>
<th>KSHV Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>163-I01</td>
<td>Index</td>
<td>Male</td>
<td>17 mo</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>A5</td>
</tr>
<tr>
<td>163-I02</td>
<td>Index</td>
<td>Female</td>
<td>7 mo</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>163-H02</td>
<td>Mother(^a)</td>
<td>Female</td>
<td>30 yr</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>A5</td>
</tr>
<tr>
<td>163-H03</td>
<td>Sister</td>
<td>Female</td>
<td>12 yr</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>163-H04</td>
<td>Brother</td>
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<td>9 yr</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>A5</td>
</tr>
<tr>
<td>163-H05(^b)</td>
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<td>Male</td>
<td>45 yr</td>
<td>+</td>
<td>+</td>
<td>N/A</td>
<td>-</td>
<td></td>
</tr>
<tr>
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<td>Male</td>
<td>12 mo</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>A5</td>
</tr>
<tr>
<td>310-H02</td>
<td>Mother(^a)</td>
<td>Female</td>
<td>28 yr</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>A5</td>
</tr>
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<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>310-H04</td>
<td>Brother</td>
<td>Male</td>
<td>4 yr</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>A5</td>
</tr>
<tr>
<td>310-H05</td>
<td>Brother</td>
<td>Male</td>
<td>7 yr</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>A5</td>
</tr>
<tr>
<td>402-I01</td>
<td>Index</td>
<td>Male</td>
<td>21 mo</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>B</td>
</tr>
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<td>-</td>
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<td>+</td>
<td>+</td>
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<tr>
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<td>Mother(^a)</td>
<td>Female</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>B</td>
</tr>
<tr>
<td>402-H03</td>
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<td>Male</td>
<td>32 yr</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>488-I01</td>
<td>Index</td>
<td>Male</td>
<td>8 mo</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>B</td>
</tr>
<tr>
<td>488-H02</td>
<td>Mother(^a)</td>
<td>Female</td>
<td>30 yr</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>A5</td>
</tr>
<tr>
<td>488-H03</td>
<td>Brother</td>
<td>Male</td>
<td>8 yr</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>488-H04</td>
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<td>Female</td>
<td>5 yr</td>
<td>-</td>
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<td>+</td>
<td>+</td>
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<tr>
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<td>Male</td>
<td>21 mo</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>B</td>
</tr>
<tr>
<td>519-H02</td>
<td>Mother(^a)</td>
<td>Female</td>
<td>24 yr</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>519-H03</td>
<td>Brother</td>
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<td>3 yr</td>
<td>-</td>
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Table 2.1 (continued). Demographic Characteristics of Complete Households with K1 Sequence Data from Index Child and At Least One Other Household Member

<table>
<thead>
<tr>
<th>Study ID</th>
<th>Relation to Index Child</th>
<th>Sex</th>
<th>Age at Baseline</th>
<th>HIV-1 Status</th>
<th>KSHV Serostatus</th>
<th>KSHV Buccal Shedding</th>
<th>KSHV K1 Sequence</th>
<th>KSHV Genotype</th>
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<tbody>
<tr>
<td>565-H02</td>
<td>Mother&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Female</td>
<td>29 yr</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>A5</td>
</tr>
<tr>
<td>565-H03</td>
<td>Brother</td>
<td>Male</td>
<td>7 yr</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>565-H04</td>
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<td>Female</td>
<td>4 yr</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>602-I01</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
<td>A5</td>
</tr>
<tr>
<td>602-H02</td>
<td>Mother&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Female</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>602-H03</td>
<td>Father</td>
<td>Male</td>
<td>47 yr</td>
<td>+</td>
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<tr>
<td>602-H04</td>
<td>Brother</td>
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<td>10 yr</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td>-</td>
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<td>-</td>
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</tr>
<tr>
<td>611-I01</td>
<td>Index</td>
<td>Male</td>
<td>18 mo</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>B</td>
</tr>
<tr>
<td>611-H02</td>
<td>Mother&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Female</td>
<td>29 yr</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>B</td>
</tr>
<tr>
<td>638-I01</td>
<td>Index</td>
<td>Male</td>
<td>18 mo</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>A5</td>
</tr>
<tr>
<td>638-H02</td>
<td>Mother&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Female</td>
<td>18 yr</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>A5</td>
</tr>
<tr>
<td>638-H03</td>
<td>Grandmother</td>
<td>Female</td>
<td>45 yr</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>638-H04</td>
<td>Uncle</td>
<td>Male</td>
<td>4 yr</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>638-H05</td>
<td>Aunt</td>
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<td>23 yr</td>
<td>-</td>
<td>+</td>
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<tr>
<td>638-H06</td>
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<td>638-H07</td>
<td>Cousin</td>
<td>Male</td>
<td>2 yr</td>
<td>-</td>
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</table>

Abbreviations: HIV-1, human immunodeficiency virus type 1; KSHV, Kaposi’s sarcoma-associated herpesvirus; mo, month; yr, year; N/A, not applicable.

<sup>a</sup>Primary caregiver to index child.

<sup>b</sup>Buccal swabs were not collected from this individual.
Table 2.2. K1 Evolution Over Time

<table>
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<tr>
<th>Study ID</th>
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<th>Samples</th>
<th>Timespan (Months)</th>
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<td>-</td>
</tr>
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<td>16</td>
<td>-</td>
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<tr>
<td>638-I01</td>
<td>A5</td>
<td>7</td>
<td>28</td>
<td>-</td>
</tr>
<tr>
<td>565-H02</td>
<td>A5</td>
<td>7</td>
<td>32</td>
<td>-</td>
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<td>219-H03</td>
<td>B</td>
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</tr>
<tr>
<td>488-H02</td>
<td>A5</td>
<td>8</td>
<td>40</td>
<td>-</td>
</tr>
</tbody>
</table>

*Variation was not sustained over time.*
Figure 2.1

- Total Individuals: n = 455
  - 137 Households
    - KSHV ORF26 DNA Detected: n = 94
      - 43 Households
        - K1 Genotyping: n = 31
          - 16 Households
            - Household Transmission Analysis: n = 24
              - 9 Households
            - Longitudinal KSHV Quantification: n = 25
              - N/A
            - K1 Genetic Diversity Analysis: n = 18
              - N/A
Figure 2.2
Figure 2.4

(a) Study ID vs. Months after first time point.

(b) Mean KSHV genome copies/10^4 Cells (Log).

Legend:
- Black: Undetectable
- Yellow: <10^5
- Red: 10^5 - 10^6
- Green: >10^6
- Gray: Missing
CHAPTER 3

EFFECTS OF ANTIRETROVIRAL THERAPY ON KAPOSI’S SARCOMA-ASSOCIATED HERPESVIRUS TRANSMISSION AMONG HIV-INFECTED ZAMBIAN CHILDREN.

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ABSTRACT

**Background.** The risk of Kaposi’s sarcoma associated herpesvirus (KSHV) acquisition among children is significantly increased by HIV infection. Antiretroviral therapy (ART) was recently made widely available to HIV-infected children in Zambia. However, the impact of early ART on KSHV transmission to HIV-infected children is unknown.

**Methods.** We enrolled and followed a cohort of 287 HIV-exposed, KSHV-negative children under 12 months of age from Lusaka, Zambia to identify KSHV seroconversion events. Potential factors associated with KSHV infection—with an emphasis on HIV, ART, and immunological measures—were assessed through structured questionnaires and blood analyses. Incidence rate, Kaplan-Meier, and multivariable Cox regression models were used to assess differences in time-to-event (KSHV seroconversion) between groups. All statistical tests were two-sided.

**Results.** During follow-up, 151 (53%) children underwent KSHV seroconversion. Based on 3552 months of follow-up, we observed similar KSHV incidence rates between HIV-infected and uninfected children. Among HIV-infected children, ART-naïve children had significantly increased risk of KSHV acquisition (adjusted hazard ratio [AHR]: 5.04, 95% confidence interval [CI]: 2.36-10.80). Time-updated CD4+ T-cell percentage was also significantly associated with risk of KSHV acquisition (AHR: 0.82, 95% CI: 0.74-0.92), such that each 5% increase of CD4+ T-cells represented an 18% decrease in risk of acquiring KSHV.
Conclusions. Our data suggest that early ART and prevention of immune suppression reduce the risk of KSHV acquisition among HIV-infected children in an area where both viruses are highly endemic. This study highlights the importance of programs in Africa to provide children with ART immediately after HIV infection is diagnosed.
Kaposi’s sarcoma (KS) is one of the most common malignancies in many countries of sub-Saharan Africa—where approximately 84% of global cases occur [1]. An endemic form of KS was first identified in these countries in the 1960s, presenting in male adults but rarely in women and young children [2, 3]. Subsequently, an HIV-associated form of KS, known as epidemic or AIDS-KS, emerged in both adults and children in parallel with the HIV/AIDS epidemic [4]. For example, in Zambia, KS accounted for approximately 25% of all pediatric malignancies by 1992, with a peak incidence between one and two years of age, making it the most common childhood cancer [5]. The introduction of antiretroviral therapy (ART) has decreased the incidence of AIDS, and consequently epidemic KS, in resource-rich and -limited countries alike [6]. Nevertheless, ART coverage in resource-limited countries of sub-Saharan Africa remains low at approximately 37% [7]. Hence, KS continues to be a substantial source of morbidity and mortality among HIV-infected children and adults in this region [1, 8-10].

All forms of KS, along with the lymphoproliferative malignancies primary effusion lymphoma and multicentric Castleman’s disease, are etiologically linked with Kaposi’s sarcoma-associated herpesvirus (KSHV; or human herpesvirus-8, HHV-8) [11-13]. Global KSHV seroprevalence is uneven; it is low in the United States and Western Europe, moderate in the Mediterranean, and high in sub-Saharan Africa [14-16]. Concordantly, in a previous prospective cohort study, we observed that KSHV infection is common among Zambian children as approximately 40% of children acquired KSHV by four years of age [17]. KSHV DNA is frequently detected in saliva of infected
individuals—implicating salivary exchange as the major route of transmission to children [18, 19]. Indeed, we recently reported that specific child feeding behaviors are associated with early childhood infection [20]. Counselling and educating caregivers regarding KSHV transmission and feeding habit changes may therefore reduce early childhood infection. However, in the absence of a KSHV vaccine, counselling alone cannot be anticipated to eliminate viral transmission and the risk of developing KS. This underscores the urgent need to prevent transmission of KSHV in the pediatric population, thereby reducing the burden of KS in both children and adults.

We previously observed that children infected with HIV had a five-fold higher risk of acquiring KSHV compared to HIV uninfected children [17]. However, this cohort study was conducted before the widespread use of ART in Zambia. We hypothesized that HIV-induced immunosuppression predisposed children to infection by KSHV; thus prevention of CD4+ T-cell depletion and subsequent immune suppression by early ART should reduce KSHV incidence. The Zambian government recently increased ART availability and, in accordance with the World Health Organization (WHO) recommendations, the current policy is to provide HIV-infected children with ART after confirmatory diagnosis regardless of CD4+ T-cell status [21, 22]. Additionally, the University Teaching Hospital (UTH) in Lusaka was one of the first hospitals to successfully implement a routine HIV counselling and testing program for children [23]. These programs supported investigating the relationship between immune suppression and KSHV transmission. In the present study, we have evaluated the impact of ART on KSHV acquisition in a prospective cohort of young Zambian children.
METHODS

Study Setting and Cohort

The current study is part of an ongoing observational cohort following children born to HIV-infected mothers to investigate the effect of ART on KSHV. Screening and enrollment was conducted between December 2009 and June 2012 at the UTH in Lusaka, Zambia. Local community workers recruited and informed potential participants about the study aims [24]. Interested mothers who visited the study clinic and provided written, informed consent to participate were given monetary compensation for transportation costs. Mothers were also counselled about HIV and KSHV infections and ways to prevent transmission. Eligibility criteria for enrollment was as follows: 1) child was less than 12 months of age, 2) child was negative for KSHV antibodies in plasma and KSHV DNA in the oral cavity, 3) child HIV status was confirmed, and 4) mother was HIV-infected. All eligible mother-child pairs returned within a week for enrollment and were scheduled for follow-up every three months for up to 48 months after enrollment. This study was approved by the Institutional Review Board of the University of Nebraska and the University of Zambia Biomedical Research Ethics Committee.

Data Collection and Laboratory Testing

Data and biological specimen collection. Trained interviewers conducted intake interviews with each mother using structured questionnaires at all study visits. The self-reported questionnaires collected data pertaining to socio-demographics, medical history, laboratory results, and ART status, adherence, and regimen. Blood samples and
oral swabs were collected from all children during a free-of-charge medical examination at each study visit. Plasma and oral swabs were shipped to the University of Nebraska-Lincoln for KSHV antibody and DNA detection, respectively.

**HIV diagnosis and blood testing.** HIV diagnosis was conducted at the UTH laboratory in Lusaka. Since all children were under 12 months of age, HIV testing was performed using DNA PCR of dried blood spots. Confirmation of mother HIV status was performed using two rapid tests—Abbott RealTime HIV-1 Qualitative (Abbott Laboratories) and Unigold Recombigen HIV-1/2 (Trinity Biotech) according to manufacturer’s protocols. Whole blood samples of HIV-infected children were analyzed at the UTH clinic to determine the percentage of total T-cells (CD3+) that were CD4+ or CD8+, using a FacsCount Cell Analyzer (BD Biosciences) according to the manufacturer’s protocol. Blood chemistry and full blood count were also conducted for each child.

**KSHV detection.** Plasma samples were tested for KSHV antibodies using a monoclonal antibody-enhanced immunofluorescence assay (mIFA) previously standardized in our laboratory [20]. Briefly, plasma samples were diluted 1:40 in PBS and incubated on stimulated and fixed BC3 cells. Mouse monoclonal anti-human IgG (CRL-1786; American Type Culture Collection) was used as a secondary antibody and DyLight 488-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) as the tertiary antibody. A plasma sample was considered KSHV positive if two readers independently determined the sample to be positive on two separate mIFAs. Oral swabs collected at screening were tested for the presence of KSHV DNA by PCR and Southern
blot analysis, as described previously [19, 25], to verify KSHV-negative status at study entry.

Statistical Analysis

Categorical data were summarized by count and percentages using Chi-square test to determine significance, and continuous variables were summarized by median and interquartile range (IQR) using Wilcoxon rank-sum test for significance. Independent variables analyzed for all children, and HIV-infected children only, are detailed in Tables 3.1 and 3.2. Generalized symptoms include any of the following: fever, sore throat, rash, diarrhea, vomiting, mouth sores, sneezing, or cough. Missing CD4% or CD8% data were imputed using that child’s median value from other visits. Additional independent variables considered for HIV-infected children taking ART were age at ART initiation (in months) and ART regimen (three nucleoside reverse transcriptase inhibitors (NRTIs), two NRTIs and one non-nucleoside reverse transcriptase inhibitors (NNRTIs), or two NRTIs and one protease inhibitor (PI)). Logistic regression was used to measure associations between KSHV seroconversion and generalized symptoms or full blood count data at either the KSHV seroconversion visit or study censor date.

All time-to-event data were right-censored at 24 months of follow-up. Crude KSHV incidence rate per 100 child months was calculated for all variables. Cumulative incidence curves were generated depicting the probability of KSHV infection as a function of age for the total cohort, HIV-uninfected vs. HIV-infected children, and HIV-uninfected vs. HIV-infected children stratified by ART status. Univariate Cox proportional
hazards modeling was used to assess the risk of KSHV infection for all variables, including those associated with caregivers, for the HIV-infected children and children taking ART. The proportional hazards assumption was verified using the empirical score process. Variables that were significant in the univariate analysis were then considered for multivariate Cox proportional hazards models using forward stepwise selection. Percentages of CD4+ and CD8+ T-cells collected at each study visit were analyzed as time-updated data. Adjusted hazard ratios (AHR) along with corresponding 95% confidence intervals (CI) are reported. Statistical tests were two-sided and p-values less than or equal to 0.05 were considered significant. Analyses were performed using statistical packages SAS (v9.3, SAS Institute) and SPSS (v22, IBM).

RESULTS

Initially, 688 children from Lusaka, Zambia were screened for HIV and KSHV infections. A total of 324 eligible children were enrolled for follow-up, of which 287 returned for at least one follow-up visit (Figure 3.1). Reasons for exclusion and loss to follow-up are detailed in Figure 3.1.

Table 3.1 summarizes the demographics of the current study cohort (N = 287), including HIV-uninfected (n = 196) and infected (n = 91) children. Maternal characteristics differed significantly between HIV-uninfected and infected children, as did child age (Table 3.1). Since the aim of our study was to evaluate the impact of ART on KSHV acquisition, we first investigated whether there were any differences in overall health status between HIV-infected and uninfected children at study enrollment. We
found that all clinical characteristics were similar between HIV-infected and uninfected children, with the exception of small yet statistically significant differences in hematocrit and monocyte levels (Table 3.2).

During the follow-up period, 151 (52.6%) children underwent KSHV seroconversion. We did not detect any associations between KSHV seroconversion and recent generalized symptoms when analyzed individually or as a group (data not shown). Analysis of full blood count data revealed a significant association between KSHV seroconversion and higher total lymphocyte percentage (median [IQR]: 61 [53 – 68] vs 55 [47 – 62], Odds Ratio: 1.05, 95% CI: 1.03 – 1.08). Higher lymphocyte counts suggest that acute KSHV infection occurred recently, despite the lack of distinguishable clinical symptoms associated with KSHV seroconversion.

The entire cohort contributed 3552 months of follow-up, with a median follow-up time from enrollment to seroconversion or censor date of 10.5 months (IQR: 4.5-22.8). Overall, we calculated an incidence rate of 4.25 KSHV infections per 100 child-months (Table 3.3). We did not observe any socio-demographic, medical, or maternal characteristics to be significantly associated with an increased rate of KSHV infection. We also determined that the rates of KSHV infection were similar in HIV-infected and uninfected children (Table 3.3 and Figure 3.2A). However, when HIV-infected children were stratified by ART status prior to KSHV seroconversion or censor date, ART-naïve children had a significantly increased rate of KSHV acquisition compared to HIV-uninfected children (Incidence Rate Ratio (IRR): 5.97, 95% CI: 3.13-11.41). In contrast, the HIV-infected children taking ART acquired KSHV at a rate indistinguishable from HIV-
uninfected controls (IRR: 0.99, 95% CI: 0.68-1.43; Table 3.3 and Figure 3.2B). The cumulative probability of KSHV infection was also significantly higher in ART-naïve HIV-infected children when age of the child was analyzed as the time-dependent variable (p-value < 0.001; Figure 3.3).

Among HIV-infected children, baseline demographic, maternal, and clinical characteristics were similar between ART-treated and ART-naïve children, with the exception of age and age-related variables (Table 3.4). In multivariate Cox proportional hazard analysis among HIV-infected children, we found that the absence of ART significantly increased the risk of KSHV acquisition (AHR: 5.04, 95% CI: 2.36-10.80). Time-updated CD4+ T-cell percentage was also significantly associated with risk of KSHV acquisition (AHR: 0.82, 95% CI: 0.74-0.92), such that each 5% increase of CD4+ T-cells represented an 18% decrease in risk of acquiring KSHV (Table 3.5). In order to investigate for possible associations of KSHV infection with the age at which ART was initiated or the ART regimen received, we analyzed data from HIV-infected children actively taking ART. The distribution of specific ART regimens among the HIV-infected children are presented in Figure 3.4, and we found no differences in KSHV acquisition by ART regimen. Similar to all children infected with HIV, however, we found that higher CD4% in children taking ART was associated with a decreased risk of KSHV infection (AHR: 0.83, 95% CI: 0.73-0.93). The observed risks of KSHV infection were similar whether or not age and gender were included in the models (Table 3.5).
DISCUSSION

This is the first study to investigate the effect of ART on KSHV incidence in HIV-infected children. Several cross-sectional studies conducted in sub-Saharan Africa have demonstrated higher KSHV seroprevalence among HIV-infected children compared to HIV-uninfected children [26-28]. Our previous longitudinal cohort study in Zambia further established that HIV-infected children were five-fold more likely to acquire KSHV [17]. However, these studies were conducted prior to the introduction of ART in sub-Saharan Africa. In the present study, we observed similar rates of KSHV acquisition between HIV-infected and uninfected children—likely due to the implementation of effective ART among HIV-infected children in Zambia. Consistent with this concept and our previous findings, we also observed that HIV-infected ART-naive children were 5-fold more likely to acquire KSHV compared with HIV-infected children taking ART.

ART success is routinely assessed by CD4+ T-cell levels. Since CD4 count varies in young children, due to normal infant lymphocytosis or factors such as malnutrition and infections, we used CD4 percentage as a more reliable measure for immune status [29]. Although very few HIV-infected children in our cohort were classified with advanced immunosuppression by WHO CD4% categories, we did detect significantly lower risk of KSHV infection among children with higher CD4+ T-cell percentages. This data supports the hypothesis that the risk of acquiring KSHV among HIV-infected children is related to CD4+ T-cell depletion and subsequent HIV-induced immune dysfunction. The specific immune dysfunction that would confer susceptibility to KSHV infection is unknown.

Activated B-cells support KSHV infection and replication, whereas resting B-cells do not
B-cell hyperactivity, functional reduction of cytotoxic T-lymphocytes, and the pro-inflammatory state triggered by HIV may create an environment conducive for KSHV infection. Initiation of ART reduced immune activation in a different cohort of HIV-infected Zambian children [31]. Furthermore, early ART significantly reduced all-cause mortality and HIV disease progression in the Children with HIV Early Antiretroviral (CHER) trial [32]. Coupled with the present data, it is likely that ART initiation within weeks of HIV infection, as was the case in our cohort, may protect and maintain normal immune functions, thereby reducing KSHV incidence and ultimately the burden of KS.

Alternatively, ART might be directly inhibiting KSHV acquisition. A recent study revealed that nelfinavir inhibited KSHV replication in vitro, but all other antiretrovirals tested—including multiple NRTIs, NNRTIs, and PIs—had no effect [33]. Nelfinavir was not prescribed to any children in the present cohort; nevertheless, ART was still associated with lower risk of KSHV acquisition. Consequently, whether any ART components can directly prevent new KSHV infection or establishment of latency is unclear. Longitudinal follow-up of children after recent KSHV acquisition will allow us to delineate the impact of ART on KSHV latency and replication during the early stages of infection in HIV-infected children.

Our cohort study has several strengths, which include prospective design, regular and frequent follow-up visits, and a large number of HIV-infected children with access to ART. However, there are some limitations. Many children were not enrolled in the study due to evidence of prior KSHV seroconversion, therefore, the incidence measures described in this report are likely to underrepresent the true incidence among
HIV-exposed children. Additionally, our mIFA is unable to distinguish between a primary or secondary seroconversion, as it detects IgG antibodies but not IgM. However, we have several other reasons to be confident that the seroconversion event represents primary KSHV acquisition: we enrolled very young children, KSHV was not detected in saliva of children at enrollment, and we observed an association between KSHV seroconversion and higher lymphocyte percentage. A final limitation was the low number of ART-naïve children in our cohort. This was expected, because ART was offered to all HIV-infected children as part of the study—consistent with strong ethical practices. Nevertheless, there were still a number of caregivers who decided not to start ART for their children, regardless of being counseled about the importance of early treatment. Although many factors related to the mother were not associated with KSHV acquisition, it is possible that these caregivers may have performed behaviors that we did not assess, such as specific feeding habits, further increasing risk of KSHV transmission. Despite these limitations, we still observed a large and significant increase in risk of KSHV acquisition among ART-naïve children. Our findings are directly applicable to HIV-infected children in Zambia, a resource-limited country with high KSHV/HIV prevalence and early childhood acquisition. Our findings may also be applicable to other countries in the region, but not in other settings where KSHV/HIV prevalence or ART coverage differs substantially. Treatment with ART is not directly applicable to HIV-uninfected children at risk for KSHV infection, although reducing the overall number of KSHV infections and transmission sources may in turn reduce the risk of KSHV transmission to HIV-uninfected children.
We did not detect associations between KSHV infection and the age of the child at ART initiation or type of ART regimen in Cox proportional hazard analysis. These data should not be interpreted that ART-related variables are unimportant for reducing KSHV incidence, but rather that our cohort was not designed to investigate these variables. Children in our cohort were recruited and followed at very young ages, therefore restricting the ART initiation age range that could be analyzed. Additionally, although three major ART regimens were represented in our cohort, over 80% of the children were taking a two NRTI and one NNRTI regimen. Numerous studies have demonstrated that ART can reduce progression of KS [34], and one suggests PI-based regimens may be more effective than others [35]. Therefore, our observation that ART is also associated with a lower risk of acquiring KSHV warrants further research into whether particular ART regimens are more effective at preventing new KSHV infections.

Since a vaccine against KSHV is not available, and efforts to develop one are limited [36], it is paramount to develop alternative strategies to prevent KSHV acquisition during early childhood. Our data suggest that ART and prevention of immune suppression play an important role in reducing the incidence and risk of KSHV acquisition among HIV-infected children in an area where both viruses are highly endemic. The WHO is currently advocating ART programs for HIV-infected pregnant women and their children to prevent new HIV infections and increase treatment retention [37]. Our data highlight the importance of these efforts and additional programs to provide ART to children as soon as HIV infection is diagnosed, not only to
reduce HIV-associated morbidity, but also help prevent KSHV infection and reduce the burden of KS among HIV-infected children.

FUNDING

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NOTES

The funders had no role in the design of the study, the collection, analysis, or interpretation of the data, the writing of the manuscript, or the decision to submit the manuscript for publication. We declare that we have no conflicts of interest.

We thank all children and caregivers for their participation in the study, as well as community health workers and laboratory staff at UTH in Lusaka for their contributions to recruitment, data management, and sample processing.

VM, CK, JW, CM, and CW conceived and designed the study. LNO, VM, and CG collected data, LNO and VM analyzed data and performed statistical analyses, and LNO wrote the first draft of the report. All authors interpreted the data, revised the drafts, and approved the final version.
REFERENCES


FIGURE LEGENDS

**Figure 3.1.** Flow chart summarizing the recruitment and enrollment procedures for the mother-child pair cohort in Lusaka, Zambia, 2009 – 2012.

**Figure 3.2.** Kaplan-Meier plots estimating the probability of remaining KSHV-free since enrollment in a longitudinal cohort of 287 children from Lusaka, Zambia, 2009 – 2012. (A) Probabilities stratified by child HIV status. (B) Probabilities stratified by child HIV and ART status. Vertical lines indicate censoring.

**Figure 3.3.** Cumulative incidence analysis estimating the probability of KSHV infection since birth in a longitudinal cohort of 287 children from Lusaka, Zambia, 2009 – 2012. (A) Total cohort incidence. (B) Incidence stratified by child HIV status. (C) Incidence stratified by child HIV and ART status. Vertical lines indicate censoring.

**Figure 3.4.** Distribution of ART regimen of 74 HIV-infected children in a longitudinal cohort of 287 children from Lusaka, Zambia, 2009 – 2012. ABC = abacavir; AZT = zidovudine; D4T = stavudine; NVP = nevirapine; 3TC = lamivudine.
<table>
<thead>
<tr>
<th>Characteristic*</th>
<th>Whole Cohort (N = 287)</th>
<th>Children without HIV (n = 196)</th>
<th>Children with HIV (n = 91)</th>
<th>p-value</th>
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<tbody>
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<td>Age at Enrollment (Months)</td>
<td>7 (5 - 11)</td>
<td>6 (4 - 9)</td>
<td>10 (6 - 12)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>136 47.4%</td>
<td>91 46.4%</td>
<td>45 49.5%</td>
<td>0.63</td>
</tr>
<tr>
<td>Male</td>
<td>151 52.6%</td>
<td>105 53.6%</td>
<td>45 50.5%</td>
<td></td>
</tr>
<tr>
<td>Household Members</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>≤ 3</td>
<td>47 16.4%</td>
<td>29 14.8%</td>
<td>18 19.8%</td>
<td>0.48</td>
</tr>
<tr>
<td>4 - 5</td>
<td>148 51.5%</td>
<td>101 51.5%</td>
<td>47 51.6%</td>
<td></td>
</tr>
<tr>
<td>≥ 6</td>
<td>92 32.1%</td>
<td>66 33.7%</td>
<td>25 28.6%</td>
<td></td>
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<tr>
<td>Monthly Household Income</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>≤ $30</td>
<td>72 25.1%</td>
<td>38 19.4%</td>
<td>34 37.4%</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>&gt; $30</td>
<td>202 70.4%</td>
<td>152 77.5%</td>
<td>50 54.9%</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>13 4.5%</td>
<td>6 3.1%</td>
<td>7 7.7%</td>
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</tr>
<tr>
<td>Mother Employment</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Unemployed</td>
<td>193 67.2%</td>
<td>124 63.3%</td>
<td>69 75.8%</td>
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</tr>
<tr>
<td>Employed</td>
<td>94 32.8%</td>
<td>72 36.7%</td>
<td>22 24.2%</td>
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</tr>
<tr>
<td>Mother Education</td>
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<tr>
<td>0 - 7 years</td>
<td>172 59.9%</td>
<td>117 59.7%</td>
<td>55 60.4%</td>
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</tr>
<tr>
<td>≥ 8 years</td>
<td>115 40.1%</td>
<td>79 40.3%</td>
<td>35 39.6%</td>
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</tr>
<tr>
<td>Mother Breastfeeding†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>14 4.9%</td>
<td>11 5.6%</td>
<td>3 3.3%</td>
<td>0.56</td>
</tr>
<tr>
<td>Ever</td>
<td>273 95.1%</td>
<td>185 94.4%</td>
<td>88 96.7%</td>
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<tr>
<td>Months of Breastfeeding</td>
<td>7.8 (5.2 - 11.8)</td>
<td>6.8 (5.0 - 10.1)</td>
<td>11 (6.3 - 14.1)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Mother ART</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>126 43.9%</td>
<td>62 31.6%</td>
<td>64 70.3%</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Ever</td>
<td>161 56.1%</td>
<td>134 68.4%</td>
<td>27 29.7%</td>
<td></td>
</tr>
<tr>
<td>Mother start ART‡‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Before pregnancy</td>
<td>95 33.1%</td>
<td>87 44.4%</td>
<td>8 8.8%</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>During pregnancy</td>
<td>34 11.8%</td>
<td>31 15.8%</td>
<td>3 3.3%</td>
<td></td>
</tr>
<tr>
<td>After pregnancy</td>
<td>32 11.1%</td>
<td>16 8.2%</td>
<td>15 17.6%</td>
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<td>Months Mother on ART†</td>
<td>17 (8 - 36)</td>
<td>24 (9 - 48)</td>
<td>7 (4 - 10)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

* Continuous variables are represented as median (IQR) and categorical variables as count (percentages).
† Fischer’s exact test used.
‡‡ n = 161 (Only mothers with history of ART).
<table>
<thead>
<tr>
<th>Characteristic*</th>
<th>Whole Cohort (N = 287)</th>
<th>Children without HIV (n = 196)</th>
<th>Children with HIV (n = 91)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height (cm)</td>
<td>63 (60 - 69)</td>
<td>63 (60 - 69)</td>
<td>63.5 (60 - 69)</td>
<td>0.86</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>7 (6.0 - 8.1)</td>
<td>7 (6.0 - 8.2)</td>
<td>6.9 (5.9 - 7.8)</td>
<td>0.55</td>
</tr>
<tr>
<td>Developmentally Appropriate†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>5 1.7%</td>
<td>3 1.5%</td>
<td>2 2.2%</td>
<td>0.65</td>
</tr>
<tr>
<td>Yes</td>
<td>282 98.3%</td>
<td>193 98.5%</td>
<td>89 97.8%</td>
<td></td>
</tr>
<tr>
<td>Generalized Symptoms‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>239 83.3%</td>
<td>164 83.7%</td>
<td>75 82.4%</td>
<td>0.79</td>
</tr>
<tr>
<td>Yes</td>
<td>48 16.7%</td>
<td>32 16.3%</td>
<td>16 17.6%</td>
<td></td>
</tr>
<tr>
<td>Alanine Aminotransferase (U/L)</td>
<td>14.3 (10.4 - 21.1)</td>
<td>13.4 (9.5 - 19.5)</td>
<td>15.1 (10.7 - 21.6)</td>
<td>0.14</td>
</tr>
<tr>
<td>Aspartate Aminotransferase (U/L)</td>
<td>40.3 (32.7 - 52.4)</td>
<td>41.1 (32.8 - 52.9)</td>
<td>39.1 (31.6 - 49.5)</td>
<td>0.55</td>
</tr>
<tr>
<td>White Blood Cell (x10³/L)</td>
<td>10.1 (8.0 - 12.7)</td>
<td>10.1 (8.0 - 12.4)</td>
<td>10.1 (8.1 - 12.8)</td>
<td>0.97</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>9.9 (9.1 - 10.9)</td>
<td>9.9 (9.1 - 10.6)</td>
<td>10.0 (9.1 - 11.5)</td>
<td>0.13</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>30.8 (28.3 - 32.7)</td>
<td>31.2 (28.8 - 32.7)</td>
<td>30.0 (27.2 - 32.0)</td>
<td>0.01</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>64.2 (57.8 - 70.8)</td>
<td>65.0 (57.8 - 71.1)</td>
<td>63.1 (59.1 - 69.6)</td>
<td>0.44</td>
</tr>
<tr>
<td>Monocyte (%)</td>
<td>8.4 (6.8 - 10.6)</td>
<td>8.8 (7.0 - 10.9)</td>
<td>7.9 (6.6 - 9.4)</td>
<td>0.005</td>
</tr>
<tr>
<td>Child ART</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>- -</td>
<td>-</td>
<td>17 18.7%</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>- -</td>
<td>-</td>
<td>74 81.3%</td>
<td></td>
</tr>
<tr>
<td>CD4 (%)</td>
<td>- -</td>
<td>-</td>
<td>40.0 (31.0 - 57.0)</td>
<td>-</td>
</tr>
<tr>
<td>CD8 (%)</td>
<td>- -</td>
<td>-</td>
<td>56.0 (47.0 - 57.0)</td>
<td>-</td>
</tr>
<tr>
<td>CD4:CD8 Ratio</td>
<td>- -</td>
<td>-</td>
<td>0.74 (0.50 - 1.02)</td>
<td>-</td>
</tr>
<tr>
<td>Immunosuppression Status§</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not Significant</td>
<td>- -</td>
<td>-</td>
<td>62 68.1%</td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>- -</td>
<td>-</td>
<td>18 19.8%</td>
<td></td>
</tr>
<tr>
<td>Advanced</td>
<td>- -</td>
<td>-</td>
<td>11 12.1%</td>
<td>-</td>
</tr>
</tbody>
</table>

* Continuous variables are represented as median (IQR) and categorical variables as count (percentages).
† Fischer’s exact test used.
‡ Includes fever, sore throat, rash, diarrhea, vomiting, mouth sores, sneezing, or cough.
§ Based on CD4% according to age appropriate WHO categories [29].
Table 3.3. Crude incidence rate of KSHV per 100 child-months since time of enrollment in a longitudinal cohort of 287 children from Lusaka, Zambia, 2009 – 2012

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of Children</th>
<th>KSHV Positive Children</th>
<th>%</th>
<th>KSHV-free child months</th>
<th>Incidence Rate per 100 Child-Months</th>
<th>Incidence Rate Ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>287</td>
<td>151</td>
<td>52.6</td>
<td>3552</td>
<td>4.25</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mother ART</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>126</td>
<td>71</td>
<td>56.3</td>
<td>1431</td>
<td>4.96</td>
<td>Ref.</td>
<td></td>
</tr>
<tr>
<td>Ever</td>
<td>161</td>
<td>80</td>
<td>49.7</td>
<td>2120</td>
<td>3.77</td>
<td>0.76</td>
<td>(0.55, 1.05)</td>
</tr>
<tr>
<td>Child HIV Status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>196</td>
<td>100</td>
<td>51.0</td>
<td>2512</td>
<td>3.98</td>
<td>Ref.</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>91</td>
<td>51</td>
<td>56.0</td>
<td>1040</td>
<td>4.90</td>
<td>1.23</td>
<td>(0.88, 1.73)</td>
</tr>
<tr>
<td>Child HIV/ART Status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV(-)</td>
<td>196</td>
<td>100</td>
<td>51.0</td>
<td>2512</td>
<td>3.98</td>
<td>Ref.</td>
<td></td>
</tr>
<tr>
<td>HIV(+)/ART(+)</td>
<td>74</td>
<td>39</td>
<td>52.7</td>
<td>989.6</td>
<td>3.94</td>
<td>0.99</td>
<td>(0.68, 1.43)</td>
</tr>
<tr>
<td>HIV(+)/ART(-)</td>
<td>17</td>
<td>12</td>
<td>70.6</td>
<td>50.47</td>
<td>23.78</td>
<td>5.97</td>
<td>(3.13, 11.41)</td>
</tr>
</tbody>
</table>
### Table 3.4. Demographic and child clinical characteristics of 91 HIV-infected children, by child ART status, in a longitudinal cohort of 287 children from Lusaka, Zambia, 2009 – 2012.

<table>
<thead>
<tr>
<th>Characteristic*</th>
<th>Children taking ART (n = 74)</th>
<th>Children not taking ART (n = 17)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at Enrollment (Months)</td>
<td>11 (8 - 12)</td>
<td>6 (4 - 8)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>38</td>
<td>51.4%</td>
<td>7</td>
</tr>
<tr>
<td>Male</td>
<td>36</td>
<td>48.6%</td>
<td>10</td>
</tr>
<tr>
<td>Household Members†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 3</td>
<td>14</td>
<td>18.9%</td>
<td>4</td>
</tr>
<tr>
<td>4 - 5</td>
<td>38</td>
<td>51.4%</td>
<td>9</td>
</tr>
<tr>
<td>≥ 6</td>
<td>22</td>
<td>29.7%</td>
<td>4</td>
</tr>
<tr>
<td>Monthly Household Income†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ $30</td>
<td>24</td>
<td>32.4%</td>
<td>10</td>
</tr>
<tr>
<td>&gt; $30</td>
<td>45</td>
<td>60.3%</td>
<td>5</td>
</tr>
<tr>
<td>Unknown</td>
<td>5</td>
<td>6.8%</td>
<td>2</td>
</tr>
<tr>
<td>Mother Employment†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unemployed</td>
<td>56</td>
<td>75.7%</td>
<td>13</td>
</tr>
<tr>
<td>Employed</td>
<td>18</td>
<td>24.3%</td>
<td>4</td>
</tr>
<tr>
<td>Mother Education</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 - 7 years</td>
<td>47</td>
<td>63.5%</td>
<td>8</td>
</tr>
<tr>
<td>&gt; 8 years</td>
<td>27</td>
<td>36.5%</td>
<td>9</td>
</tr>
<tr>
<td>Mother Breastfeeding†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>3</td>
<td>4.0%</td>
<td>0</td>
</tr>
<tr>
<td>Ever</td>
<td>71</td>
<td>96.0%</td>
<td>17</td>
</tr>
<tr>
<td>Months of Breastfeeding</td>
<td>12 (7 - 16)</td>
<td>6 (6 - 8)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Mother ART†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>49</td>
<td>66.2%</td>
<td>15</td>
</tr>
<tr>
<td>Ever</td>
<td>25</td>
<td>33.3%</td>
<td>2</td>
</tr>
<tr>
<td>Mother start ART‡‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before pregnancy</td>
<td>7</td>
<td>28.0%</td>
<td>1</td>
</tr>
<tr>
<td>During pregnancy</td>
<td>2</td>
<td>8.0%</td>
<td>1</td>
</tr>
<tr>
<td>After pregnancy</td>
<td>16</td>
<td>64.0%</td>
<td>0</td>
</tr>
<tr>
<td>Months Mother on ART‡</td>
<td>7 (4 - 10)</td>
<td>-§</td>
<td>-</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>67 (61 - 70)</td>
<td>63 (57 - 67)</td>
<td>0.04</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>7.3 (6.1 - 8.5)</td>
<td>6.5 (5.8 - 6.9)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Children taking ART (n = 74)</th>
<th>Children not taking ART (n = 17)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Developmentally Appropriate†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>2</td>
<td>1</td>
<td>0.47</td>
</tr>
<tr>
<td>Yes</td>
<td>72</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Generalized Symptoms§</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>54</td>
<td>10</td>
<td>0.25</td>
</tr>
<tr>
<td>Yes</td>
<td>20</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Alanine Aminotransferase (U/L)</td>
<td>14.5</td>
<td>12.4</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>(10.1 - 22.8)</td>
<td>(9.1 - 21.9)</td>
<td></td>
</tr>
<tr>
<td>Aspartate Aminotransferase (U/L)</td>
<td>39.7</td>
<td>39.2</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>(31.2 - 59.0)</td>
<td>(29.4 - 56.0)</td>
<td></td>
</tr>
<tr>
<td>White Blood Cell (x10^3/L)</td>
<td>10.8</td>
<td>8.9</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>(8.3 - 15.3)</td>
<td>(7.7 - 16.1)</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>9.5</td>
<td>9.9</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>(8.6 - 11.0)</td>
<td>(9.3 - 11.2)</td>
<td></td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>29.1</td>
<td>29.5</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>(26.9 - 31.0)</td>
<td>(25.5 - 31.0)</td>
<td></td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>63.1</td>
<td>64.3</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>(56.1 - 68.8)</td>
<td>(60.5 - 71.1)</td>
<td></td>
</tr>
<tr>
<td>Monocyte (%)</td>
<td>8.7</td>
<td>8.1</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>(6.6 - 9.9)</td>
<td>(7.5 - 9.8)</td>
<td></td>
</tr>
<tr>
<td>CD4 (%)</td>
<td>41.5</td>
<td>37</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>(32.0 - 57.0)</td>
<td>(27.0 - 46.0)</td>
<td></td>
</tr>
<tr>
<td>CD8 (%)</td>
<td>54.5</td>
<td>57</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>(46.0 - 57.0)</td>
<td>(55.0 - 60.0)</td>
<td></td>
</tr>
<tr>
<td>CD4:CD8 Ratio</td>
<td>0.78</td>
<td>0.64</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>(0.54 - 1.19)</td>
<td>(0.47 - 0.82)</td>
<td></td>
</tr>
</tbody>
</table>

* Continuous variables are represented as median (IQR) and categorical variables as count (percentages).
† Fischer’s exact test used.
‡ n = 27 (Only mothers with history of ART).
§ ART duration only available from one mother.
|| Includes fever, sore throat, rash, diarrhea, vomiting, mouth sores, sneezing, or cough.
¶ Based on CD4% according to age appropriate WHO categories [29].
Table 3.5. Multivariate cox regression models for risk of KSHV acquisition since time of enrollment in a longitudinal cohort of 287 children from Lusaka, Zambia, 2009 – 2012

<table>
<thead>
<tr>
<th></th>
<th>Model 1*</th>
<th></th>
<th>Model 2*</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard Ratio</td>
<td>95% CI</td>
<td>p-value</td>
<td>Hazard Ratio</td>
</tr>
<tr>
<td>HIV+ Children</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absence of ART</td>
<td>5.04</td>
<td>(2.36, 10.80)</td>
<td>&lt;0.001</td>
<td>4.34</td>
</tr>
<tr>
<td>CD4% (5% intervals)</td>
<td>0.82</td>
<td>(0.74, 0.92)</td>
<td>&lt;0.001</td>
<td>0.83</td>
</tr>
<tr>
<td>Age at Enrollment (Months)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.96</td>
</tr>
<tr>
<td>Male Gender</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.33</td>
</tr>
<tr>
<td>ART+ Children</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4% (5% intervals)</td>
<td>0.83</td>
<td>(0.73, 0.93)</td>
<td>0.002</td>
<td>0.85</td>
</tr>
<tr>
<td>Age at Enrollment (Months)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.98</td>
</tr>
<tr>
<td>Male Gender</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.72</td>
</tr>
</tbody>
</table>

* Model 1 was adjusted for ART status and CD4% among HIV+ children and CD4% alone among ART+ children. Model 2 was also adjusted for age and gender.
Figure 3.1

Children recruited in Lusaka, Zambia from 2009 - 2012
Under 12 months of age and born to HIV-infected mothers
N = 688

Excluded  n = 364
- Ineligible  n = 333
  - Mother not HIV-infected  n = 5
  - Child HIV status indeterminate  n = 3
  - Child KSHV seropositive  n = 301
  - Child KSHV DNA in saliva  n = 0
  - Child KSHV status indeterminate  n = 24
- Eligible but not interested  n = 31

Children enrolled  N = 324

Lost to follow up  n = 37
- Child Died  n = 11
- Mother Died  n = 3
- Migrated  n = 11
- Withdrew  n = 12

Final cohort of KSHV-negative children  N = 287
Figure 3.2

A

Follow-up time (months)

Percent KSHV-free

HIV(-)
HIV(+)

Number at Risk
HIV(-) 196 133 97 70 27
HIV(+) 91 53 39 27 12

p = 0.0239

B

Follow-up time (months)

Percent KSHV-free

HIV(-)
HIV(+) ART(+)
HIV(+) ART(-)

Number at Risk
HIV(-) 196 133 97 70 27
HIV(+) ART(+) 74 51 39 27 12
HIV(+) ART(-) 17 2 0 0 0

p < 0.001
Figure 3.3

A

B

C
Figure 3.4

![Bar chart showing the number of children on ART regimens based on KSHV status.](image-url)
CHAPTER 4

LONGITUDINAL ANALYSIS OF THE ANTI-KSHV HUMORAL IMMUNE RESPONSE AFTER PRIMARY INFECTION DURING EARLY CHILDHOOD

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²Department of Pediatrics and Child Health, University Teaching Hospital, 10101 Lusaka, Zambia

*Current Address: Department of Epidemiology, College of Public Health, University of Nebraska Medical Center, Omaha NE 68198
ABSTRACT

Kaposi’s sarcoma-associated herpesvirus (KSHV) is the etiologic agent for Kaposi’s sarcoma (KS)—one of the most common pediatric cancers in sub-Saharan Africa—however, the factors that lead to disease progression are not fully understood. HIV infection, immunosuppression, and high KSHV viral load increase the risk of developing KS, suggesting that the loss of an effective anti-KSHV immune response may be an important risk factor. However, very little is known about the KSHV-specific immune response prior to KS and less is known about the anti-KSHV immune response during the very early stages of infection. We therefore prospectively followed a cohort of 86 Zambian children for two years after primary KSHV seroconversion to characterize the humoral immune response during the early stages of KSHV infection. Plasma, peripheral blood mononuclear cells (PBMCs), and oral swabs were collected from patients every three months and analyzed for KSHV-specific antibodies and presence of viral DNA. At the time of primary KSHV seroconversion HIV-infected children undergoing antiretroviral therapy (ART) had similar KSHV antibody titers compared to HIV-uninfected children; however, HIV-infected ART-naïve children had a more robust KSHV antibody response. The presence of KSHV antibodies in infected children was not consistent over time. We observed an approximately 40% KSHV seropositive rate among infected children at time points after primary seroconversion, indicating that seroreversion is common after primary KSHV infection. Moreover, the longitudinal humoral antibody response to KSHV detected in study participants was highly variable and did not correlate with available clinical information, HIV status, or presence of KSHV
Collectively, our data suggest that the humoral immune response may not be an important factor in controlling KSHV viral replication after infection. This study also highlights the importance of longitudinal studies for assessing the prevalence of KSHV infection, as patients frequently serorevert after primary infection and may be missed in cross-sectional studies.
INTRODUCTION

Kaposi’s sarcoma-associated herpesvirus (KSHV), or human herpesvirus-8 (HHV-8), is the etiologic agent for Kaposi’s sarcoma (KS)—one of the most common cancers in many countries of sub-Saharan Africa [1, 2]. KS was endemic in sub-Saharan Africa prior to the HIV/AIDS epidemic, but KS cases drastically increased thereafter [3, 4]. For example, by 1992, KS accounted for approximately 25% of all childhood cancers diagnosed in Zambia, making KS the most common pediatric malignancy [4]. Indeed, HIV infection is now known to significantly increase the risk of developing KS [5]. The increased risk is likely imparted by the immune dysregulation caused by HIV infection. This is most significantly evinced by the observation that risk of developing KS increases for every decline of 50 CD4\(^+\) T-cells per microliter of blood in AIDS patients [6]. The introduction of antiretroviral therapy (ART) has decreased the incidence of AIDS, and concurrently AIDS-associated KS, in resource-rich and -limited countries alike [7]. Moreover, 81% of patients treated by ART without KS-specific chemotherapy exhibit regression of KS [8]. Taken together, these data indicate that HIV-induced immune dysregulation plays an important role in progression to KS.

High KSHV load in peripheral blood is correlated with development and progression of KS in HIV-infected adults [9-11], indicating that immune control of virus replication is important to prevent disease. Recent data from our laboratory demonstrated that prevalence of KSHV neutralizing antibodies as well as titer of both total and neutralizing antibodies are higher in HIV-infected KS patients compared to asymptomatic KSHV-infected individuals [12]. Moreover, antibodies against KSHV
antigens are strongly associated with risk of KS, and this risk increases with increasing antibody titer among HIV-infected individuals [10]. Together, this may suggest that the immune response is unable to control KSHV infection close to the time of KS onset in HIV-infected adults. Current reports on the effect of ART on the anti-KSHV humoral immune response indicate increases in antibodies against both lytic and latent antigens after ART initiation [13, 14], but whether these antibodies are neutralizing and effective in reducing viral replication is not known.

While the effect of HIV and ART on the KSHV immune response has been studied around the onset of KS in adults, it is possible that a robust immune response against KSHV following primary infection could minimize viral replication and prevent the development of KS. However, very little is known about the KSHV-specific immune response soon after primary infection. This is, in part, because KS may take years to develop after primary KSHV infection or never occur. Furthermore, primary infection of KSHV is difficult to ascertain among adult populations because clinical symptoms have not been clearly identified [15-17] and loss of detectable antibodies can occur among infected individuals [18, 19]. We previously observed that KSHV infection is common during early childhood in Zambia, as approximately 40% of children acquired KSHV by four years of age [18]. Therefore, young children represent an ideal cohort to study primary KSHV infection because the timing of primary infection can be ascertained due to young age.

We previously reported that, prior to the widespread introduction of ART in sub-Saharan Africa, children infected with HIV had a five-fold higher risk of acquiring KSHV
More recently, we observed that after ART became available the rates of KSHV acquisition between HIV-infected and –uninfected children were similar [17]. Moreover, absence of ART and low CD4 T-cell percentages significantly increased the risk of KSHV acquisition among HIV-infected children. We hypothesized that HIV-infected children on ART, in addition to HIV-uninfected children, have a more robust KSHV-specific humoral immune response following primary KSHV infection and therefore lower levels of KSHV replication compared to HIV-infected ART-naive children. This in turn would decrease the risk of disease progression to KS among HIV-infected children. In the present study, we have evaluated the impact of HIV and ART on the KSHV-specific humoral immune response over two years in a prospective cohort of Zambian children who were identified to have recently undergone primary KSHV infection.

METHODS

Study Participants.

We previously reported the prospective analysis of an observational cohort of 287 children, recruited between December 2009 and June 2012 at the UTH in Lusaka, Zambia to investigate the effect of ART on KSHV acquisition [17]. From the date of primary KSHV seroconversion identified in the previous study, we prospectively followed 86 children at three month intervals for a total of 24 months. Data and biological specimen collection was conducted at each study visit, as described previously [17]. Plasma, peripheral blood mononuclear cells (PBMCs), and oral swabs were shipped to the University of Nebraska-Lincoln for KSHV antibody and DNA detection. This study
was approved by the Institutional Review Board of the University of Nebraska and the University of Zambia Biomedical Research Ethics Committee.

**KSHV antibody detection and titer.**

Plasma samples were tested for presence of binding KSHV antibodies using a monoclonal antibody-enhanced immunofluorescence assay (mIFA) previously standardized in our laboratory [17]. Briefly, plasma samples were diluted 1:40 in PBS and incubated on stimulated and fixed BC3 cells. Mouse monoclonal anti-human IgG (CRL-1786; American Type Culture Collection) was used as a secondary antibody and DyLight 488-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) as the tertiary antibody. A plasma sample was considered KSHV positive if two readers independently determined the sample to be positive on two separate mIFAs. Plasma samples found to be positive for KSHV antibodies at the 1:40 dilution were then further titrated by 2-fold dilutions to 1:5120 and tested using the mIFA. The inverse of the most dilute positive sample represents the antibody titer.

**KSHV neutralization assay.**

The presence of neutralizing antibodies in samples positive for KSHV binding antibodies was investigated using a KSHV neutralization assay previously standardized in our laboratory [12]. This assay utilizes a recombinant KSHV virus that expresses GFP (rKSHV.219) to monitor the level of infection. Briefly, heat inactivated plasma (56 °C for one hour) was incubated at a 1:50 dilution with rKSHV.219 virus at 37 °C for one hour.
HEK293 cells were infected with the virus-plasma mixture by centrifugation (400 g, 20 min) and subsequently incubated at 37 °C for 72 hours. The level of infection, as measured by the number of GFP expressing cells, was quantified by flow cytometry (BD Biosciences). Plasma samples were analyzed in triplicate and a sample was considered positive for the presence of neutralizing antibodies when 50% or higher inhibition of KSHV infectivity was observed as compared to negative control plasma.

**KSHV DNA detection in oral swabs and PBMCs.**

DNA from oral swab and PBMC samples was extracted using the Puregene DNA Extraction Kit (Qiagen), as per the manufacturer’s protocol. Purified DNA was then subjected to a duplex, nested PCR analysis to amplify human β-actin and KSHV ORF26. For first round PCR, primers specific to β-actin (Actin1 [5’-TTCTACAATGAGCTGCGTGT-3’] and Actin2 [5’-GCCAGACAGCACTGTGTTGG-3’]) and ORF26 (KS-1 [5’-AGCCGAAAGATTCCACCAT-3’] and KS-2 [5’-TCCGTGTGTCTACGTCCAG-3’]) were used with 200ng of genomic DNA as template. Second round PCR was performed using a nested primer set specific to ORF26 (KS-4 [5’-CGAATCCAACGGATTTGACCTC-3’] and KS-5 [5’-CCCATAATGACACATTTGGTGGA-3’]) with 2μL of first round PCR product as template. Each PCR reaction was performed in a total volume of 25 μl using 0.4 μM of each specified primer and TaKaRa Ex Taq DNA polymerase kit (TaKaRa Biotechnology) according to the manufacturer’s protocol, with the exception of 2.5 units enzyme. All reactions were performed using the following conditions: 95°C for 5 min, 35 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec, and one cycle of 72°C for 7 min.
Statistical analyses.

Prior to statistical analyses, KSHV antibody titer was log$_2$ transformed. Statistical significance of antibody titers between study groups was calculated using two-sided Student’s t-test or one-way ANOVA with Tukey’s multiple comparisons test using GraphPad Prism 5.04 (Graph Pad Software).

RESULTS

Characteristics of the study cohort.

Table 4.1 describes the characteristics of the 86 children followed in the current study, by HIV and ART status at the time of KSHV seroconversion. Briefly, the median age for HIV-uninfected (n = 57), HIV-infected ART treated (n = 23), and HIV-infected ART-naïve (n = 6) children was 15, 21, and 7 months, respectively. The observed difference in age is consistent with our previous finding that HIV-infected ART-naïve children acquire KSHV at a significantly increased rate, and therefore a younger age, compared to HIV-infected children on ART and HIV-uninfected children [17]. The median CD4 T-cell percentage and HIV viral load was 40% and 4.0 log copies/mL, respectively, for HIV-infected children on ART. Prior to KSHV seroconversion, the median time of ART was 11.7 months. For HIV-infected children who were ART-naïve at KSHV seroconversion, the median CD4 T-cell percentage and HIV viral load was 25% and 4.6 log copies/mL, respectively (Table 4.1).
Longitudinal antibody response to acute KSHV infection.

The main goal of the current study was to characterize the KSHV-specific humoral immune response after primary infection. Therefore, we measured the presence and titer of KSHV binding antibodies, at three month intervals, for 24 months after KSHV acquisition. We observed that the presence of KSHV antibodies in infected children was not consistent over time. We detected KSHV antibodies in only 335 of 718 follow-up plasma samples, for an overall detection rate of 46.6%, indicating that seroreversion occurs frequently after primary KSHV infection. Within the first three months after the KSHV primary seroconversion time point (100% seropositive), only 42% of children had detectable KSHV antibodies. At each subsequent follow-up interval, the percentage of children with detectable KSHV antibodies remained at approximately 40% (Figure 4.1).

The titers of longitudinal antibody responses to KSHV observed in study participants varied greatly. We observed KSHV antibody profiles in children that ranged from stable low titers to stable high titers, in addition to profiles that progressively changed over time. Several children also displayed profiles where antibody titers frequently alternated between detectable and undetectable levels. Figure 4.2 displays longitudinal KSHV antibody titer profiles from five children that are representative of the antibody patterns observed in the cohort. Changes in KSHV antibody titer over time did not correlate with longitudinal data collected for full blood count (white blood cell count, hemoglobin level, hematocrit, lymphocyte percentage, and monocyte percentage), generalized symptoms (e.g. fever, rash, cough, and diarrhea), malaria
infection, HIV infection, or CD4⁺:CD3⁺ T-cell percentage. Additionally, the proportion of children with highly variable KSHV antibody titers compared to those with more stable titers over time were similar between HIV-infected and HIV-uninfected children (34.5% vs 40.0%).

To examine the role of HIV-infection and ART status on the KSHV-specific antibody response, longitudinal KSHV antibody titers were summarized by calculating the geometric mean titer (GMT) of all time points for each child and compared by HIV and ART status at the time of KSHV seroconversion (Table 4.1 and Figure 4.3). The median of longitudinal GMT for HIV-infected ART-naïve children was marginally higher compared to HIV-infected children on ART and HIV-uninfected children (Table 4.1). However, the log₂ transformed longitudinal GMTs were not significantly different (p-value = 0.28) due to small sample size of ART-naïve children (Figure 4.3).

Among all children with detectable KSHV antibodies, the GMT for each follow-up interval was consistent—remaining between 89 and 138 (Figure 4.4A). Interestingly, however, the maximum antibody titer decreased over time from 2560 to 320, consistent with a persistent infection that enters latency. At the time of seroconversion, the KSHV antibody GMT among HIV-infected ART-naïve children was significantly higher, at 640, compared to 90 and 105 among HIV-infected children on ART and HIV-uninfected children, respectively (Figure 4.4B). The KSHV antibody GMT between groups at all other follow-up intervals were similar, with the exception of nine months post-seroconversion (HIV+: 160 vs. HIV-: 75, p-value <0.05) (Figure 4.4B). Of note, by six months after KSHV
seroconversion only one HIV-infected child remained ART-naïve and by nine months all HIV-infected children were taking ART.

Whether a KSHV-specific antibody response during the early stages of infection also correlates with development of a neutralizing antibody response is unknown. Hence, we screened KSHV seropositive samples for the presence of neutralizing antibodies. Of the 86 children, and 335 samples with detectable KSHV binding antibodies, only one child developed neutralizing antibodies. The child’s longitudinal KSHV binding antibody profile is displayed in Figure 4.2E. Corresponding with an increase in total KSHV antibody, neutralizing antibodies were detected at 15, 18, and 21 months after primary KSHV seroconversion, but had declined by 24 months post-seroconversion. This child was HIV-infected and had been taking ART for 12 months before KSHV seroconversion. However, there were no known characteristics that distinguished this child from other HIV-infected children taking ART.

**Detection of KSHV DNA during acute infection.**

We also sought to determine whether levels of KSHV DNA detected in the oral cavity and PBMCs correlated with the KSHV humoral immune response between HIV-uninfected and –infected children. The overall KSHV DNA detection rate among all samples was very low—approximately 7% in oral swabs and 4% in PBMCs—and consequently KSHV DNA detection did not correlate with longitudinal KSHV humoral immune response. Additionally, due to low KSHV DNA detection rate and few number of ART-naïve children, there were insufficient sample numbers to adequately assess
differences in KSHV DNA detection by ART status. Therefore, whether KSHV DNA was detected at any follow-up time point for each child was measured and compared by HIV status. The KSHV detection rates per child in both oral swab and PBMC samples were similar between HIV-uninfected and –infected children, 28% vs. 30% and 14% vs 16%, respectively (Table 4.2). This is consistent with similar longitudinal KSHV antibody levels regardless of child HIV status.

**DISCUSSION**

An immune response that is effective at controlling virus replication during infection can prevent clinical disease progression in the infected individual, as well as reduce the potential for transmission to other individuals. Among KSHV-infected individuals, HIV-induced immune dysregulation and the loss of an effective KSHV-specific immune response are hypothesized to contribute to KS development. However, very little is known about the immune response to KSHV infection prior to KS, let alone the immune response during the early stages of infection. It is possible that a robust and early immune response against KSHV could minimize the infection and prevent the development of KS. Although primary KSHV infection is difficult to determine among adult populations, young children represent an ideal cohort to study because the timing of primary KSHV infection can be ascertained due to young age. In the present study, we have evaluated the impact of HIV and ART on the KSHV-specific humoral immune response over two years in a prospective cohort of Zambian children who recently underwent primary KSHV infection.
Among the KSHV-infected children followed in the present cohort, we only
detected KSHV antibodies in approximately 40% at any given follow-up interval after
KSHV acquisition. This further confirms observations from our previous cohort [18] that
KSHV seroreversion is common. Seroreversion could be due to complete loss of
antibodies against KSHV or a reduction to levels undetectable by current serological
assays. KSHV serological assays have variable sensitivity levels [20]; hence, a highly
sensitive, ‘gold standard’ serological assay to detect KSHV antibodies is needed. In the
absence of a ‘gold standard’ test, however, the presence of KSHV seroreversion calls
attention to the importance of longitudinal studies. With approximately 60% of KSHV-
infected children having undetectable antibody levels after primary seroconversion,
cross-sectional studies will substantially underestimate KSHV infection rates.

Evaluation of KSHV-specific antibody titers revealed high variability over time
among the children analyzed. Furthermore, we did not detect correlations between
KSHV antibody titer and available clinical information, HIV status, or presence of KSHV
DNA. Our findings are consistent with a recent study that investigated early,
asymptomatic infection by Epstein-Barr Virus (EBV)—a closely related
gammaherpesvirus [21]. The study, conducted among children from The Gambia,
showed that EBV-specific antibody titers were variable after primary infection and did
not correlate with EBV load. Furthermore, within our cohort KSHV neutralizing
antibodies only developed in one child, despite the observation that total KSHV-specific
antibody titers could be high.
This suggests that the antibody response against gammaherpesviruses, including KSHV, may not be an important component of the initial immune response to control virus replication.

Cellular immunity is known to be important against other herpesviruses [22, 23]. Indeed, EBV-specific CD8+ T-cell levels detected in recently infected Gambian children decreased over time in correlation with EBV load [21]. T-cell mediated-immunity may similarly play an important role in controlling KSHV in children during the early stages of infection. Cytotoxic T-lymphocyte (CTL) responses against KSHV have been detected in healthy KSHV-seropositive adults [24, 25], and several epitopes within KSHV proteins gB, K8.1, ORF73/LANA, and K12 can induce both monofunctional and polyfunctional CTL responses [26]. T-cells in tonsillar explants have also been shown to prevent KSHV replication and promote latency in infected B-cells [27]. This presents an alternative, noncytolytic mechanism of cell-mediated control of KSHV infection. However, the importance of these responses to control virus replication in vivo and their relation to disease progression is yet to be determined.

A primary objective of the current study was to evaluate the role of HIV and ART on the humoral immune response against KSHV. To this end, the KSHV antibody GMT among HIV-infected ART-naïve children was significantly higher at the time of KSHV seroconversion. Conversely, the longitudinal KSHV GMT did not significantly differ by child HIV or ART status. Although the difference was not significant, except at nine months after seroconversion, HIV-infected children actively taking ART had marginally higher KSHV GMT compared to HIV-uninfected children at several time points. It is
possible that polyclonal B-cell activation and a pro-inflammatory state triggered by HIV infection may have increased the production of anti-KSHV antibodies. One limitation to this analysis was the very low number of ART-naïve children; by nine months after KSHV seroconversion all children were taking ART. Therefore longitudinal analysis of the effect of ART on the KSHV-specific humoral immune response could not be conducted. This was expected, because all children were offered and advised to take ART as part of the cohort design and local standard of care. To more adequately evaluate the role of ART on the KSHV humoral immune response, a similar study using samples collected before the widespread use of ART in Zambia would need to be conducted.

The present cohort study has several strengths, including detailed demographic and clinical information, prospective design, and short follow-up intervals. However, an important limitation to the study is the lack of a KSHV-specific IgM analysis. We have several reasons to conclude that the primary IgG seroconversion event is representative of KSHV acquisition, including young age of the children and absence of KSHV in saliva at study entry. Nonetheless, KSHV-specific IgM responses will provide further details into the early immunological events following KSHV infection and may correlate with clinical data or HIV/ART status. We are therefore currently developing a sensitive and reliable assay to assess the KSHV-specific IgM responses.

In summary, the current study describes the humoral immune response against KSHV following primary infection in young children. We show that KSHV-specific antibody titers are highly variable over time and do not correlate with available clinical information, HIV/ART status, or KSHV replication. Collectively, these data suggest that
the humoral immune response may not be an important factor in controlling KSHV infection, but that other mechanisms of immunity, such as cell-mediated, should be further investigated.
REFERENCES


FIGURE LEGENDS

Figure 4.1. KSHV seroprevalence rate among KSHV-infected children at three month intervals after primary seroconversion.

Figure 4.2. Longitudinal KSHV antibody titers over twenty-four months were calculated for 86 children that underwent primary KSHV seroconversion. The inverse of the highest sample dilution determined to be positive by KSHV mIFA represents the antibody titer. Graphs display the longitudinal profiles from five children that are representative of the patterns observed: (A) Stable low titer, (B) stable high titer, (C) decreasing over time, (D) inconsistent, and (E) increasing over time. The number and percentage of children that each graph represents are noted.

Figure 4.3. Longitudinal KSHV antibody titers were summarized by calculating the geometric mean of titers from all follow-up time points for each child. Summary data were log$_2$ transformed and geometric mean titer (GMT) was compared by child HIV and ART status at the time of KSHV seroconversion. One-way ANOVA revealed no significant difference (p-value = 0.28).

Figure 4.4. Geometric mean titer (GMT) of KSHV antibodies among KSHV seropositive children at three month intervals after primary seroconversion. (A) Columns represent KSHV antibody GMT with 95% confidence interval of all KSHV seropositive children. Diamonds (♦) represent the maximum antibody titer for that follow-up time point. (B)
Columns represent antibody GMT with 95% confidence interval of KSHV seropositive children by HIV and ART status. One-way ANOVA with Tukey’s multiple comparisons test was used to compare KSHV GMT at intervals 0, 3, and 6. Two-sided Student’s t-test was used to compare KSHV GMT at intervals 9 – 24. *** denotes p-value <0.001. * denotes p-value <0.05.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HIV- (n = 57)</th>
<th>HIV+/ART+ (n = 23)</th>
<th>HIV+/ART- (n = 6)</th>
</tr>
</thead>
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<tr>
<td>Gender</td>
<td></td>
<td></td>
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<tr>
<td>Male</td>
<td>30 (52.6)</td>
<td>13 (56.5)</td>
<td>2 (33.3)</td>
</tr>
<tr>
<td>Female</td>
<td>27 (47.4)</td>
<td>10 (43.5)</td>
<td>4 (66.7)</td>
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<tr>
<td>Age (months)</td>
<td>15 (11 - 19)</td>
<td>21 (15 - 25)</td>
<td>7 (7 - 10)</td>
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<tr>
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<td>3.6 (1.7 - 35.2)</td>
<td>12.7 (4.3 - 201.8)</td>
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<td>4.6† (4.2 – 5.0)</td>
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<tr>
<td>CD4%*</td>
<td>-</td>
<td>40 (35 - 57)</td>
<td>25 (20 - 40)</td>
</tr>
<tr>
<td>Months on ART</td>
<td>-</td>
<td>11.7 (6.1 - 20.5)</td>
<td>-</td>
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</tbody>
</table>

†n = 2; missing 4 samples
* Wilcoxon rank-sum test p-value <0.05
Table 4.2. Children with detectable KSHV DNA at any time point after KSHV seroconversion

<table>
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<tr>
<th>KSHV DNA detected in</th>
<th>HIV- (n = 57)</th>
<th>HIV+ (n = 29)</th>
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<tr>
<td>Buccal swab</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>40 70%</td>
<td>21 72%</td>
</tr>
<tr>
<td>Yes</td>
<td>17 30%</td>
<td>8 28%</td>
</tr>
<tr>
<td>PBMC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>48 84%</td>
<td>25 86%</td>
</tr>
<tr>
<td>Yes</td>
<td>9 16%</td>
<td>4 14%</td>
</tr>
</tbody>
</table>
Figure 4.1

<table>
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<th>Months after primary KSHV seroconversion</th>
<th>0</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>12</th>
<th>15</th>
<th>18</th>
<th>21</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent children KSHV seropositive (%)</td>
<td>100</td>
<td>42</td>
<td>45</td>
<td>38</td>
<td>39</td>
<td>35</td>
<td>40</td>
<td>40</td>
<td>38</td>
</tr>
<tr>
<td>Number of KSHV(+) children</td>
<td>86</td>
<td>33</td>
<td>35</td>
<td>30</td>
<td>31</td>
<td>28</td>
<td>30</td>
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<tr>
<td>Total Children</td>
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<td>78</td>
<td>78</td>
<td>80</td>
<td>79</td>
<td>75</td>
<td>78</td>
<td>86</td>
</tr>
</tbody>
</table>
Figure 4.3
CHAPTER 5

WHOLE-GENOME SEQUENCING OF KSHV FROM ZAMBIAN KAPOSI’S SARCOMA BIOPSIES REVEALS UNIQUE VIRAL DIVERSITY

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Data from this chapter is currently undergoing peer-review.

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ABSTRACT

Kaposi’s sarcoma-associated herpesvirus (KSHV) is the etiological agent for Kaposi’s sarcoma (KS). Both KSHV and KS are endemic in sub-Saharan Africa where approximately 84% of global KS cases occur. Nevertheless, whole-genome sequencing of KSHV has only been completed using isolates from Western countries—where KS is not endemic. The lack of whole-genome KSHV sequence data from the most clinically important geographical region, sub-Saharan Africa, represents an important gap as it remains unclear whether genomic diversity has a role on KSHV pathogenesis. We hypothesized that distinct KSHV genotypes might be present in sub-Saharan Africa compared to Western countries. Using a KSHV-targeted enrichment protocol followed by Illumina deep-sequencing, we generated and analyzed sixteen unique Zambian, KS-derived, KSHV genomes. We enriched KSHV DNA over cellular DNA 1,851 to 18,235-fold. Enrichment provided coverage levels up to 24,740-fold; therefore, supporting highly confident polymorphism analysis. Multiple alignment of the sixteen newly sequenced KSHV genomes showed low level variability across the entire central conserved region. This variability resulted in distinct phylogenetic clustering between Zambian KSHV genomic sequences and those derived from Western countries. Importantly, the phylogenetic segregation of Zambian from Western sequences occurred irrespective of inclusion of the highly variable genes K1 and K15. We also show that four genes within the more conserved region of the KSHV genome contained polymorphisms that partially, but not fully, contributed to the unique Zambian KSHV whole-genome
phylogenetic structure. Taken together, our data suggest that the whole KSHV genome should be taken into consideration for accurate viral characterization.

IMPORTANCE

Our results represent the largest number of KSHV whole-genomic sequences published to date and the first time that multiple genomes have been sequenced from sub-Saharan Africa, a geographic area where KS is highly endemic. Based on our new sequence data, it is apparent that whole-genome KSHV diversity is greater than previously appreciated and differential phylogenetic clustering exists between viral genomes of Zambia and Western countries. Furthermore, individual genes may be insufficient for KSHV genetic characterization. Continued investigation of the KSHV genetic landscape is necessary in order to effectively understand the role of viral evolution and sequence diversity on KSHV gene functions and pathogenesis.
INTRODUCTION

Kaposi’s sarcoma-associated herpesvirus (KSHV), or human herpesvirus-8 (HHV-8), is the etiologic agent for all forms of Kaposi’s sarcoma (KS) [1]. KS manifests as an endothelial tumor primarily on the skin but can also involve mucosal membranes and visceral organs. Among the HIV-uninfected population KS is rare worldwide; however, HIV infection and immunosuppression greatly increase the risk of developing KS [2]. In sub-Saharan Africa, HIV is epidemic and KSHV is endemic. Accordingly, KS is one of the most common cancers in sub-Saharan Africa and this region accounts for 84% of global KS cases [3]. Two other HIV-associated lymphoproliferative malignancies (primary effusion lymphoma [PEL] and multicentric Castleman’s disease [MCD]), as well as the KSHV inflammatory cytokine syndrome (KICS) are also associated with KSHV infection [4-6]. However, the role of KSHV genetic variation on pathogenesis and disease presentation is unknown. Therefore, as a first step, it is necessary to analyze KSHV genetic variation in sub-Saharan Africa at the whole-genome level.

KSHV is a human gamma-herpesvirus with a largely conserved double-stranded DNA genome of approximately 140 kilobases. However, the extreme 5’ and 3’ termini are disproportionately variable compared to the central region of the KSHV genome and both have been used to categorize KSHV into different genotypes [7, 8] The 5’ end encodes the K1 gene and can be separated into five distinct genotypes (A, B, C, D, E), differing by up to 30% at the amino acid level. At the nucleotide level, 85% of polymorphisms within K1 are nonsynonymous, suggesting that strong selective pressure acts on the gene [7]. The 3’ terminus of the KSHV genome encodes the K15 gene.
Sequence analysis of K15 supports additional categorization of KSHV sequences into P, M, or N alleles, with up to 70% inter-allele divergence at the amino acid level [8, 9]. In addition, nine discrete loci (∼5.6% of the genome) within the central, more conserved, region of the KSHV genome also contain polymorphisms, albeit at a much lower rate. Together, twelve KSHV genotypes have been proposed based on these 11 discrete loci [9]. However, the remaining KSHV genes, representing more than 90% of the genome, have not been used to further characterize KSHV genetic structure and diversity due to a lack of high coverage, whole-genome, viral sequences.

Presently, only six KSHV whole-genome sequences are available. The first complete, and most extensively annotated genome, GK18, was generated from a classic KS lesion from a Greek patient (AF148805.2). The nearly complete, ‘KS’ genome was sequenced using shotgun sequencing of fragments obtained after Sau3A digestion of DNA from AIDS-associated KS biopsies (U93872.2). Additionally, three genomic KSHV sequences were generated from KSHV-infected PEL cell lines, BC-1 (U75698.1), JSC-1 (GQ994935.1), and BCBL-1 (HQ404500.1) [10-12]. The sixth and most recently sequenced KSHV genome, DG-1 (JQ619843.1), was the first to be completed using Illumina next-generation sequencing technology and the first obtained from virus in patient plasma [13]. Despite these significant efforts, all current genomic KSHV sequences were generated from samples obtained in Western countries where KSHV is not endemic. The lack of whole-genome KSHV sequence data from sub-Saharan Africa—the geographical region most relevant to KSHV infection—represents an important gap in genetic characterization for this pathogen as it remains unclear whether a correlation
exists between whole-genome sequence diversity and KSHV pathogenesis. A recent study of Epstein Barr Virus (EBV) whole genomes revealed significant levels of sequence diversity in isolates from nasopharyngeal carcinoma (NPC) clinical samples in a region with high NPC prevalence [14]. This further suggests that a thorough characterization of KSHV whole-genomes needs to be conducted—including isolates from sub-Saharan Africa—as a first step to investigate possible relationships between genomic diversity and pathogenesis.

In the current study, we sought to test the hypothesis that distinct whole-genome KSHV variants are present in sub-Saharan Africa compared to Western countries. We also investigated whether diversity within the central region genes may contribute to viral characterization. To this end, we generated and analyzed KSHV whole-genome sequences derived from KS skin lesions of sixteen different Zambian patients. Using a biotinylated RNA-library as bait, KSHV sequences were preferentially enriched over human genomic DNA present in tumor samples and the KSHV-enriched DNA was sequenced using Illumina deep-sequencing technology. Polymorphism and phylogenetic analyses were then performed to measure KSHV genome-wide diversity. Our results represent the largest number of KSHV whole-genomic sequences published to date and the first time that multiple genomes have been sequenced from sub-Saharan Africa, a geographic area where KS is highly endemic.
METHODS

KS sample collection.

KS biopsies were obtained from patients upon disease presentation at the skin clinic of the University of Zambia, University Teaching Hospital. The biopsies were collected as part of KS diagnosis and residual tissue samples were used for the current study. All patients provided written, informed consent to participate in the study. Collection of biopsies was approved by the Institutional Review Board of the University of Nebraska and the University of Zambia Biomedical Research Ethics Committee.

Sample DNA preparation.

Total DNA was extracted from frozen KS tumor biopsies using the Gentra Puregene Tissue Kit according to manufacturer’s protocol (Qiagen). Purified DNA samples were analyzed via Qubit broad-range dsDNA kit, Nanodrop spectrometer, and agarose gel electrophoresis to measure concentration, protein contamination, and level of degradation. All samples were of high quality and usable for downstream applications.

KSHV viral load.

The number of KSHV genomes in each KS biopsy sample was quantified before enrichment using the Bio-Rad QX100 droplet digital PCR (ddPCR) system. Human β-globin and KSHV ORF26 were amplified using primers and probes described previously [15]. Each 20μL ddPCR reaction contained 1X ddPCR Supermix (Bio-Rad), 900nM forward
and reverse primer, 250nM Taqman probe, and 6 or 60ng of genomic DNA. Droplet generation, amplification, and reading were carried out according to the manufacturer’s protocol. Amplification conditions for β-globin were as follows: 95°C for 10 min, 40 cycles of 94°C for 30 sec and 60°C for 60 sec, and 98°C for 10 min. KSHV ORF26 amplification conditions were similar with the exception of a 55°C elongation temperature. All samples were run in triplicate and the mean KSHV copy number per cellular equivalent was calculated.

**Library preparation, Target Enrichment, and Illumina sequencing.**

Sample library preparation and target enrichment were performed using the SureSelectXT Target Enrichment System (Agilent) according to the manufacturer’s Illumina paired-end sequencing library protocol. Briefly, 120bp overlapping RNA baits were custom designed at 5x coverage in conjunction with Agilent SureDesign based on the KSHV GK18 sequence (AF148805.2). Baits with high homology to human DNA were excluded from the RNA-library. For each sample, 3μg of purified KS tissue biopsy DNA was sheared and Illumina specific adapters were added. The DNA-libraries were then hybridized for KSHV-specific enrichment, index tagged, and pooled at equimolar amounts for sequencing. Next-generation sequencing was performed using an Illumina HiSeq with 100bp paired-end reads on two separate runs at the University of Nebraska DNA Sequencing Core.
**Guided assembly of KSHV genomes.**

Output reads from the Illumina HiSeq 2500 were filtered using Trimmomatic [16]. Reads were trimmed on both 5’ and 3’ extremities using a quality (Q) threshold keeping only bases with Q>=30. All reads shorter than 101bp long were filtered out, thus retaining only full-size reads of high quality. The dataset was controlled for quality using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), both before and after the filtering steps.

High quality paired-end reads were aligned to the KSHV genome (GK18, accession: AF148805.2) using Bowtie2 version 2.2.1 [17]. First, reads were assembled one sample at a time using the Columbus extension of Velvet 1.2.10 [18] and the corresponding initial alignment. A k-mer size of 91 was found to produce the best results after multiple trials of different values. Both average and minimum k-mer coverage were determined on a sample-by-sample basis by first performing a ‘blank’ assembly with the ‘exp_cov’ and ‘cov_cutoff’ parameters set on ‘auto’ and then looking at the k-mer coverage of the largest contigs produced matching the reference sequence. Afterwards, the ‘cov_cutoff’ parameter was set on a tenth of the ‘exp_cov’ value for the final Velvet assembly. Next, a second assembly was produced for each sample using MIRA [19] for correction purposes. The same reference sequence, mapping, and accurate flags were described for Velvet assembly, and lossless digital normalization (-ldn) was activated to reduce the dataset to a memory-manageable size.
Correction, scaffolding and annotation.

The viral contigs in both assemblies were selected and ordered by aligning them to the reference sequence using Mauve [20]. We also manually merged neighbor contigs in the Velvet assemblies exhibiting a 10 to 90nt long overlap with each other. For each sample, a multiple alignment comprising the GK18 reference sequence, the Velvet assembly, and the MIRA assembly was performed using Kalign2 [21]. To generate the final sequences, conserved regions between the Velvet and MIRA assemblies were accepted and the discrepancies were manually corrected by comparing the original read alignments to input sequences. We then used Tablet [22] and Jalview [23] for visualization of reads and genome alignment respectively.

After determining the final assembly, each new genome was annotated. Given the close proximity to the KSHV reference sequence, the annotation of each new genome was carried out by transferring the available KHSV annotation in Genbank using RATT [24]. However, splicing junctions were unsuccessfully transferred for the K8 and K15 genes. Manual correction was therefore performed for both genes because the K8 exons/introns annotated in the GK18 Genbank entry do not accurately reflect previously reported splicing [25], and the K15 gene features many exons that RATT could not transfer.

Comparative and phylogenetic sequence analysis.

The assembled KSHV genomes were aligned using Kalign2. The multiple alignment was then used to generate whole-genome maximum likelihood phylogenetic
trees using PhyML [26] with 1000 bootstrap replicates, and the trees were visualized using MEGA6 [27]. Genome-wide mutations were visualized using the mVista software [28], with a 100bp scanning window. Multiple amino acid alignments were generated for each KSHV gene and inspected for high level of nonsynonymous mutations. Maximum likelihood phylogenetic trees were generated using PhyML and amino acid highlighter plots were generated to visualize the specific mutations using the Highlighter tool as part of the Los Alamos National Laboratory HIV sequence database (www.hiv.lanl.gov). Reference sequences used for K1 genotyping were as follows:

AF133038 (A1), AF130305 (A2), U86667 (A3), AF133039 (A4), AF178823 (A5), AF133040 (B1), AY042947 (B2), AY042941 (B3), DQ309754 (B4), AF133041, (C1), AF133042 (C3), AF133043 (D1), AF133044 (D2), and AF220292 (E). Reference sequences used for K15 classification were AAD46505.1 (P), AAD45296.1 (M), and a personal communication from Gary Howard (N).

**RESULTS**

*Summary of sequencing data.*

In the present study, we analyzed the sequence diversity of KSHV whole genomes acquired from KS skin biopsies of sixteen Zambian patients—11 males and 5 females. Clinical data collected for each patient is summarized in Table 5.1. Total DNA was extracted from the biopsy samples and KSHV burden in tumor tissue ranged from 0.21 to 17.16 copies per cell (Table 5.2). In order to efficiently sequence the KSHV DNA, which was present as a small proportion of total tumor DNA (0.0006% - 0.05%), we used
a custom biotinylated RNA-library (Agilent) to hybridize and selectively enrich the KSHV DNA for sequencing with an Illumina HiSeq.

We first tested the efficiency of our whole protocol, from DNA enrichment to genome sequencing, on three samples (ZM116, ZM117, and ZM118). This process resulted in up to 12,107-fold enrichment of KSHV DNA over cellular DNA with 62% of the total sequence reads mapped to the KSHV reference sequence (GK18). The GK18 sequence was selected because it is currently the most comprehensively annotated KSHV sequence and also served as the reference for generating the RNA bait library employed in the current study. After our initial results, we continued with the remaining 13 samples in a second run of DNA enrichment and deep-sequencing. A total of 528,849,840 paired-end reads, 101bp long, were produced from both HiSeq runs with an average of 49% of the sequence reads mapped to KSHV. Together, we obtained a mean enrichment of KSHV DNA over cellular DNA of 8,437-fold (range 1,851 - 18,235-fold) (Table 5.2). Thus, we were able to filter the dataset at a high quality threshold and still maintain high coverage (mean: 8,437-fold; range: 786 - 24,740-fold) (Table 5.2).

The assembly of each viral genome was conducted in a two-step approach: first the sequence reads were assembled using Velvet and the KSHV reference sequence; then this initial assembly was manually corrected with the help of a second assembly generate using MIRA. After manual fusion of contigs exhibiting large overlaps in the initial Velvet assembly, each genome featured an average of 4 contigs. This is consistent with the presence of 3 major repeat regions in KHSV that were hard to resolve using short-read sequencing technology alone. After manual correction with the MIRA
assembly, we were able to reduce this value to an average of 2 contigs per genome. All genomes were correctly sized with an average of 137Kb, corresponding to the size of previously published KSHV genomes from Western sources. Apart from the repeat regions, most genomes (12 of 16) had uniform read coverage. However, four genomes (ZM091, ZM095, ZM116, and ZM124) showed a few regions with coverage of up to 3 times the sample average. Nevertheless, these discrepancies did not hinder the assembly process.

*KSHV whole-genome variability analysis.*

The sixteen newly assembled and annotated KSHV genomic sequences, in addition to the six previously sequenced KSHV genomes, were used for multiple alignment and analyzed phylogenetically. Gaps and/or repeated regions of each genome, including the reference sequence, were masked. Figure 5.1 presents an unrooted maximum likelihood tree depicting the relative phylogenetic distance between samples. Although the overall identity at the nucleotide level is very high among the 22 genomes, distinct phylogenetic clusters are evident. All previously published KSHV genomes from Western countries clustered together, while the isolates from Zambia appear to form two separate clusters and contain much more variability among isolates. Isolate ZM004 diverged substantially from all other sequences and therefore was used to root subsequent whole-genome cladograms. The distinct phylogenetic clusters with higher variability among Zambian isolates can also be seen in the ZM004-rooted cladogram (Figure 5.2A).
The K1 and K15 genes are known to vary greatly among KSHV isolates and K1 subtypes are associated with specific geographical regions. Therefore, we investigated whether variability in K1 or K15 correlated with the KSHV-whole genome variability we detected. To this end, we performed a multiple alignment of all 22 KSHV genomic sequences without the K1 or K15 genes and generated a ZM004-rooted cladogram. Despite removing the highly variable genes, the topology of the phylogenetic tree remained similar to that of the whole-genome KSHV cladogram (Figures 5.2A and 5.2B). The only difference was a slight restructuring among the Western isolates, most likely because the BC-1 isolate contains the K15 M allele. Moreover, when nucleotide phylogenetic trees were generated from the K1 and K15 sequences, the tree topology and sample clustering did not correlate with the whole-genome phylogenetic analysis (Figures 5.2C and 5.2D). Together this indicates that the phylogenetic clustering detected at the whole-genome level is a function of variability in the central region of the KSHV genome.

We then sought to investigate whether variability in the central region of the Zambian KSHV isolates could be accurately characterized by individual genes or if consideration of the whole region is required. The distribution of nucleotide variability among the central region of all 22 KSHV genomes compared to GK18 was visualized using mVista (Figure 5.3). We did not find any areas of high nucleotide variation, other than the K1 and K15 genes; rather, we detected low-level variation throughout the entire central region when the Zambian isolates were compared to GK18 (Figure 5.3).
The total number of mutations compared to the GK18 sequence, including deletions, insertions, and substitutions, for each of the sixteen Zambian KSHV genomes is summarized in Table 5.2. We did not identify any correlations between the Zambian KSHV isolate sequence variation and clinical data. Additionally, we did not detect any intra-subject sequence variation, indicating that KSHV within each KS tumor was clonal.

*KSHV coding region mutations.*

Given that the phylogenetic segregation between Western and Zambian KSHV genomic sequences was due to low level variation across the central region of the genome, we inspected all coding sequences for nonsynonymous mutations compared to the GK18 reference genome. Out of the 84 annotated coding regions, we identified six KSHV genes with high levels of nonsynonymous mutations—four within the central conserved region (K4.2, K8.1, K11/VRIF2, and K12/Kaposin) and two previously known to have high variability (K1 and K15). Among the genes within the central region of the KSHV genome, K4.2 contained the highest level of nonsynonymous mutations compared to GK18. Phylogenetic analysis of the K4.2 gene revealed similar clusters for samples ZM004, ZM114, and ZM130 compared to the whole-genome analysis, but not for the remaining samples (Figure 5.4A). ZM091, ZM095, and ZM118 were very similar to GK18, with only three amino acid substitutions, whereas the remaining K4.2 sequences contained more than 20 substitutions and/or significant truncations at the C-terminal end of the coding region (Figure 5.4B). Sample ZM124 contained a 25 nucleotide deletion in the K8.1 coding sequence resulting in a frameshift mutation that produced a
stop codon very early in the gene (Figure 5.5B). Additionally, multiple amino acid substitutions, insertions, and deletions were identified within the K11/vIRF2 and K12/Kaposin genes (Figures 5.5C and 5.5D).

Since K1 and K15 have previously been demonstrated to be highly variable and are frequently used for KSHV genotyping, we generated amino acid maximum likelihood phylogenetic trees, including reference sequences, to determine the K1 and K15 genotypes of the sixteen Zambian KSHV isolates (Figure 5.6). All K1 sequences clustered with genotypes A5 (n = 1) or B (n = 15), consistent with previous K1 genotyping of samples from Zambia [15]. Within the B genotype, nine samples clustered with the sub-genotype B1, two with B3, and four with B4. The majority of the Zambian KSHV isolates contained the K15 P allele, however, two isolates (ZM095 and ZM128) contained the rare N allele. This is the first time the K15 N allele has been identified in Zambia.

**DISCUSSION**

Exploring relationships between KSHV sequence polymorphism and disease pathogenesis requires a more complete perspective of the magnitude and breadth of viral sequence diversity in geographical regions with the highest KSHV disease prevalence. However, little is known regarding the whole-genome diversity of KSHV, as only six complete genomes have been sequenced. Moreover, none of those previously published KSHV genomic sequences derive from sub-Saharan Africa—where prevalence of KSHV and KS is the highest. In the current study, we report the enrichment, sequencing, assembly, and analysis of sixteen unique Zambian KSHV genomes isolated
from KS tumors in adults. This study is the first to utilize SureSelect target capture technology to enrich and sequence KSHV DNA from clinical KS biopsies. The data generated represents the largest number of KSHV whole-genomic sequences published to date. Additionally, this study is the first to compare multiple KSHV genomes from a common geographical region that is endemic for KS.

Due to the low ratio of KSHV:human DNA in each tumor preparation, targeted enrichment of KSHV DNA was required prior to Illumina deep-sequencing. The enrichment protocol we employed resulted in 1,851 - 18,235-fold KSHV enrichment. This supported an average coverage depth of 8,437-fold, therefore, allowing high confidence in downstream polymorphism analyses. To further improve the accuracy of our whole-genome assemblies, we used MIRA assembly software to corroborate the Velvet-generated assemblies for each genome. Despite its overall accuracy, Velvet still produced a few clearly misassembled regions or unjustified gaps. Therefore, the two-step assembly enabled us to capitalize on Velvet’s accuracy while correcting nearly all misassembled regions and gaps with MIRA.

Given the extremely low error-rate of herpesvirus polymerases [29], it is not surprising that previous comparisons of the six KSHV whole-genomes revealed a high level of sequence conservation [13]. The genomic conservation might also be anticipated because the sequences were all generated from US or European patient samples, despite derivation from distinct clinical presentations. Multiple alignment of the sixteen newly sequenced Zambian KSHV genomes also showed high conservation. However, a low level variability across the central conserved region resulted in distinct
phylogenetic clustering between the genomic sequences of Zambian KSHV isolates and those from Western countries.

For EBV, the divergent EBNA-3 alleles correlate with whole-genome clustering and serve as adequate surrogates to distinguish between EBV type-1 and -2 [14, 30], but this has not previously been investigated for KSHV. The genes at either termini of the KSHV genome, K1 and K15, have been previously shown to contain higher levels of polymorphism than the rest of the genome. Our Zambian KSHV sequence data also revealed high levels of sequence diversity at these loci. Nevertheless, when K1 and K15 were excluded from the whole-genome multiple alignment and subsequent phylogenetic analysis the topology of the phylogenetic tree did not change. Moreover, the topology of the KSHV whole-genome phylogenetic tree did not correlate with those of phylogenetic trees generated from K1 or K15 alone, suggesting that these genes are poor surrogates for measuring whole-genome KSHV diversity. Conversely, K4.2 phylogenetic analysis showed partial, but not full, correlation to whole-genomic clustering. This suggests that K4.2 may contribute to viral genomic characterization, but again, this single gene does not adequately characterize the whole-genome diversity we detected. Taken together, our data suggest that the whole KSHV genome should be taken into consideration for accurate viral characterization.

Although single gene polymorphisms did not adequately represent the genome-wide KSHV diversity, we identified several nonsynonymous mutations within KSHV protein coding regions that could potentially effect the viral phenotype. Interestingly, all six genes with significant levels of nonsynonymous mutations were gene products
uniquely encoded by KSHV. Five of the variant genes identified in this study are directly immunomodulatory genes and therefore, may lead to differential effects on the host immune response. Of interest, the coding sequences of K4.2 contained the highest level of nonsynonymous variation. K4.2 interacts with pERP1 to inhibit immunoglobulin secretion and increase calcium influx [31]. The amino acid domains of K4.2 that are important for these functions are unknown, hence the newly identified variability may be important for interaction with pERP1 or other cellular functions. This is currently under investigation.

K8.1 protein is not known to be directly immunomodulatory, but this virion membrane-associated glycoprotein does have important effects on the KSHV viral life cycle. K8.1 is utilized for KSHV attachment to target cells and induces VEGF expression upon binding [32, 33] Additionally, K8.1 is involved in virus egress from infected cells [33]. Despite these functions, a K8.1-null mutant virus can still infect HEK293 cells, indicating that K8.1 function is potentially dispensable in vitro [34]. The nucleotide sequence from sample ZM124 predicted a truncation in the amino acid sequence before the transmembrane domain of K8.1. If the protein was expressed as predicted, it is not clear what effect a K8.1-deficient virion and/or virion-independent, soluble K8.1 would have in the context of an in-vivo infection. K8.1 is highly immunogenic compared to other KSHV proteins [35], and exogenous expression of soluble K8.1 induces an interferon response [36]. Together, the ZM124 predicted K8.1 may elicit a modified innate and humoral immune response.
Three primary K15 alleles have been previously identified, P, M, and N. There is little variation within allele groups but extreme divergence across alleles. The P and M alleles, for example, have only 30% identity [8]. We identified, for the first time, two KSHV isolates from Zambia that contain the rare K15 N allele. Although the N allele is highly divergent from P and M, the signaling motifs for SH2 and TRAF are conserved in all K15 alleles. Recently, it was shown that the K15-P allele activates the alternative NF-κB signaling pathway by direct recruitment of NF-κB inducing kinase (NIK) to a distinct signal sequence [37]. However, this sequence is not conserved in either the M or N alleles. This genetic variation may lead to altered levels of K15-induced NF-κB activation and subsequently functional differences between alleles, thus further functional analyses of these K15 alleles is warranted.

In summary, we successfully enriched KSHV from a background of human DNA from KS biopsies using targeted RNA baits. Analyses of the sequences identified a low level variability across the KSHV central conserved region that resulted in distinct phylogenetic clustering between the genomic sequences of KSHV from Zambia and Western countries. Moreover, four genes within this region had significant levels of polymorphisms but did not adequately characterize the whole-genome diversity we detected. Based on the new sequence data in the present study, it is apparent that whole-genome KSHV diversity is greater than previously appreciated. Although the observed phylogenetic clustering between Western and Zambian KSHV genomic sequences could represent distinct subtypes, more whole-genome sequences are required from additional regions to infer distinct viral subtypes specific to any
geographical region. Continued investigation of the KSHV genetic landscape is necessary in order to effectively understand the role of viral evolution and sequence diversity on KSHV gene functions and pathogenesis.

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REFERENCES


FIGURE LEGENDS

**Figure 5.1.** Unrooted nucleotide maximum likelihood phylogenetic tree of six previously published KSHV whole-genome sequences and 16 new KSHV whole-genome sequences from Zambian KS biopsies. Phylogenetic tree was generated using PhyML with 1000 bootstrap replicates and visualized using MEGA6.

**Figure 5.2.** Maximum likelihood phylogenetic tree analysis of 22 KSHV whole genomes and nucleotide sequence of K1 and K15 genes. All phylogenetic trees were generated using PhyML with 1000 bootstrap replicates and visualized using MEGA6. (A) KSHV whole genome cladogram rooted on sample ZM004. (B) ZM004-rooted cladogram of KSHV whole genome with K1 and K15 gene sequences removed. (C and D) Midpoint-rooted cladogram of KSHV K1 (C) and K15 (D) sequences from 22 KSHV whole genome sequences.

**Figure 5.3.** Distribution of nucleotide variation within the central region of the KSHV genome as compared to the reference sequence GK18. The figure was generated using mVista software with a 100bp scanning window. The curve for each sequence represents up to 10% nucleotide variation within that window. Topological phylogenetic tree of sequences was generated using PhyML and visualized using MEGA6. Of note, multiple regions within the DG-1 sequence appear to have significant nucleotide diversity compared to GK18; however, these regions represent gaps in the published sequence due to low coverage that could not be masked.
Figure 5.4. Amino acid polymorphisms within the K4.2 gene. (A) Maximum likelihood phylogenetic tree of K4.2 amino acid sequence generated using PhyML with 1000 bootstrap replicates and visualized using MEGA6. (B) Amino acid highlighter plot of K4.2 generated using the Highlighter tool as part of the Los Alamos National Laboratory HIV sequence database.

Figure 5.5. Amino acid polymorphisms of three other KSHV central region genes. (A) Amino acid highlighter plot coloring scheme. Amino acid highlighter plots were generated for KSHV genes K8.1 (B), vIRF-2 (C), and K12 (D) using the Highlighter tool as part of the Los Alamos National Laboratory HIV sequence database.

Figure 5.6. KSHV K1 and K15 subtyping. Phylogenetic analyses using amino acid sequences for K1 (A) and K15 (B) from the 22 KSHV genomic sequences and prototypic reference sequences. Maximum likelihood phylogenetic trees were generated using PhyML with 1000 bootstrap replicates and visualized using MEGA6.
Table 5.1. Clinical information for 16 KS patients

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Figure 5.1

[Diagram showing a phylogenetic tree with various labeled nodes and branches, including labels such as BCBL-1, GK18, DG-1, KS, JSC-1, ZM116, ZM18, ZM123, ZM106, ZM124, ZM091, ZM121, ZM108, ZM17, ZM102, ZM027, ZM114, ZM130, ZM004, ZM095, ZM128.]

Scale: 0.001
Figure 5.2

A

B

C

D
Figure 5.4
Figure 5.5
Kaposi’s sarcoma-associated herpesvirus (KSHV) is the etiologic agent for all forms of Kaposi’s sarcoma (KS)—one of the most common cancers in many countries of sub-Saharan Africa [1, 2]. KS was endemic in sub-Saharan Africa prior to the HIV/AIDS epidemic, but KS cases drastically increased thereafter [3, 4]. Specifically, KS was the most common childhood malignancy in Zambia by 1992, accounting for approximately 25% of all pediatric cancers [5]. Concordantly, our laboratory previously observed that KSHV infection is common among Zambian children as approximately 40% of children acquired KSHV by four years of age [6]. KSHV DNA is frequently detected in saliva of infected individuals—implicating salivary exchange as the major route of transmission to children [7, 8]. However, additional factors associated with the transmission of KSHV to children are poorly understood.

The overall objective of this dissertation research was to determine the factors that may affect KSHV transmission to young children in a region where KSHV is endemic and HIV is epidemic. Important factors that may influence KSHV transmission include those associated with 1) the source of transmission (donor), 2) the newly infected individual (recipient), and 3) the virus itself. This project, therefore, employed a multifaceted design—utilizing three separate Zambian cohorts while employing molecular and epidemiological techniques—to investigate the range of factors that affect KSHV transmission to children.
Chapter 2 examines factors of transmission that are associated with the donor; focusing on household members as potential sources of KSHV transmission. Utilizing a cohort specifically designed to investigate KSHV transmission among household members in Zambia, we described 31 KSHV-infected individuals from 16 households with amplifiable KSHV K1 DNA. Molecular analysis of K1 sequence provided evidence that (1) early childhood KSHV transmission occurs from both within and outside the household, (2) intra-household transmission can occur via non-maternal sources, (3) superinfection did not occur, and (4) the dominant K1 sequence within an individual did not rapidly evolve over time. Additionally, quantitative PCR analysis of oral swab samples revealed that KSHV shedding in the oral cavity is highly variable over time.

The studies described in Chapters 3 and 4 investigate immune function and antiretroviral therapy (ART) as factors of KSHV transmission related to the recipient. We enrolled and followed a cohort of 287 HIV-exposed, KSHV-negative children under 12 months of age from Lusaka, Zambia to identify KSHV seroconversion events. Potential factors associated with KSHV infection—with an emphasis on HIV, ART, and immunological measures—were assessed through structured questionnaires and blood analyses. In Chapter 3 we reported 151 children that underwent KSHV seroconversion. We observed similar KSHV incidence rates between HIV-infected and uninfected children. This is in contrast to our previous observation, from before the widespread use of ART, that HIV-infected children had a five-fold higher risk of acquiring KSHV [6]. Further analysis in Chapter 3 revealed two key findings among HIV-infected children: (1) ART-naïve children had significantly increased risk of KSHV acquisition (adjusted hazard
ratio [AHR]: 5.04, 95% confidence interval [CI]: 2.36-10.80, p-value <0.001 ) and (2) time-updated CD4+ T-cell percentage was significantly associated with risk of KSHV acquisition (AHR: 0.82, 95% CI: 0.74-0.92, p-value <0.001), such that each 5% increase of CD4+ T-cells represented an 18% decrease in risk of acquiring KSHV.

Chapter 4 extended the cohort study from Chapter 3, following 86 newly KSHV-infected children for two years after primary seroconversion. The objective was to characterize the humoral immune response against KSHV during the early stages of infection and investigate the impact of HIV and ART status on the immune response. At the time of seroconversion, HIV-infected ART-naïve children had a more robust KSHV antibody response, while HIV-infected children taking ART had similar KSHV antibody titers compared to HIV-uninfected children. It is possible that KSHV replicates more efficiently in HIV-infected, ART-naïve children therefore eliciting a higher antibody response. However, the number of ART-naïve children in this cohort was very low and this hypothesis could not be investigated. Moreover, the longitudinal antibody response to KSHV detected in study participants was highly variable and did not correlate with clinical data, HIV/ART status, or KSHV replication. Collectively, these data suggest that the humoral immune response may not be an important factor in controlling KSHV infection, but that other mechanisms of immunity, such as cell-mediated, should be further investigated.

Next-generation, deep sequencing of KSHV viral genomes was used in Chapter 5 to assess KSHV diversity in the endemic setting of Zambia compared to non-endemic settings of Western countries. This is the first step to investigate possible relationships
between genetic variations and functional modifications in viral products that may alter pathogenesis and transmission. In this project, we generated and analyzed sixteen unique Zambian, KS-derived, KSHV genomes. Analyses of the sequences identified a low level variability across the KSHV central conserved region that resulted in distinct phylogenetic clustering between the genomic sequences of KSHV from Zambia and Western countries. Moreover, four genes within this region had significant levels of polymorphisms. Interestingly, these gene products are unique to KSHV and have immunomodulatory effects. Whether the detected polymorphisms translate into functional modifications is unknown and is a potential source of future investigation. Continued investigation of the KSHV whole-genomic landscape among different geographical locations, age groups, and disease manifestations is necessary in order to effectively understand the role of viral evolution and sequence diversity on KSHV gene functions, pathogenesis, and transmission.

Based on the current findings, further studies of the immune response against KSHV during early infection are warranted. As the IgG antibody response against KSHV did not correlate with clinical factors or KSHV replication following infection (discussed in Chapter 4), a more comprehensive analysis into the production of all antibody isotypes—particularly IgM and IgA—against KSHV will provide further details into the early humoral immunological events. In a similar vein, determining the specific KSHV proteins that elicit antibody responses during the early stages of infection in childhood compared to those elicited during asymptomatic infection in adulthood and disease progression could be of clinical use. It would be of further interest to investigate which
proteins elicit neutralizing antibodies vs. possible decoy responses. Labo et al. [9] recently expressed nearly 85% of all KSHV proteins and developed a magnetic bead-based assay to delineate antibody responses to specific KSHV proteins. This could prove to be a useful serological tool for future investigations.

Since cell-mediated immunity plays an important role during herpesvirus infection, further investigation of the CD8+ T-cell response against KSHV is also needed. CTL responses against KSHV have been detected in healthy KSHV-seropositive adults [10]. However, which epitopes elicit the most effective responses to control virus replication and whether the responses identified are similar in populations where KSHV is endemic is unknown. Consequently, the importance of CTL responses to control KSHV replication in vivo and their relation to disease progression is yet to be determined.

In summary, these results present a deeper understanding of the multiple epidemiological, immunological, and viral factors related to KSHV transmission among young children in Zambia—a region where KSHV is endemic and HIV is epidemic. As a vaccine against KSHV is not currently available, it is paramount to understand the factors associated with transmission so that alternative strategies can be developed to prevent KSHV acquisition during early childhood. The findings from this study will be important for developing and establishing such behavioral and clinical strategies to help reduce KSHV spread among young children. This, in turn, will help reduce the burden of KS among children and adults in endemic settings. Finally, KSHV-specific immunological
data obtained during the early phases of infection may be useful for future development of a KSHV vaccine.
REFERENCES


