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THE EPIDEMIOLOGY OF HUMAN HERPESVIRUS-8: TRANSMISSION OF
INFECTION TO CHILDREN IN ZAMBIAN HOUSEHOLDS

By

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THE EPIDEMIOLOGY OF HUMAN HERPESVIRUS-8: TRANSMISSION OF
INFECTION TO CHILDREN IN ZAMBIAN HOUSEHOLDS

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University of Nebraska, 2013

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Human Herpes virus-8 (HHV-8) is the known etiologic agent for several malignant pathologies, including Kaposi's sarcoma (KS), the most common tumor in children in sub-Saharan Africa. Saliva is implicated as the culprit of transmission; however there is a paucity of information regarding transmission to young children. In this study, we investigated the hypothesis that household behaviors exposing the susceptible child to saliva increase the risk of transmission of HHV-8 to that child.

To test our hypothesis a large prospective cohort study in Lusaka, Zambia, enrolling 464 young children and their households, was followed for 48 months. Socio-demographics, health histories, feeding and child-rearing behaviors were assessed.

At enrollment, 75 HHV-8 positive children were analyzed for existing risk factors contributing to HHV-8 seropositivity. Analysis for independent variables found that for each additional HHV-8 positive household member, there was 2.5 greater odds for the child to be HHV-8 positive ($P < 0.01$). Of the household saliva sharing risk behaviors, a primary caregiver testing temperature of food prior to feeding to a child posed a 2.4 greater odds ($P = 0.01$). A child having been breastfed offered a decreased risk (OR 0.3, $P < 0.01$).

Of the enrolled children, 270 children were available for longitudinal analysis. Of these, 137 children seroconverted to become HHV-8 positive, an incidence of 29.94 per

100 child-years. Most significant independent behavioral risk factors for HHV-8 seroconversion included increased risk with current breastfeeding (HR 2.1, $P = 0.002$), increased risk with use of pacifier (HR 9.0, $P = 0.01$), and greater risk with household members blowing on food prior to sharing with child (HR 2.8, $P = 0.05$). Other studies in this report include development of an HHV-8 serodiagnostic assay.

Taken together, this report describes the epidemiology of HHV-8 transmission to a susceptible child within households, and the association with saliva sharing behaviors. Our results support the need for increased public awareness and education regarding risks of viral transmission with child-rearing behaviors that expose the child to saliva.

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TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGEMENTS	iv
TABLE OF CONTENTS	vi
CHAPTER 1: LITERATURE REVIEW	1
THE HERPESVIRUSES	1
HERPESVIRUS CLASSIFICATIONS	2
<i>Alphaherpesviruses</i>	3
<i>Betaherpesviruses</i>	3
<i>Gammaherpesviruses</i>	4
EPSTEIN BARR VIRUS	5
HUMAN HERPESVIRUS-8	6
<i>HHV-8 Genome organization</i>	6
<i>Latent phase of infection</i>	7
<i>Lytic phase of infection</i>	8
<i>HHV-8 immune responses</i>	9
<i>Immune modulation by HHV-8</i>	10
<i>Epigenetics</i>	13
<i>Pathogenic Mechanisms of HHV-8</i>	15
<i>Diseases caused by HHV-8</i>	16
<i>Transmission of HHV8</i>	22
<i>HHV-8 Diagnostics</i>	26
<i>HHV-8 in Zambia</i>	28
STUDY AIMS	29
REFERENCES	32
TABLES	43
CHAPTER 2: THE ZAMBIA CHILDREN’S KS-HHV8 STUDY: RATIONALE, STUDY DESIGN, AND STUDY METHODS	44
ABSTRACT	45
INTRODUCTION	46
MATERIALS AND METHODS	48
<i>Study population and site</i>	48
<i>Screening for enrollment</i>	48
<i>Enrollment and follow-up</i>	50
<i>Data management</i>	52
DISCUSSION	57
REFERENCES	61
TABLES AND FIGURES	63
CHAPTER 3: RISK FACTORS FOR EARLY CHILDHOOD INFECTION OF HUMAN HERPESVIRUS-8 IN ZAMBIAN CHILDREN: THE ROLE OF EARLY CHILDHOOD FEEDING PRACTICES	70
ABSTRACT	71
INTRODUCTION	72
MATERIALS AND METHODS	74
<i>Study design and population</i>	74
<i>Data collection and measures</i>	75
<i>Laboratory testing</i>	75
<i>Data analysis</i>	77

	vii
RESULTS.....	78
DISCUSSION.....	81
REFERENCES.....	86
TABLES AND FIGURES.....	89
CHAPTER 4: CHILDHOOD FEEDING PRACTICES AS A RISK FOR CHILDHOOD HHV-8	
ACQUISITION.....	96
ABSTRACT.....	96
INTRODUCTION.....	97
METHODS.....	99
<i>Study setting</i>	99
<i>Data collection and measures</i>	100
<i>Laboratory testing</i>	101
<i>Data analysis</i>	102
RESULTS.....	104
DISCUSSION.....	109
REFERENCES.....	114
TABLES AND FIGURES.....	116
CHAPTER 5: DEVELOPMENT OF AN IMMUNOFLUORESCENT ASSAY USING	
RECOMBINANT PROTEINS EXPRESSED IN INSECT CELLS FOR THE SCREENING AND	
CONFIRMATION OF HUMAN HERPESVIRUS 8 ANTIBODIES.....	126
ABSTRACT.....	127
INTRODUCTION.....	128
MATERIALS AND METHODS.....	130
RESULTS.....	134
DISCUSSION.....	137
REFERENCES.....	142
TABLES AND FIGURES.....	146
CHAPTER 6: CONCLUDING REMARKS.....	151
REFERENCES.....	156

CHAPTER 1: LITERATURE REVIEW

THE HERPESVIRUSES

The *Herpesviridae* is one of the largest families of viruses, with more than 100 members isolated from almost all mammalian species (reviewed in [1]). Assignment of a virus into the herpesvirus family is based on morphology of the virus particle and genomic similarity. Under an electron microscope, the virions of different members of the *Herpesviridae* family are indistinguishable. Each consists of four well-defined components including the core, capsid, tegument, and envelope. The core contains a double-stranded DNA genome, which is located inside an icosadeltahedral capsid that is approximately 100 nm in diameter and contains 162 capsomeres (reviewed in [1]). A layer of proteins collectively known as the tegument is located between the capsid and the viral envelope. The variation in size of mature herpes viruses (120 to 300 nm) is mostly due to differences in the size of the individual viral teguments. The tegument arrangement is typically asymmetrical with some herpetic members showing less ambiguous tegumental structures than others. Structurally the tegument connects the capsid to the envelope, while functionally acting as a reservoir for viral proteins which are required during the initial phases of viral infection [2]. Several herpesviral tegument proteins have been shown to play a role in evading the host immune response. For example, the p65 protein of human cytomegalovirus blocks the presentation of viral immediate-early proteins by MHC class I molecules, and interferes with NK cell responses by interacting with an NK cell-activating receptor [3].

The outermost framework of the herpes virion is the envelope, which is derived from cell nuclear membranes and is impregnated with various viral glycoproteins. By

interacting with cellular receptors during early phases of infection, these glycoproteins help to determine host range of specific herpesviruses. All herpesvirus infections have a limited cellular tropism within their specific host, determining their sites of infection and latency. Most herpesviruses recognize multiple cellular receptors, and the majority makes initial contact with cells by binding to glycosaminoglycans such as heparin sulfate on the cell surface, followed by interaction with other specific cellular receptors [4].

Some important herpesviruses of domestic animals include Suid herpesvirus 1, the cause of pseudorabies; bovine herpesvirus 1, the cause of infectious bovine rhinotracheitis; feline herpesvirus 1, the cause of feline viral rhinotracheitis; and equine herpesviruses, which can cause diseases of the respiratory, reproductive, and nervous system of horses. Marek's disease of chickens is caused by an oncogenic herpesvirus that can cause T cell lymphomas, along with other syndromes [5].

Eight viruses in the family *Herpesviridae* are of specific interest because of their capability to cause disease in humans (see table 1). Clinically significant human herpesviruses include the herpes simplex viruses (HSV-1 and HSV-2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), and Kaposi's sarcoma-associated herpesvirus (KSHV), also known as HHV-8.

HERPESVIRUS CLASSIFICATIONS

Herpesviruses have been classified into three subfamilies, α , β and γ , by the Herpesvirus Study Group of the International Committee on the Taxonomy of Viruses. Membership into a particular subfamily is based on its biologic properties. See Table 1 for short description of the herpesvirus subfamilies, associated diseases in humans, cellular tropism and the means of transmission in humans. Although classification does

not rely on DNA sequence homology, sequence analysis of the DNA polymerase gene and the glycoprotein B gene have allowed researchers to identify and appropriately characterize the viral subfamilies [6]

Alphaherpesviruses

The *alphaherpesvirinae* subfamily is divided into two genera; *Simplexvirus*, which includes herpes simplex virus-1 (HSV-1), herpes simplex virus-2 (HSV-2) and *Varicellovirus*, which includes varicella-zoster virus (VZV), also called human herpesvirus-3 (HHV-3). Alphaherpesviruses are known for causing painful fluid-filled epithelial vesicular lesions, HSV-1 is the causative agent for oro-facial fever blisters, also known as common cold sores. The primary route of HSV-1 transmission is via saliva; however it can also be transmitted via an oro-genital route, causing genital lesions [7, 8]. HSV-2 is known as a sexually transmitted disease, responsible for genital herpes. A risk of transmission to newborns also exists correlating to maternal viral shedding at delivery (reviewed in [8]). VZV causes varicella (chicken pox), and herpes zoster (shingles). After primary infection (chicken pox syndrome), VZV maintains latency in sensory ganglia and can reactivate later in life, producing herpes zoster [9].

Alphaherpesviruses have a short replicative cycle in the host, grow and spread rapidly in cell culture, and share the establishment of latent infections in sensory ganglia. Members of this subfamily are often referred to as neurotropic herpes viruses [6].

Betaherpesviruses

The *betaherpesvirinae* subfamily consists of two genera; *Cytomegalovirus*, which includes human herpesvirus-5 or cytomegalovirus (CMV), and *Roseolovirus*, which includes human herpesvirus-6 (HHV-6A and HHV-6B) and human herpesvirus-7 (HHV-

7). HHV-6 and HHV-7 are ubiquitous in the general population, but cause disease in immunocompromised patients and in infants. The disease *Exanthema subitum*, also known as roseola infantum can be caused by both HHV-6 strains and HHV-7 [10]. This disease occurs in young children and is characterized by a generalized macular or popular rash on the face or trunk, accompanied by high fever [11]. Although usually mild, convulsions and encephalopathy may accompany the infection [12].

CMV is considered to be ubiquitous in adults, and infection can be unnoticed in healthy individuals. However life threatening diseases can develop for immune compromised individuals infected with CMV, such as HIV-positive individuals, organ transplant recipients, and newborn infants [13]. Congenitally acquired CMV is perhaps the most clinically significant form, resulting in infant mortality or permanent disabilities such as hearing loss and developmental disabilities (reviewed in [14]). CMV transmission is known to occur via the sexual route in adults, (reviewed in [14]), and through breastfeeding to young children [15].

Betaherpesviruses have a fairly restricted host range, with a long reproductive cycle, both in cell culture and within the infected host. These viruses are cytopathic in culture; infected cells demonstrate morphological changes and cytomegalia (cellular enlargement) [6].

Gammaherpesviruses

The *gammaherpesvirinae* subfamily includes HHV-8 and human herpesvirus-4 (HHV-4) or Epstein-Barr virus (EBV), the only other gamma herpesvirus infecting humans. Gammaherpesviruses have a restricted host range, viral replication generally restricted to either T or B-cell lymphocytes, and latent infections in lymphoid tissues [6].

The gammaherpesviruses subfamily contains two genera, *Lymphocryptovirus* (LCV) (gamma-1), and *Rhadinovirus* (RDV) (gamma-2). EBV is the only LCV and HHV-8 is the only RDV discovered known to infect humans. LCV is found only in primates, but RDV can be found in both primates and other mammals [6].

EPSTEIN BARR VIRUS

Epstein-Barr virus is implicated in numerous clinical diseases, including infectious mononucleosis, Burkitt's lymphoma, Hodgkin's disease, B-lymphoproliferative disease, nasopharyngeal carcinoma, and oral hairy leukoplakia. There is a well-established relationship between EBV and oncogenesis. Several genes encoded by EBV are essential for inducing transformation of B cells, including EBNA-1, EBNA-2, EBNA-3A, EBNA-3C and LMP-1 [16]. The cellular transformation is a complex process, involving cooperative interactions between several viral proteins, however the pathogenesis is thought to also include genetic aberrations, as occurs in Burkitt's lymphoma in which the c-myc gene is translocated [17].

Epstein-Barr virus routes of transmission have been well established, and serve as a prototype when studying HHV-8 transmission. Viral DNA is readily isolated in saliva and salivary exchange is the accepted mode of transmission for EBV. Transmission occurs via intimate kissing, sharing drinking glasses, and children sharing of toys. Viral shedding in saliva has been demonstrated to persist for an extended length of time post infection. Fafi-Kremer et al. (2005) demonstrated that in patients evaluated after infectious mononucleosis, EBV viral DNA persisted for 6 months post disease onset, whereas viral load decreased considerably from PBMC's and rapidly disappeared from plasma [18].

HUMAN HERPESVIRUS-8

HHV-8 is the most recently discovered human herpesvirus family member, the etiologic agent for Kaposi's sarcoma (KS). HHV-8 was initially isolated from KS lesions in patients with acquired immunodeficiency syndrome (AIDS) using representational difference analysis [19]. This virus was further characterized using an infected B-cell line derived from an AIDS-related lymphoma patient and a genomic library made from a KS lesion to be a gamma-2 herpesvirus [19, 20].

HHV-8 has sequence homology and genomic organization that is similar to both EBV and another well-known Rhadinovirus, herpesvirus saimiri, a T-cell tropic virus known to cause lymphoma in primate hosts such as squirrel monkeys, but also has many unique sequences [19, 21].

Strain variation has been found among HHV-8 isolates. Five HHV-8 variants (groups A to E) have been identified based on analysis of gene products from two HHV-8 genes, ORF-K1 and ORF-15. Groups A and C predominate in Europe and North America, B is dominant in Africa, D and E are found in the Pacific Islands and Amerindian populations (reviewed in [22]).

HHV-8 Genome organization

The HHV-8 viral DNA genome consists of a single, long, unique region containing all the coding regions and is similar to the genome of herpesvirus saimiri. The coding region is flanked by variable numbers of direct terminal repeats. HHV-8 possesses approximately 26 core genes, which are highly conserved across the alpha-, beta-, and gammaherpesviruses. These genes are responsible for gene regulation, nucleotide metabolism, DNA replication, and virion maturation and structure (reviewed

in [1]). HHV-8 also has at least 12 human host gene homologs, not shared by other human herpesviruses. Some of these genes retain host function, or have been modified and implicated in oncogenesis. These include genes encoding viral Bcl-2, cyclin D, interleukin-6, G-protein-coupled receptor, and ribonucleotide reductase [23] (reviewed in [1]). In addition HHV-8 has a number of genes and regulatory proteins that interact with the host immune response, functioning in evasion of host cellular defense mechanisms [24].

Latent phase of infection

A hallmark of all herpesviruses is that after infecting a host cell, herpesviruses cycle between two phases: lytic and latent. HHV-8 generally enters the lytic cycle immediately following primary infection, and then rapidly enters the latent phase. In the latent phase, the viral genome assumes a circular conformation known as an episome. In the latent phase, viral replication is suppressed, resulting in the formation of a quiescent state of dormancy, with which minimal gene expression occurs. HHV-8 is able to establish a predominantly latent, life-long infection in host monocytes, dendritic cells, B lymphocytes, and endothelial cells.

As with EBV, infection of B cells by HHV-8 triggers the expression of several latency-specific genes. These genes encode proteins that function primarily in the maintenance of episomal viral genome in latently infected cells to transform the cells to ensure the long-term survival in a short-lived cell. Latently expressed genes include ORF71, which encodes a homologue of the antiapoptotic factor vFLIP, and ORF73, the latent nuclear antigen (LANA) which functions to maintain genome integrity and episomal persistence [25].

Lytic phase of infection

For viral propagation to occur, the virus should undergo reactivation from the latency into the lytic phase, when active viral replication occurs and newly synthesized virions are released into the extracellular space. The herpesvirus lytic replicative phase can be divided into four phases, immediate early (IE), early (E), partial late, and late, depending on the transcription pattern prior or after lytic life cycle induction (reviewed in [1]).

Immediate-early (IE) or α genes require no prior viral protein synthesis. These genes are involved in transactivating transcription from other viral genes. The most notable HHV-8 IE gene, ORF50 encodes R transcriptional activator (Rta), which functions in the earliest phases of reactivation from latency into the lytic cycle [25].

Early or β genes, are expressed independently of viral DNA synthesis. Encoded by K2, vIL-6 is a homolog to the cellular IL-6, and stimulates multiple cellular pathways to induce cell proliferation, immunomodulation, and anti-apoptotic activity [26]. Polyadenylated nuclear (PAN) RNA stabilizes unspliced transcripts, increasing the abundance of RNA in the nucleus and blocking the assembly of mRNA-protein complexes [27].

Partial late or γ_1 genes are expressed in concert with the beginning of viral DNA synthesis. This is a subset of late genes, and their transcription rate is enhanced during DNA synthesis. ORF18, which encodes a transfactor that is essential for late gene transcription of gammaherpesviruses, is considered to be a γ_1 gene [28].

Late or γ_2 gene expression is totally dependent upon synthesis of viral DNA. Virion structural genes encoding for capsid proteins and envelope glycoproteins are

considered as late genes. K8.1 is a late gene that encodes a viral envelope glycoprotein at a late phase of infection [29].

HHV-8 immune responses

Neutralizing antibody (nAb) responses against HHV-8 have been investigated in several systems. A 2004 study compared levels of HHV-8 infection in dermal microvascular endothelial cells after incubation with serum from HHV-8 seropositive individuals, versus HHV-8 negative controls. Using an indirect immunofluorescence assay, these investigators reported significant inhibition of HHV-8 by seropositive sera diluted at 1:10 or 1:50, but not at 1:500. Depletion of the IgG fraction of the seropositive sera by protein A resulted in a loss of the HHV-8 neutralizing activity [30].

Another group reported similar results using HHV-8 infectivity assays on 293 cells, and also found that patients with KS had significantly lower nAb titers compared to other HHV-8 infected groups, suggesting a possible role for nAb in protecting against progression from latent HHV-8 infection to KS [31]. In contrast, Inoue et al. (2004) found no significant difference in the levels of nAbs in HHV-8 positive individuals with KS (24%) compared to those without KS (31%) [32].

Because of the association between HIV and HHV-8, several studies have explored the question of whether CD4⁺ T cell levels influence immune responses to HHV-8. Among HIV-infected individuals, Kimball et al. (2004) reported that those with KS had lower CD4⁺ T cell counts and higher levels of anti-HHV-8 antibodies than those without KS [31]. This suggests that HHV-8 replication may be limited by CD4⁺ T cell activity. Another study similarly found that HHV-8 infected people with declining CD4⁺

T cell counts were associated with increasing antibody reactivity against the HHV-8 Orf 65 minor capsid antigen [33].

HHV-8 infection also stimulates cytotoxic T lymphocyte (CTL) responses. A 1999 study demonstrated that seven out of seven HHV-8 positive, KS negative individuals exhibited MHC class I-restricted CTL-activity against three HHV-8 antigens, the K8.1 and K1 lytic proteins, and the K12 latent protein. In contrast, only two of six HHV-8 positive individuals with KS had detectable CTL responses against these antigens. In one patient, whose KS had resolved under HAART therapy, CTL activity was restored [34]. In an investigation of HIV-negative subjects that seroconverted to HHV-8, Wang et al. (2001) observed distinct CD8+, HLA class I restricted responses and increases in the interferon-gamma (IFN- γ) response to at least three of five lytic HHV-8 antigens in each of the five subjects. They also observed that CTL reactivity declined after several years, probably as HHV-8 entered a more latent state after primary infection [35].

Immune modulation by HHV-8

As is the case for all herpesviruses, one of the major immune evasion strategies used by HHV-8 is the establishment of latency, during which the majority of viral genes are not expressed. This allows the virus to “hide” from most host immune mechanisms during this part of its replication cycle. When the virus is in the lytic phase, however, other active mechanisms of immune evasion take place. Of the 86 genes encoded by the HHV-8 genome, at least 22 may have immunomodulatory effects [36].

As part of innate immunity, the complement system may play a role in early defenses against HHV-8. The ORF4 gene of HHV-8 encodes a protein called KSHV

complement-control protein (KCP), also called kaposica, which inhibits complement activation by two separate mechanisms [37]. KCP accelerates the decay of the C3 convertase enzyme complex generated by the classical pathway of complement activation, and it also acts as a cofactor for factor I-mediated inactivation of C3b and C4b.

HHV-8-associated diseases, especially KS, are associated with alterations in secretion of a variety of cytokines. An early component of innate immune responses to viruses is the secretion of type I interferons (IFNs). Cellular responses to type I IFNs involve the IFN-regulatory factor (IRF) family of transcription factors, which increases expression of MHC class I molecules and activates NK cells. HHV-8 encodes four vIRF genes with homology to cellular IRFs [23, 38]. These vIRF proteins (vIRF-1, -2, -3, and -4) generally inhibit the activities of various cellular IRFs, plus they may have other immunomodulatory activities, such as suppression of the IFN-induced, double-stranded RNA-activated protein kinase R (PKR) [39] and inhibition of Fas ligand (CD95L) expression [40].

As mentioned earlier in this review, tegument proteins of herpesviruses can also be involved in immunomodulation. An example of this is the HHV-8 ORF45 (tegument) protein, which blocks phosphorylation and nuclear translocation of IRF-7 [41, 42]. The introduction of the ORF45 protein into the cell as part of the virion ensures that it is active at the very earliest phases of infection, when deregulating IRF-7. The inactivation of cellular IRF-7 seems to be a key event in HHV-8 replication, as evidenced by the observation that the HHV-8 replication and transcription activator (RTA) protein, which

regulates the switch between latency and lytic replication, also negatively regulates IRF-7 by targeting it for proteasome-mediated degradation [43].

HHV-8 also encodes a homologue of human IL-6. IL-6 is normally synthesized by mononuclear phagocytes, vascular endothelial cells, and other cells in response to microbial infection and to other cytokines. Binding of IL-6 to the IL-6 receptor can have several effects, including the synthesis of acute-phase proteins, stimulation of B lymphocytes, and upregulation of apoptotic antagonists such as BCL-X_L [44]. In 1996, Moore et al. reported the discovery of a HHV-8 homologue of IL-6 (vIL-6) which shares 25% identity with the human cytokine [45]. In some infected individuals, secretion of vIL6 may contribute to HHV-8-associated MCD and perhaps PEL by promoting cell survival, driving proliferation and preventing apoptosis [46].

Due to the central role of CTLs in eliminating virus-infected cells in an MHC class I-dependent manner, a large number of viruses interfere with presentation of cytosolic antigens by MHC class I molecules. This is especially true for viruses, such as herpesviruses, that establish persistent or latent infections, [47]. HHV-8 encodes two proteins named modulator of immune recognition 1 and 2 (MIR1 and MIR2) [48]. During lytic replication, these proteins increase endocytosis of surface MHC class I molecules, leading to their degradation in endolysosomes [49-51]. This mechanism differs from other known viral inhibitors of MHC class I expression, which interfere with the synthesis or assembly of MHC class I chains or retain them in the ER [47]. HHV-8 MIR2 also reduces surface expression of at least two key molecules involved in T cell function - B7-2 (CD86) and ICAM-1 (CD54). B7-2 is an important T cell costimulatory molecule that expressed by antigen-presenting cells, and ICAM-1 is an intercellular adhesion

molecule that interacts with LFA-1. Therefore, MIR2 not only inhibits the MHC-dependent presentation of antigen to T cells, but also interferes with other cellular interactions that are essential for proper T cell function [49].

A more recently described immune evasion strategy employed by HHV-8 is a modification or reshaping of the host cell receptors for natural killer (NK) cells [24]. Natural killer cells are known for their ability to directly lyse infected cells and secretion of cytokines which limit viral replication, therefore playing a key role in early control of viral infections. Dupuy et al. (2012) described the capability of HHV-8 in decreasing expression of NKp30 and NKp46, both which are cellular receptors responsible for activating NK cells. Also demonstrated was a down-regulation of the inhibitory receptor CD161. It has been suggested that the loss of this receptor might result in the accumulation of a population of NK cells with a lower activation threshold, which could lead to the elimination of activated dendritic cells, establishing a defective antiviral adaptive response [24].

Epigenetics

Epigenetics refers to modifications of DNA that affect gene expression, with no change in nucleotide sequence. Epigenetic influences on disease have become an important area of study in understanding factors that play a critical role in disease development. Transcription of a gene is controlled by local chromatin structure. Epigenetic modifications to the chromatin structure, either by changes to histones, or the DNA strand itself, can change gene expression. Epigenetic modifications mostly include chromatin remodeling, histone acetylation, and DNA methylation. These all induce changes in gene expression that may persist even after removal of the initiating agent,

creating the potential to influence the health of future generations. One of the most notable studies of epigenetics and disease was completed by Kaati et al. (2002). They found by examining paternal grandfathers who experience a shortage of food at a critical time in their growth and development, presented a decreased risk of death from cardiovascular disease or diabetes in their grandchildren [52]. These findings suggest that diet can cause changes to genes that are inheritable and these alterations can affect susceptibility to certain diseases.

Oncogenic viruses are known to cause epigenetic modification. An example of DNA methylation and implication in oncogenesis is seen in EBV. The EBV latency-associated virus gene products in B cells lead to epigenetic repression and CpG methylation of the pro-apoptotic gene Bim, inhibiting tumor suppression [53]. HHV-8 is considered to be an oncogenic virus, in which its ability to maintain latency is implicated in its oncogenesis. Chromatin remodeling and demethylation of the HHV-8 lytic switch gene Rta (ORF50) promoter, controls viral lytic phase entry; hyperacetylation of the latent replication origin controls latent cycle replication [54]. This switch between latent and lytic phases of HHV-8 infection leads to active viral replication, cellular transformation, or oncogenesis. In most primary effusion lymphoma (PEL) cell lines, HHV-8 is in the latent phase, but the lytic cycle can be induced by treatment with the DNA methyltransferase inhibitor 5-Azacytidine (5-AzaC), the HDAC inhibitor sodium butyrate (NaB), and the HAT inducer tetradecanoylphorbol acetate (TPA) [55, 56]. Chen et al. (2001) also showed that treatment with 5-AzaC, and therefore demethylation of the HHV-8 Orf50 promoter, caused lytic reactivation accompanied by IE, early, and late gene expression [55]. Orf50/RTA expression may be activated by physiological

conditions, such as hypoxia, or pharmaceutical agents, leading to viral replication and disease pathogenesis [57, 58].

This reprogramming of host cell DNA has important implications not only for understanding viral persistence and pathogenesis of herpesviral associated diseases, but also has potential significance in epidemiology and transmission. In transmission studies, this potential influence of epigenetics raises many questions. What are these changes, and how does the environment play a role? When considering viral pathogenesis, it is especially interesting to determine if the epigenetic influence on disease may also be an influencing factor on risk of transmission.

Epigenetic initiators are not well defined, but in human disease, many different environmental influences have been implicated as sufficient to initiate epigenetic changes, such as stress, diet, hormones, toxins, ageing, and genetic influences [59]. All of these influences may play a role in viral ability to infect, persist, and replicate, and therefore help explain populations at risk for disease, as well as potentially transmission.

Pathogenic Mechanisms of HHV-8

HHV-8 is considered to be an oncogenic virus because of its involvement in cancer related etiologies such as B-cell lymphomas, and Kaposi's sarcoma (KS). Much of the pathogenic mechanisms of HHV-8 has been learned from studying KS development. The classic hallmark of KS tissue is the appearance of characteristic spindle shaped cells. Early histochemistry and ultrastructural studies revealed that proliferating KS spindle tumor cells are of endothelial origin, confirmed by more recent use of genomic technologies [60, 61]. Circulating blood mononuclear and endothelial "Progenitor cells" when infected with HHV-8 are reprogrammed to resemble lymphatic

endothelium, which upregulates several lymphatic associated genes such as lymphatic vessel endothelial receptor 1 (LYVE1) podoplanin, and vascular endothelial growth factor receptor 3 (VEGFR3) [62] (reviewed in [61]).

Worldwide, HHV-8 seroprevalence far exceeds the incidence of KS. This indicates that HHV-8 infection alone appears to be insufficient for the development of KS, suggesting that other cofactors are involved in the causal association, such as epigenetic initiators as discussed earlier, co-morbidities, or host immune suppression. Of most significance, is the apparent reliance of KS progression on host immune dysfunction and the local inflammatory milieu. HHV-8 encodes a variety of gene products, some involved in cell survival processes such as transformation, proliferation, cell signaling, antiapoptosis and angiogenesis, and involvement in immune modulation, including cytokine production, and immune evasion [60, 63]. All these mechanisms may be involved in the promotion of oncogenesis and viral persistence.

Diseases caused by HHV-8

HHV-8 was first identified in 1992 by Chang and Moore (1994) in a biopsy of an endothelial tumor lesion from an AIDS patient [19]. HHV-8 has currently been determined to be the causative agent in distinctive pathologies: Kaposi's sarcoma (KS), body cavity-based B-cell lymphoma (BCBL), primary effusion lymphoma (PEL), and multicentric Castleman's disease.

Kaposi's Sarcoma

Kaposi's sarcoma (KS) is an atypical form of cancer that develops in supportive connective tissue such as cartilage, bone, fat, muscle, blood vessels, and fibrous tissue. Histopathologically, the lesions are classified as endothelial neoplasms containing not

only the primary neoplastic proliferating spindle-shaped cells of endothelial origin, but also an abundance of extravasated erythrocytes and infiltrating inflammatory cells. KS was first described in 1872 by the Hungarian dermatologist, Moritz Kohn Kaposi, as a relatively uncommon tumor of very limited prevalence found almost exclusively in elderly men of Italian or Eastern European Jewish ancestry [64]. Years later, another epidemiological form was reported in young adult and prepubescent equatorial black Africans. In 1969, a third form of KS was documented as associated with immunosuppressant therapy. However, it was not until 1982 when a sudden increase in the prevalence of KS cases, affecting a large variety of individuals who were not previously associated with any known epidemiological cluster, that KS began to elicit amplified concern in the medical community. This novel form of KS was much more aggressive, with a clinical course often characterized as disseminating and fulminating. Eventually it was linked to a disease model later denoted as acquired immunodeficiency syndrome (AIDS).

These separate manifestations of KS tumors have been shown to be essentially identical from the histopathological standpoint. However clinical symptoms, age and the course of the disease are distinguishable from each other, leading to classifications designated as classic, endemic, AIDS-associated, and iatrogenic.

Classic Kaposi's Sarcoma

The terms classic, Mediterranean, and sporadic KS all refer to the same form of KS. This is a rare cancer demonstrating ethno-geographical predominance in Eastern Europe, and the Mediterranean region with high incidence in Italy, Turkey, Greece, and Israel [65]. Classic KS historically has an ethno-geographical predominance in the

Mediterranean region, with up to a 10-fold higher incidence than in the rest of Europe and the United States (U.S.). In general, the average onset of the disease is between forty to seventy years of age, predominantly occurring in men with sex ratios estimated to range from 3:1 to 10:1 [61, 66]. Classic KS tumors are described as one or more asymptomatic lesions that occur in the dermal layer of the skin and appear as either brown, blue, purple, or red blotches or nodules. The lesions are usually localized to one or both lower extremities and frequently involve the ankles and soles of feet, but mucosal and visceral lesions may develop [61, 65]. Mortality rates for classic KS are low, and although the skin lesions are normally disfiguring, they often are not disabling. Kaposi's sarcoma lesions can persist for ten to fifteen years, with indolent growth of primary tumors and a gradual slow growth of additional lesions. Complications most commonly seen include venous congestion and dependant pedal edema secondary to the slowing of circulation caused by physical pressure from local tumor infiltration, and infiltration of surrounding tissues and lymphatics [67].

In chronic cases, visceral lesions can develop in lymph nodes, along the gastrointestinal tract, and in other organs. Generally, these lesions are asymptomatic, most often discovered only at autopsy, although gastrointestinal bleeding can occur [67, 68].

Endemic Kaposi's sarcoma

In some equatorial countries of Africa, KS has existed for several decades, documented as early as 1953 as a relatively commonly occurring neoplasm in native populations [69]. This form of KS is known as endemic KS, and reflects the KS disease that exists in Africa, not associated with the HIV/AIDS epidemic. From 1968 to 1970,

KS was associated with approximately 6.6% of all cancers seen in Ugandan males, reflecting endemic KS incidence prior to the HIV epidemic [70]. The epidemiology differs than that of classic KS in the population demographics. The median age of classic KS is 65 years, but the median age of endemic KS is 40 years. The disease is also seen in both children and adults, with a higher male to female ratio of 15:1 (reviewed in [71]).

Two forms of endemic KS have been described; a local aggressive form and a more rare lymphadenopathic form. The local aggressive form is very aggressive, characterized by granulating chancroid sores and a fungating tumor that can penetrate from the skin to the underlying bone [72]. The lymphadenopathy form is less frequent, and develops primarily in prepubescent children [63]. The generalized lymphatic involvement in the lymphadenopathic form is usually associated with visceral organs, and is very aggressive [61]. In these cases, a 100% fatality rate within three years of diagnosis has been reported [73].

Transplant-Immunosuppressive Therapy-Related Kaposi's sarcoma

Kaposi's sarcoma that develops due to the immunosuppressive therapy prescribed for post-transplant patients to prevent graft rejection is referred to as immunosuppression-associated or iatrogenic KS. Post-transplant KS can result in rejection of the graft and death of the patient [26]. Penn et al. conducted a study of 356 post-transplant patients with KS. Of these 40% had visceral involvement and 17% of these died due to KS [26].

The risk of KS associated with transplants is exemplified by iatrogenic KS prevalence rates of 500 to 1,000-fold higher in solid-organ transplant recipients than in the general population, with 46% of all cases developing in the first year [74, 75]. Risk varies geographically, ranging from 0.4% of transplant patients in the U.S. and Western

Europe, to 5.3% of renal transplant patients in Saudi Arabia [26, 76]. This ethnogeographic variation reflects the HHV-8 seroprevalance in the general population of different countries [26].

Iatrogenic KS appears more frequently in renal transplant patients as compared to other grafted organs (reviewed in [1]). Presumably this is due to the types of drugs used frequently in kidney transplant patients, including cyclosporine, which was demonstrated to reactivate HHV-8 in vitro [77, 78]. The influence of cyclosporine was demonstrated in a study in which eight of eleven iatrogenic KS patients had complete regression of visceral and cutaneous tumor progression within six months of cessation of cyclosporine [79].

AIDS-Related Epidemic Kaposi's sarcoma

The beginning of the AIDS epidemic was ushered in by the appearance of a fulminant and disseminated form of KS, first observed in U.S. immuno-compromised homosexual males in 1981 [80-82]. Subsequently, KS became the most common neoplasm occurring in patients with AIDS [77].

The underlying cause of AIDS was determined in 1985, originally named the T cell leukemia type III virus, later to be renamed the human immunodeficiency virus (HIV) [83-85]. HIV infects CD4 + cells, leading to cell death. The resulting immune dysregulation results in an immune-compromised state that predisposes the host to a variety of opportunistic infections and neoplasms, including KS.

AIDS-KS is a much more aggressive tumor than the other forms of KS and can disseminate into the viscera with higher mortality rates [86]. The aggressive nature of AIDS-KS has been hypothesized to be due to the HIV encoded protein, Tat. Tat is a

transactivator of HIV viral genes, and also some host cell genes. Tat can act extracellularly, inducing angiogenesis and inflammation in endothelial cells, contributing to KS pathogenesis (reviewed in [87]).

As with the other forms of KS, AIDS-KS has different associated risks among HIV positive individuals. In Western countries including the U.S., AIDS-KS predominantly is found in HIV positive homosexual or bisexual men, and less commonly in heterosexual individuals [77]. Studies in the U.S. have shown that HIV-infected homosexual men have a greater than 10,000 times risk of developing KS than the general population [88].

The impact of the AIDS epidemic on incidence rates of KS in African countries has been significant. The increase in incidence does not exhibit the same gender and sexual orientation preference exhibited in developed countries, with increased incidence rates in both men, and women, as well as in children. In Uganda, KS accounted for approximately 6.6% of all cancers prior to the onset of the AIDS epidemic; however data compiled from 1981-91 attributed 48.6% of all cancers in males, making it the leading cancer in males. In women, during this same time period, KS was the second most frequent cancer, at 17.9%. In children KS increased by an additional 40% [70]. AIDS-related KS risk associations in Africa include better education, an affluent life style, urban as opposed to rural living, travel away from home, and co-infections with sexually transmitted diseases [89].

By the mid 1990's, the incidence of AIDS-KS in HIV positive individuals decreased significantly due to the introduction of highly active antiretroviral therapy (HAART) [90, 91]. HAART represents the use of a combination of antiretroviral

pharmaceutical therapies, sometimes termed a drug cocktail. It combines protease inhibitors with reverse transcriptase inhibitors, which helps to prevent the emergence of drug-resistant HIV strains and decrease viral load. The success of HAART results in increased T-lymphocyte survival, decreased patient mortality rates, and a reduction in AIDS defining diseases such as KS. In a study done on 14,183 people diagnosed with AIDS during 1990-2000 in San Francisco, CA, factors associated with a longer KS survival time included HAART use for 6 or more months (HR=0.37, 95% CI 0.31-0.45) [92].

Transmission of HHV8

Transmission of HHV-8 in adults has been suggested to occur via blood, bodily fluids, and sexual contact. In industrialized countries, sexual transmission is highest in the population of men who have sex with men (MSM). A study conducted on a San Francisco cohort of MSM, HHV-8 found significant rates of sexual transmission [93]. Engels et al (2007) in a study using the NHANES III cohort examined HHV-8 status on 13,894 adults over 18 years of age for associated risk factors. Their findings showed that among MSM seroprevalence increases with the number of previous male partners, and an association exists with HHV-8 seropositivity and duration of heterosexual activity and lifetime number of heterosexual partners. However, no associations with sexual risk factors were found among women [94]. In contrast, a cohort in Zimbabwe of heterosexual men found that HHV-8 infection was not associated with any sexual risk factors other than with HIV-1 infection [95]. Taken together, these studies suggest that sexual transmission may occur, but is more likely to occur in MSM rather than

heterosexual sexual contact. Sexual transmission does not explain the high prevalence of HHV-8 in endemic populations, and does not explain the risk of transmission to children.

Blood and blood products have been explored as a potential route of transmission. Occurrence of HHV-8 transmission in intravenous drug users gives evidence that transmission via blood is possible [96-98]. Disparity among several studies suggests that transmission of HHV-8 via transfusion of blood or blood products is possible, but unlikely [99-101]. The reports of transmission via blood transfusion products vary greatly with geographical area. Incidence rates are low in North America, and higher in areas of endemic HHV-8. A Ugandan study demonstrated that 41 of 991 blood transfusion recipients seroconverted to be HHV-8 positive post transfusion, with an increase in risk noted in those receiving HHV-8 seropositive blood [102]. It is likely that the risk varies not only with population prevalence, but also with the differences in collection and storage of blood products. Such studies are difficult to conduct due to the co-morbidities of transfusion recipients that may bias results, as well as the anonymous nature of the blood donation, making it difficult to follow-up to confirm serodiagnostic tests.

In children, there is a paucity of data regarding when and how transmission occurs. HHV-8 has been shown to productively infect placental cells in vitro, confirming the possibility of mother to child vertical transmission [103]. In addition, epidemiologic studies have shown HHV-8 transmission from mother to child vertically during pregnancy, but the incidence is found to be low [104, 105]. The increase in the prevalence of HHV-8 throughout early childhood prior to onset of sexual activity suggests the possibility of additional routes of transmission via mother to child routes,

most likely during early childhood [106-108], or between household contacts and among children, possibly via saliva [109-111]. Another source to consider is breast milk. Although breast milk is known to transmit other viruses such as CMV and HIV-1, previous research indicates a lack of HHV-8 DNA in breast milk, making transmission unlikely [112].

Saliva has also been implicated in the transmission of other human herpesvirus family members, including HSV-1, HHV-6, and HHV-7 [113-115]. Our lab has previously isolated, HHV-8 virus in saliva samples from seropositive mothers implicating saliva as a likely mode of HHV-8 transmission [116]. Other published studies have also indicated saliva as a mode of transmission. A South African study found that an increased prevalence of HHV-8 specific antibodies in children in mothers shedding $>5 \times 10^4$ viral DNA copies/ml of saliva, which suggests that a high viral load in saliva may be associated with transmission to the child [106].

In examining HIV status as a risk factor for transmission of HHV-8, Miller et al. demonstrated that HIV-positive individuals were significantly more likely to shed HHV-8 in their saliva than the uninfected controls (OR, 4.15 [95% CI, 1.87 to 9.19]) [117]. In the United States, saliva has been implicated as a source for transmission of HHV-8 in HIV positive individuals, because viral DNA has been detected in as many as 70% of oral cavity samples in studies involving HIV-1 infected men with KS [118]. However, our lab found no significant difference in HHV-8 viral shedding in saliva cells when comparing HIV-1 infected mothers and uninfected mothers in a mother/infant cohort from Lusaka Zambia [112]. HHV-8 infection in children was not found to be associated with either HHV-8 or HIV-1 infection of the mother [119], however we found while

following the same cohort longitudinally, that childhood HIV-1 status was a significant risk factor for the child to acquire HHV-8 infection by 48 months of age (HR, 4.60 [95%CI, 2.93,7.22]) [120].

An interesting area of study in the epidemiology of infectious diseases is genetic risk. A study in Sardinia identified an HLA predisposition in classic KS patients, identifying a positive association with types HLA-DRB1*1104, DRB1*1302, DQB1*0302, Cw7, and DQB1*0604 and a negative association with types A30, B58, Cw5, DRB1*1601, and DQB1*0502 [121]. A positive association was identified with AIDS-KS and a critical amino acid residue at position 13 in HLA-DRB1 [122]. These studies taken together with HHV-8 association with KS, has prompted studies into the genetic influence on HHV-8. In a South African study, Alkharsah et al. (2007) found a significant association with allele groups HLA-1*68, HLA-1*43, and HLA-DRB1*04 with an increased rate of viral shedding or increased viral load in a cohort of HHV-8 infected mothers [123]. However, a Ugandan group did not find any associations with types HLA-A and HLA-DRB1 alleles, and only with children with the HLA-B allele. They found a marginal association of KSHV DNA shedding in saliva but not in peripheral blood among children carrying HLA- B*4415 and marginal association of KSHV DNA shedding in peripheral blood but not in saliva among children carrying HLA- B*0801 alleles. This suggests that different populations could pose different genetic risks, however small sample size could bias their data [124]. Further longitudinal studies are needed to confirm the influence of haplotypes in increased risk of HHV-8 in populations and the subsequent transmission to susceptible children.

Whitby et al. (2007) proposed that environmental cofactors present in KS endemic regions are responsible for reactivation of HHV-8 in infected subjects, leading to high viral loads, increased viral shedding, and an increased risk of transmission. Dubbed the “oncoweed” hypothesis, this study presents *in vitro* data demonstrating an increased expression of KSHV mRNAs upon exposure to extracts from African native plants. Several of these plants are used in native populations in activities such as basket weaving or in traditional medicine [81]. This data suggests a possible role of environmental factors as an originator of epigenetic changes, leading to increased viral replication and an increased risk of transmission.

HHV-8 Diagnostics

Polymerase chain reaction (PCR)-based methodology of HHV-8 DNA detection gives the greatest specificity for diagnosis, compared to all other tests currently used for measuring HHV-8 exposure or infection. Viral genomic copy number varies according to cell type and disease status. For example, chemically infected B lymphoma cells carry 40-150 copies of HHV-8 DNA per cell genome, compared to 1-2 copies per cell in KS tissue. In terms of other body locations, viral DNA is most often found in saliva followed by peripheral blood mononuclear cells (PBMC) and occasionally in semen and the male urogenital tract.

In PEL cells, also referred to as body cavity-based lymphoma cells (BCBL), HHV-8 DNA is detected at 100% efficiency, which corresponds to 100% assay sensitivity. In comparison, PCR of KS lesions from known HHV-8 positive patients is detected in approximately 95% of all cases. This discrepancy between BCBLs and KS tissues is believed to reflect the viral copy number as well as cellular heterogeneity of the

tissue opposed to the relative homogeneity of the single B-cell typed lymphoma. In circulating PBMCs and in the plasma, detection of HHV-8 DNA in seropositive individuals is very rare, possibly because of low levels of viral replication during latent infection. Viral DNA detection has been used to analyze HHV-8 disease-associated progression. Reactivation of latent virus and proliferative infection leads to an increase in viral load in the peripheral blood stream. This increased viral load contributes to increased viral dissemination and spreading of the infection. In KS patients, an increase in plasma viral DNA, isolated from other blood products including not lysed cells, has been used as an indicator of a more advanced viral infection. Studies have also demonstrated a strong correlation between viral load within the peripheral blood, including cells, and an increased risk of KS progression. Hence, peripheral blood viral load is used to predict the pathogenic outcome of the infection by measuring active HHV-8 replication through PCR detection of viral DNA.

The low sensitivity and expense of the method limit the clinical application of viral DNA detection in population screening. Immunologic assays are the most widely used in HHV-8 diagnostics. Currently four antibody detection assays are commonly used in HHV-8 serodiagnostics: enzyme-linked immunosorbant assay (ELISA), immunofluorescent assay (IFA), Western blot, and immunohistochemistry (IHC).

Immunofluorescence assays (IFAs) are used extensively in serodiagnostics. This technique employs a target cell expressing desired viral proteins fixed and spotted onto glass slides. If antibodies specific for the expressed proteins are present in the patient's plasma, those will attach to the antigen, indicating past or present patient exposure to the virus. A detection antibody is then applied, usually a commercially available antispecies

(i.e. human) antibody with a fluorescent tag. The slides can then be viewed with fluorescent microscopy to identify seropositive patients. Target cells used in this assay are often B-cell lymphoma based cells, stimulated to express lytic phase proteins using either sodium butyrate [125], or 12-O-tetradecanoylphorbol-13-acetate [126]. Antibodies recognizing lytic proteins will result in a whole cell fluorescent staining, whereas recognitions of latent proteins results in a more restricted, punctate nuclear appearance, demonstrating recognition of the latently expressed protein, LANA.

Also commonly used in IFAs are recombinant proteins expressed in insect cells. As discussed in later in chapter 5, *Spodoptera frugiperda* (Sf9) are cells that can be used for recombinant protein production using baculovirus expressing immunogenic proteins such as K8.1, LANA, and ORF65 [126].

HHV-8 in Zambia

In sub-Saharan Africa there has been an increase in KS co-incident with the HIV epidemic and high incidence of HHV-8 infection, with seroprevalence reported to range from 20-48% [127, 128]. KS is known to be the most common tumor among HIV positive individuals [129].

A dramatic increase in incidence of KS was observed after the HIV epidemic. In children, KS was relatively rare prior to 1980, but by 1990 to 1992, post the emergence of the HIV epidemic, the incidence in children raise to constitute 20-25% of all pediatric malignancies in Zambia [130]. In this same study, pediatric KS incidence noted at the University Teaching Hospital in Lusaka, Zambia increased from 3.2% to 19% in just one decade [130].

Preliminary data from our laboratory has determined HHV-8 seroprevalence rate of Zambian children at 12, 24, 36, and 48 months of age as 15%, 20%, 18%, and 22% respectively [120]. While *in utero* HHV-8 infection is possible, it cannot account for the high HHV-8 seropositivity in children during the first 12 months of age. These findings suggest that horizontal transmission is likely the predominant mode of virus transmission in our targeted study population. The overall goal of my dissertation research is to delineate possible routes and risk factors of HHV-8 transmission within a household to a susceptible child. Although breastfeeding has been implicated in early transmission of other viral diseases such as HIV our previous research has demonstrated that HHV-8 DNA cannot be readily detected in breast milk. However, HHV-8 virus can be isolated in saliva samples obtained from seropositive mothers in our above defined cohort, implicating saliva as a likely mode of HHV-8 transmission to children, but the maternal serostatus of HHV-8 is not a significant risk factor in transmission [112].

STUDY AIMS

The hypothesis of this dissertation research is that children in Sub-Saharan Africa can be infected by HHV-8 in early childhood through saliva contact with household members. This research plan was designed to test this hypothesis. To test our hypothesis, three specific aims were pursued, they are:

1. *To determine risk factors associating with HHV-8 positive children as compared to negative children*
 - a. Evaluating questionnaires specific to eating, feeding, and social practices within households.

- b. Identifying significant behaviors or factors specific to either HHV-8 positive or negative household members.
 - c. Identifying health status associations such as child nutritional status or other co-infections as risk factors for HHV-8 infection.
 - d. Completing statistical analysis to look for significant difference.
2. *Following the child for seroconversion post-enrollment in the study.*
- a. Evaluating the uninfected child every four months to determine incidence of HHV-8 seroconversion.
3. *Determining the risk factors associated with the household and the child during seroconversion.*
- a. Assessing if HHV-8 positive households increase the risk of seroconversion of the index child
 - b. Assessing lifestyle habits or behaviors that increase exposure to saliva, blood or bodily fluids through extensive questionnaires.
 - c. Identifying potential factors that could be of interest
 - B. Socioeconomic factors
 - C. Demographic data
 - D. Co-infections
 - d. Completing statistical analysis to look for significant difference.

In chapter 2, The Zambia Children's KS-HHV-8 Study is described in detail. The study design, recruitment, enrollment and cohort description is presented. Potentials for bias are addressed, and the socio-demographics of the enrolled cohort are described.

In chapter 3, specific aim 1 is met. A baseline cross-sectional analysis is performed, examining 75 HHV-8 positive children that were enrolled into the study. Caregiver and household behaviors, health practices and child health status were analyzed. HHV-8 presences in the household as well as behaviors such as testing the temperature of food prior to feeding to a child were determined to be significant risks of HHV-8 seropositivity.

In chapter 4, specific aims 2 and 3 are met. The children enrolled in the Zambia Children's KS-HHV-8 Study are followed for the duration of 48 months. Incidence rates are calculated, and risk analysis is completed. This prospective cohort study supports the findings of the first study in significant risks associated with saliva sharing behaviors and childhood acquisition of HHV-8.

Also included in this dissertation in chapter 5 is a study that was completed in analytical methods. To complete the serodiagnostics of the children and household members, a reliable assay is required. No gold standard exists for testing for HHV-8 infection. This final chapter outlines the work that was done prior to the beginning of the Zambia Children's KS-HHV-8 study, and helps to exemplify some of the work involved in assay development.

Overall, this dissertation describes the development of an epidemiologic study to examine HHV-8 transmission within households, and the association of transmission of HHV-8 to a susceptible child with behaviors that expose a child to saliva. Taken together, they describe epidemiologic, diagnostic, and analytical methods used in the investigation of risks associated with early childhood HHV-8 acquisition.

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TABLES

Table 1. Human Herpesvirus Subfamilies, Associated Diseases in Humans, Cellular Tropism and Means of Transmission Among Humans.

Virus name	Subfamily	Major associated disease in humans	Cellular tropism	Means of Transmission
Herpes simplex virus Type I (HSV-1)	α	Oral herpes-Cold sores	Sensory Nerve Ganglia	Oral routes, sexual
Herpes simplex virus Type 2 (HSV-2)	α	Genital herpes	Sensory Nerve Ganglia	Sexual transmission
Varicella Zoster Virus (VZV) (HHV-3)	α	Chicken-pox, shingles	Sensory Nerve Ganglia	Respiratory and sexual
Epstein Barr virus (EBV) (HHV-4)	γ	Burkitt's lymphoma nasal pharyngeal carcinoma Infectious mononucleosis	B lymphocytes epithelial cells	Saliva, transfusions, tissue transplant, and congenital
Cytomegalovirus (CMV) (HHV-5)	β	Congenital disease resulting in hearing loss	CD4+ T lymphocytes	Saliva, urine, breast milk
Roseolovirus (HHV-6A) Herpes lymphotropic virus (HHV-6B)	β	<i>Exanthema subitum</i> (roseola infantum)	CD4+ T lymphocytes	Respiratory, congenital mother to child
Human herpes virus-7 (HHV-7)	β	<i>Exanthema subitum</i> (roseola infantum)	CD4+ T lymphocytes	Unclear, likely saliva
Human herpes virus-8 (HHV-8)	γ	Kaposi's sarcoma	B lymphocytes Endothelial cells	Sexual, saliva, rare congenital

CHAPTER 2: THE ZAMBIA CHILDREN'S KS-HHV8 STUDY: RATIONALE, STUDY DESIGN, AND STUDY METHODS

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ABSTRACT

The HIV epidemic in Zambia has led to a dramatic rise in the incidence of human herpesvirus 8 (HHV-8) -associated Kaposi's sarcoma in both adults and children. But there is a paucity of knowledge about the routes of HHV-8 transmission to young children. The Zambia Children's KS-HHV8 Study; a large prospective cohort study in Lusaka, Zambia was launched in 2004 to investigate the role of household members as a source of HHV-8 infection in young children and social behaviors that may modify the risk of HHV-8 acquisition. This cohort is distinct from other epidemiological studies designed to investigate HHV-8 incidence and transmission because it recruited and followed complete households in the urban Central African context. Between July 2004 to March 2007, 1,600 households were screened and 368 households comprising 464 children and 1,335 caregivers and household members were enrolled. Follow-up of this population has continued for 48 months post-recruitment, affording a unique opportunity to study horizontal transmission of HHV-8 and understand the routes and sources of transmission to young children in Zambia. We describe the study rationale, design, execution and characteristics of this cohort which provides critical data on the epidemiology and transmission of HHV-8 to young children in Zambia.

INTRODUCTION

Human herpesvirus-8 (HHV-8), also known as Kaposi's sarcoma-associated herpesvirus (KSHV), is the most recently described human herpesvirus [1]. With the emergence of the HIV epidemic, Kaposi's sarcoma (KS) has become one of the most frequently diagnosed cancers in sub-Saharan Africa [2]. The seroprevalence of HHV-8 varies greatly and is generally high in areas where classic or endemic form of KS is common [3]. One such country is Zambia, a part of the "KS belt" where endemic KS was prevalent and where significant increase in KS incidence in adults and children has coincided with the emergence of the HIV-1 epidemic [4-6]. By 1992, KS accounted for approximately 25% of all childhood cancers diagnosed in Lusaka, the capital of Zambia [7].

Routes of HHV-8 transmission and factors associated with increased risk of HHV-8 acquisition have yet to be delineated. Based on results from our previous cohort study in Zambia conducted from 1998 to 2003, we were the first to report that; 1) HHV-8 may be transmitted perinatally albeit rarely; 2) HHV-8 may be detected in saliva but not in breast milk, and; 3) early childhood infection with HHV-8 is common [8-10]. Taken together, these results suggest that the major mode of HHV-8 transmission to children is by person-to-person contact, likely via saliva. We have also reported that HHV-8 infection in young children could occur independently of the HHV-8 serostatus of the mother, suggesting the possible role of other household members in transmitting infection [10]. Therefore it is critical to evaluate the role of household contacts in transmitting infection to young children especially in HHV-8 endemic areas like Zambia. To date, there has been no cohort study designed to recruit and follow entire households to

investigate HHV-8 transmission within households. Socioeconomic factors such as low parental education, using water from a communal source and low income have been implicated as risk factors associated with HHV-8 seroprevalence; these studies were cross-sectional in design and thus unable to associate specific risk factors with incident HHV-8 infection [11-13]. Therefore, the Zambia Children's KS-HHV8 study was designed in which longitudinal 4 year follow-up of recruited households was undertaken with the specific objective of determining the rate and source of horizontal transmission of HHV-8 to young children. We aimed to investigate whether transmission of HHV-8 to young children could occur through casual, person-to-person contact within a household.

Conducting prospective cohort studies are challenging endeavors, particularly in resource poor settings such as Zambia, which has poor infrastructure, high poverty levels, and limited number of skilled personnel. The aim of this report is to describe the rationale, design, execution, logistics and characteristics of a large observational household-based cohort study in Lusaka, Zambia.

MATERIALS AND METHODS

Study population and site

The Zambia Children's KS-HHV8 study is a community-based cohort study executed by investigators of the University of Zambia Teaching Hospital (UTH) in Lusaka, Zambia in collaboration with investigators at University of Nebraska Lincoln. The study coordinating office was based at the UTH, with an independent clinic for recruitment and follow-up of study participants and a fully equipped laboratory for biospecimen processing, testing, and storage. The study office also houses the data management team and the study coordinator; together they manage the paper and electronic versions of questionnaires, study-related forms and laboratory results. All study personnel at the clinic and laboratory are Zambians and all study related discussions and decisions are made by consensus by the study coordinator, Zambian and U.S.-based study directors and the principal investigators. This study was approved by the Institutional Review Board at the University of Nebraska-Lincoln and the Ethics Board of The University of Zambia.

Screening for enrollment

Recruitment and long-term follow-up of participants in Zambia can be a difficult process. There is general disinterest concerning participation in research studies as the economic and educational status of an average household is low, with approximately 64% of Zambian households living below poverty (in 2006) [14]. Zambia is one of the poorest countries in sub-Saharan Africa with a large percentage of a highly mobile population living in unorganized and densely populated settlements called 'compounds', making it difficult to locate and track study participants. To achieve a high follow-up

rate, we tried a novel method to create interest in the study and encourage follow-up visits. Community health workers (CHW), who were trained and educated about the study goals, spread the information about the goals of the study in their community. CHWs were residents of Lusaka and were instructed to reach potential study participants not only from within their residential areas and clinics but also from areas and clinics in other compounds. CHWs provided potential study participants with the basic information about the study goals and expectations, and gave opportunity to ask questions. If interested, they were directed to visit the study office at UTH, where trained study nurses provided comprehensive information about the study and answered all relevant questions. HIV and HHV-8 counseling was also given and written informed consent was obtained to participate in the study. Upon obtaining consent, each household and each member of the household was given a unique identification number. Participation incentives included the availability of basic health care at the study clinic, counseling to all study participants, common medications at no cost and reimbursement of the travel cost to the study office and clinic.

Our objective was to enroll complete households that had at least one HHV-8 negative child (referred to as index child). The eligibility criteria were 1) having an HHV-8 negative index child between 6 and 24 months of age in a household (children younger than 24 months were screened for HHV-8 serostatus); 2) the household had to reside in Lusaka District. Our early research experience suggested that participants living outside Lusaka were difficult to recruit or follow-up because of their inability or disinterest in travelling to the study site at UTH. According to the 2000 census, 14% of the Zambian population lived in Lusaka District, we therefore decided to limit to study

participants residing in Lusaka District [15]; 3) all members of the household, up to a total of 10 members, had to agree to participate and visit the study office for scheduled follow-up visits. The size of the household was limited to 10 to help limit the number of possible contacts with the index child; 4) the child should not be seriously ill (for example, Tuberculosis, cancer, AIDS) on the day of screening to ensure timely follow-up visits. Seriously ill children were excluded only because of their inability to travel or health risks involved in travelling to the study clinic. On meeting all the eligibility criteria, all members of the household had to return to the study office for enrollment within one month of screening. In addition a small group (n=32) of HHV-8 positive children younger than 24 months of age were enrolled as a control group to investigate the risk factors for seroconversion for a baseline cross-sectional analysis.

Enrollment and follow-up

The HHV-8 negative children along with their primary caregiver were requested to return for follow-up visits every 4 months until age 48 months; the other household members were followed annually. During each visit, all the study participants received a physical exam and free medications for common ailments such as worm infestation, fever and minor aches and pains, and multivitamin supplements in case of malnourishment. During enrollment and at each follow up visit, various biologic specimens were collected as described in the next section, health assessments were completed, and questionnaires to obtain demographic and behavioral information were administered (Table 1). Throughout the duration of the study, increase in household size due to additions in the household were enrolled either as index children if under the age of two years, or as household members if older than two years. CHWs tracked study participants that did not

return for scheduled visits to remind them of their missed visits or, if they decided to withdraw, determine the withdrawal reasons.

The major outcome variable of interest was HHV-8 serostatus at each visit. Outcome assessment was done by testing plasma collected from each infant during each follow up visit for evidence of HHV-8 infection as described previously [16]. HHV-8 shedding in buccal cavity was also monitored at each visit.

Data collection instruments and exposure assessment

Structured interview questionnaires were developed to collect information on factors that may be associated with increased risk of horizontal transmission of HHV-8 to the index child. The content of these questionnaires were based on discussions with focus groups conducted in March 2004 [17]. Discussions with men and women from diverse ethnic and socioeconomic backgrounds were conducted to determine various behavioral and socio-cultural practices that could impact the risk of the index child acquiring HHV-8. The questionnaires were administered to the caregiver during enrollment and at each follow-up visit in English or the local language (translated into commonly used languages, Bemba or Nyanja, by the interviewer). Questions pertained to household living conditions (electricity, water source, toilet facilities, number of rooms/sleeping areas, household density), behaviors involving food and drinks (pre-mastication, sharing sweets and/or drinks), health and personal care practices (example, bathing habits, use of traditional medicine and the use of saliva to soothe injuries), demographic variables (sex, age, education of the primary caregiver, household size, playmates), medical history (ailments and hospitalizations) and health assessments of the primary caregiver and the child. Developmental milestones of the child were also recorded.

Data management

Printed copies of the questionnaires and laboratory test results were recorded in the database and managed locally. Databases were generated by entering each form twice and comparing both entries to identify entry errors. At the initiation of the study we employed an optical character recognition software for transferring information from each form to the database. However, character recognition and record posting problems were frequently encountered leading to extensive manual data cleaning. Subsequently, manual entry using MS Access™ forms was employed leading to significant improvement in data quality. Copies of the database were regularly provided to the study staff at University of Nebraska-Lincoln where analytic datasets were generated and are currently being used for analysis, review and publication by study investigators.

Biospecimen collection and processing

All samples collected at the clinic were processed by the study laboratory within two hours of collection. Venous blood was collected from all the study participants at every visit and plasma was separated and stored. The peripheral blood mononuclear cell (PBMC) pellet was divided in two vials; one vial was stored at -80 °C and the other vial was lysed for DNA extraction. During the screening visit, both the child and the caregiver were tested for presence of HHV-8 and HIV-1 antibodies as described previously [16]. Screening for the presence of HIV-1 antibodies was done using Abbott Determine (Abbott Laboratories, Chicago, USA) and confirmed using Unigold (Trinity Biotech, Ireland) test kits, according to Zambia Ministry of Health, HIV-1 testing guidelines. For children younger than 18 months of age, polymerase chain reaction (PCR) was performed in the study laboratory using dried blood spots. Buccal cells were also collected at each

visit by scraping the buccal cavity with a cotton swab, then stored in a collection tube containing cell lysis buffer until DNA was extracted. Buccal cell lysates were used for investigating HHV-8 shedding, frequency of shedding, and the viral load present in the oral cavity.

RESULTS

From July 2004 to March 2007, caregivers from 1,600 Zambian households, including 1,657 index children younger than 24 months (referred to as index child) visited the study clinic for participation in the study. All were residents of one of 40 townships within Lusaka District (Figure 1). While our attempt was to achieve sampling from all areas of the city, we were unable to sample equally from all residential areas especially areas with residents from higher socioeconomic status. Our goal was to recruit and retain at least 370 index children to have 85% power to detect HHV-8 seroconversion difference from 0.12 to 0.22 at a significance level of 0.05. By the end of the study period there were 464 index children and 1,335 household members enrolled (Figure 2).

During the first visit to the clinic, eligibility was determined after the caregiver provided informed consent to participate in the study. Refusal to participate at this visit was rare (0.5%) because caregivers had a basic understanding about the study through CHWs before they attended the study clinic. The most common reason for refusal was fear of blood collection. Eligibility criteria were met by participants of 1,023 households who were invited to return with the entire household within one month (Figure 2). Of these, all members of 368 households (1,799 total study participants) were enrolled and followed. In 254 households, one or more household member(s) could not attend the study clinic within the specified enrollment period, were considered incomplete households and not followed at later time points. The enrollment rate of complete households per households initially screened was 23.0%. There was variation noted in enrollment rates between townships ($P < 0.0001$), most likely as a result of distance and accessibility to the study site (data not shown).

The annual enrollment and characteristics of 464 index children and 1,335 household members during the enrollment period are summarized in Table 2. Of the 464 index children initially enrolled, 395 children (85%) returned for one or more visits. The baseline characteristics of the 368 enrolled complete households are summarized in Table 3. Homes were generally small in size (mean - 2 rooms), with mean of 4.7 household members per household. A majority of the households were of lower socioeconomic status having no electricity, running water or in-home toilet.

Table 4 summarizes the characteristics of the primary caregiver, and his/her relationship with the enrolled index child. There were a number of different household structures represented, including multiple index children in a single household. The second index child could be a younger sibling, a niece, nephew, or other extended family member living in the household. Of the 464 enrolled index children from 368 households, 97% of caregivers were mothers to these children, while others included grandmothers, aunts, father and even cousins. A majority of primary caregivers were between 20-29 years of age (54.3%, age range - 14 to 78). Households were classified as 'two parent' if both a mother and a father to at least one index child in the household were present, 'single parent' if only one parent was available in the household (all the single parents in our enrolled cohort were mothers), and if there were no parents, or if the household included another adult such as an aunt or uncle, these households were classified as 'extended family'. Nearly half (48%) of the households were classified as either 'single parent', or 'extended family', which reflects a community continuing to suffer a high adult mortality rate from HIV/AIDS and related conditions. Most of the primary

caregivers in the cohort (54%) had completed primary education; no primary caregiver reported education beyond high school.

DISCUSSION

In this report we describe the study rationale, design, and methods of the Zambia Children's KS-HHV8 study in Lusaka, Zambia. This study is the only known study that has recruited and followed complete households, in an HIV/AIDS and KS endemic area like Zambia. Our objective was to design and implement a study to examine HHV-8 incidence and factors associated with HHV-8 acquisition by young children. Collection of a wide range of demographic, medical and behavioral data and biological samples during each visit have enabled us to meet our objectives.

Several steps implemented in this study ensured its success in recruitment and follow-up. This is a second cohort study being undertaken by our group. The present study allowed us to establish a fully equipped, modern diagnostic laboratory within the premises of UTH which provided no-cost service to UTH patients, helping build positive relationships with local health officials. Additionally, the establishment of the study clinic helped to provide basic medical care to study participants who seldom have access to quality medical care, encouraging participation and high follow-up rates. The study emphasized the follow-up of children for childhood health and wellness, prevention, of common childhood illnesses, with regular household checkups having a focus on the well-being of the child, rather than only emphasizing the testing for HIV-1 infection. This was done to increase participation rates because some households are not eager to participate in HIV-1 studies as compared to studies emphasizing the general well-being of the child. Close collaboration with local CHWs was of immense help during screening and recruitment of households as education level is low in most communities in Lusaka, which made it difficult to spread the correct message regarding the objectives of the

study. CHWs helped to educate the clients about the study objectives, and addressed participant fears and questions in the local language and familiar setting, achieving high consent rates among the participants who attended the study clinic. CHWs also reduced the need for lengthy study descriptions at the clinic as these had previously been provided, allowing for more time dedicated to medical or counseling needs. Also, all study personnel in Zambia were local people who helped in establishment of contacts and gaining the trust of study participants. This was critical in achieving enrollment goals and high follow-up rates. In our experience, research studies in Zambia will greatly benefit by clearly defining and explaining the benefits to study participants. High poverty level makes it difficult to garner interest about research studies in these participants because their individual problems are so severe that they have little time, energy or resources for participating in research studies.

Cohort studies are a challenge to plan and execute, even more so in a low-resource setting such as Zambia. In our experience, high-tech equipment does not work well without constant technical support. We believe that one of the reasons behind malfunctioning of the scanning software was the lack of firewall protection to the UTH network of computers. Affordable internet in Zambia is often slow and unreliable making many tasks tedious. Also, another common problem faced by the study personnel was ensuring that the enrolled household was indeed one unit. To establish this, all relationships were asked to be clearly defined by the client. For example, a client describing her sister's son as "like a son" was further queried to understand if the client was taking care of the child briefly or that the child was indeed living with the household. The household structure in Zambia is markedly different from Western countries with a

common joint family system, leading to large household size. Addition of new household members is also common because of relatives staying for extended periods of time, as is the caring for young children whose one or both parents have died. Careful planning and explanation of definition of household is essential for such studies.

This study design had several strengths. Most epidemiological studies on HHV-8 reported to-date are cross-sectional in design and cannot provide incidence estimates during early childhood in relation to the factors that may be associated with increased incidence. Also, very few studies have focused on studying infection within households especially in endemic areas like Zambia. Unlike Western countries, where primary infection mostly occurs after adolescence, our previous studies have shown that children are infected in Zambia at a young age [10]. We have a unique opportunity to study whether the source of infection is from within or outside the household. But despite careful planning, this cohort may still have certain limitations. We were unable to recruit households from more affluent backgrounds, thus limiting the ability to generalize our findings to different living situations. However, with 64% of Zambian households living in poverty, our results will be generalizable to the majority of Zambian population. Also, since we had to limit the household size to 10, we may have missed the effect of larger household size on HHV-8 acquisition. Another potential limitation could be self-selection bias; because of provision of medical benefits, the participants in the study may have been of poorer overall health due to HHV-8 or HIV infection, and had more of an incentive to be enrolled as well as to continue follow-up. If a selection bias was present, we will still be able to assess HHV-8 acquisition, using information about overall health of individuals as predictor variables. Another limitation could be informational bias;

some of the questions regarding eating habits or personal care habits may incite a defensive response in the caregiver, resulting in answers that err towards more socially acceptable habits. To decrease this potential bias, the questionnaire was carefully worded to avoid possible negative connotation, and the nurses were instructed to ask questions in a non-judgmental manner. In addition, since participants were informed of their and their child's HHV-8 status at follow-up visits, this could potentially alter their interactions with the child. The caregiver could assume that certain behaviors that were being asked on a regular basis were important and may change their behavior. To avoid this, participants were not instructed that any questioned behaviors were known to be associated with HHV-8 transmission.

The results from this study have the potential to contribute towards the design of effective health behavior interventions for prevention of HHV-8 infection and overall community health promotion. Designing effective behavioral interventions to prevent HHV-8 transmission to young children requires us to investigate the degree to which that behavior is influenced by attitudes, perceived norms, and self-efficacy. The questionnaire data collected from study participants will be critical in understanding these potential determinants of transmission. The laboratory and virological data will help in understanding the biology of HHV-8 in the Zambian setting. To summarize, this study was designed to yield critical clinical, demographic, laboratory and behavioral data useful for answering a variety of questions about HHV-8 transmission, including: Does a child acquire HHV-8 infection from within a household? Does HIV-1 infection of the child or household member increase risk of HHV-8 acquisition in a child? What behavioral or life style risk factors increase the risk of acquiring HHV-8 by a child?

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TABLES AND FIGURES

FIGURE 1.

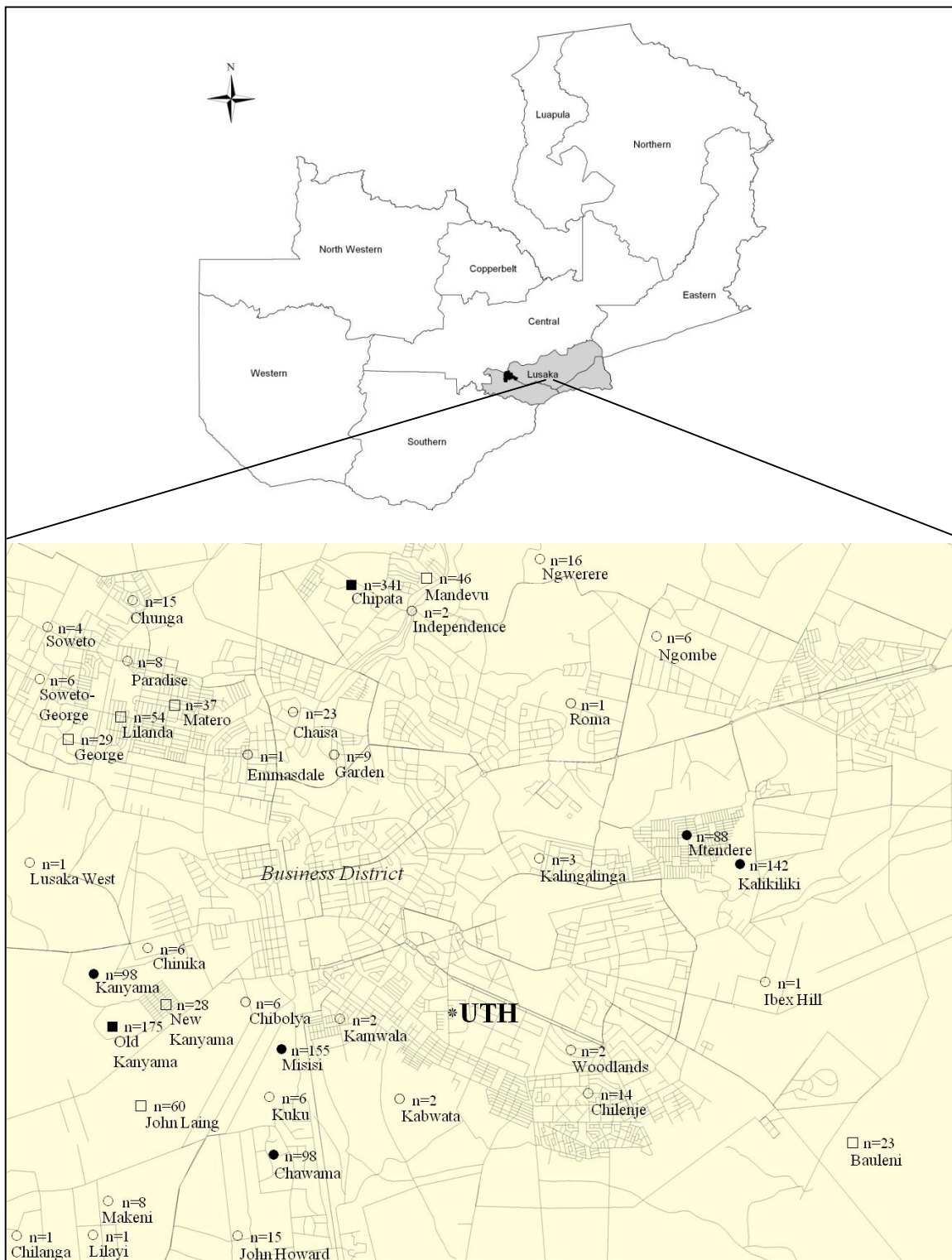


FIGURE 1. Outline Map of Zambia Depicting the Nine Provinces. City of Lusaka, the capital of Zambia, is a part of Lusaka district (shaded black). Lusaka District is a part of Lusaka province (shaded gray). Detailed Area Map of District of Lusaka, Zambia Showing the Distribution of Townships of the Households (N=1600) that were Screened for Enrollment in the Zambia Children's KS-HHV8 study, 2004-2009. No. – Number, UTH – University Teaching Hospital

Note:

Number of households (n) enrolled from each township is indicated. Symbols represent the percentage of households enrolled from each township; filled squares- $\geq 10\%$, filled circles- 5-9.9%, open squares- 1-4.9%, open circles- $< 1\%$.

FIGURE 2.

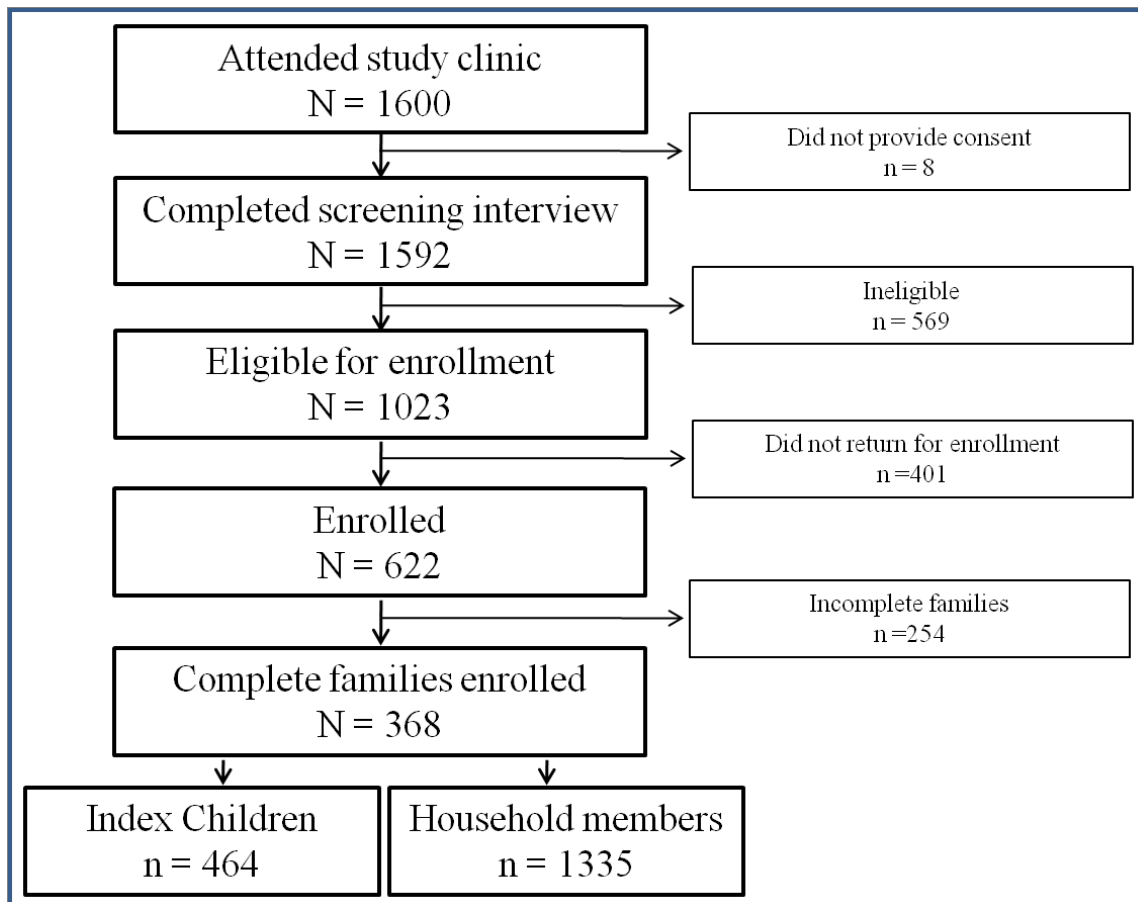


FIGURE 2. Flow Chart Describing the Recruitment of the Study Cohort for The Zambia Children's KS-HHV8 Study, Lusaka, Zambia, 2004-2009.

TABLE 1. Questionnaires, Biological Specimens Collected and Laboratory Measures for the Zambia Children’s KS-HHV8 Study, Lusaka, Zambia, 2004-2009.

Visit	Questionnaire	Samples collected	Tests performed
Screening	Screening questionnaire for eligibility	Blood	HHV-8
Enrollment	Enrollment questionnaire for: - basic medical examination - demographic characteristics - baseline data - household behavioral data -developmental milestones	Blood, buccal swab	HIV, CD4 count, HHV-8 serology, DNA shedding in buccal swabs Others, if indicated (Malaria, Syphilis)
Follow-up	Follow-up questionnaire - basic medical examination - changes in medical conditions - household behavioral data -developmental milestones	Blood, buccal swab	HIV, CD4 count, HHV-8 serology, DNA shedding in buccal swabs Others, if indicated (Malaria, Syphilis)

TABLE 2. Enrollment Characteristics of study Participants During Each Year of the Zambia Children's KS-HHV8 study, Lusaka, Zambia, 2004-2009.

	Year 2004	Year 2005	Year 2006	Year 2007	Year 2008	Total
	Number (%)	Number (%)	Number (%)	Number (%)	Number (%)	Number (%)
Index Children	n=11	n=171	n=151	n=92	n=39	n=464
Sex						
Female	6 (54.5)	81 (47.4)	62 (41.1)	46 (50.0)	24 (61.5)	219 (47.2)
Male	5 (45.5)	90 (52.6)	89 (58.9)	46 (50.0)	15 (38.5)	245 (52.8)
Age, months						
0 - 5	0	0	9	15	2	26
6-12	4	61	64	45	33	207
13-18	6	42	36	19	4	107
19-24	1	51	41	10	0	103
25-29	0	17	1	3	0	21
Household Members	n=38	n=567	n=500	n=226	n=4	n=1335
Sex						
Female	19 (50.0)	321 (56.6)	288 (57.6)	136 (60.2)	2 (50.0)	766 (57.4)
Male	19 (50.0)	246 (43.4)	212 (42.4)	90 (39.8)	2 (50.0)	569 (42.6)
Age, years						
≤ 5, Child	9	104	89	39	0	241
6-15, Youth	9	197	171	70	2	449
> 15, Adult	20	266	240	117	2	645
Total Enrolled	49	738	651	318	43	1799

TABLE 3. Characteristics of 368 Enrolled Households in the Zambia Children's KS-HHV8 study, Lusaka, Zambia, 2004-2009.

Characteristic	N (%)
Number of Household members	
≤3	87 (23.6)
4-5	175 (47.6)
>5	106 (28.8)
Number of rooms in home	
1	89 (24.2)
2-3	223 (60.6)
≥4	23 (6.3)
Electricity in home	
Yes	64 (17.4)
No	270 (73.4)
Toilet in home	
Shared outhouse (latrine)	329 (89.4)
Flush toilet in own house	6 (1.6)
Water source	
Community tap or shared with neighbors	291 (79.1)
Tap outside on property or in own house	44 (12.0)

Note: All numbers do not equal total household numbers due to missing values

TABLE 4. Characteristics of Primary Caregiver in the Zambia Children's KS-HHV8 study conducted in Lusaka, Zambia, 2004-2009.

Characteristic	N (%)
Primary caregiver relationship to index child (N=464)	
Mother	449 (96.8)
Grandmother	6 (1.3)
Aunt	7 (1.5)
Father	1 (0.2)
Cousin	1 (0.2)
Primary caregiver age (years) (N=368)	
<20	25 (6.8)
20-29	200 (54.3)
30-39	120 (32.6)
≥40	23 (6.3)
Parental caregiver status within households (N=368)	
Households with two parents	185 (50.3)
Households with single parent	130(35.3)
Households with extended family	53 (14.4)
Education of the primary caregiver [†] (N=368)	
None	33 (9.0)
Primary	200 (54.3)
Secondary	102 (27.7)

Note: All numbers do not equal total household numbers due to missing values

[†] A Primary caregiver is defined as the household member with the most contact with the child.

**CHAPTER 3: RISK FACTORS FOR EARLY CHILDHOOD
INFECTION OF HUMAN HERPESVIRUS-8 IN ZAMBIAN
CHILDREN: THE ROLE OF EARLY CHILDHOOD FEEDING
PRACTICES**

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ABSTRACT

Background: Human herpesvirus-8 (HHV-8) infection in early childhood is common throughout sub-Saharan Africa with prevalence increasing throughout childhood.

Specific routes of transmission have not been clearly delineated, though HHV-8 is present in high concentrations in saliva.

Methods: To understand the horizontal transmission of HHV-8 within households to children we enrolled for cross-sectional analysis; 251 households including 254 children, age two and under, in Lusaka, Zambia. For all children, plasma was screened for HHV-8 and HIV-1 and health and behavioral questionnaires were completed. Multi-level logistic regression analysis was conducted to assess independent factors for HHV-8 infection in children.

Results: Risk factors for HHV-8 infection included increasing number of HHV-8 positive household members [OR 2.5 (95% CI: 1.9, 3.3) $P < 0.01$] and having a primary caregiver who tested the temperature of food with their tongue prior to feeding the child [OR 2.4 (95% CI: 1.93, 3.30) $P = 0.01$]. Breastfeeding was protective against infection with HHV-8 for children [OR 0.3 (95% CI: 0.16, 0.72) $P < 0.01$].

Conclusions: These results demonstrate that exposure to HHV-8 in the household increases risk for early childhood infection with specific feeding behaviors likely playing a role in transmission.

Impact: Interventions to protect children from infection should emphasize the possibility of infection through sharing of foods.

INTRODUCTION

Human herpesvirus 8 (HHV-8) is the most recently discovered member of the gamma-herpesvirus family [1]. Also known as Kaposi's sarcoma-associated herpesvirus (KSHV), it is known to be the causative agent of Kaposi's sarcoma (KS), as well as other malignancies such as primary effusion lymphoma [2] and multicentric Castleman's disease [3]. Seroprevalence of HHV-8 depicts uneven distribution worldwide but is generally high in areas where non-HIV associated forms of KS (classic or endemic forms) are common [4]. Results of epidemiological studies on HHV-8 seroprevalence in African adults vary from 29% to 48% based on region and population group [5-7]. In children, seroprevalence differs significantly with age and between different geographical regions, and the prevalence of infection increases consistently throughout childhood and adolescence in young children in some endemic areas of Africa such as Zambia [8].

Prior to the onset of the HIV/AIDS epidemic in the mid 1980's, KS was rare in children even in KS endemic areas such as sub-Saharan Africa [9-13]. During the HIV/AIDS epidemic, KS incidence rates in Africa increased dramatically in both adults and children [14]. In Zambia, pediatric KS incidence increased from 3.2% to 19.0% as a direct result of the HIV/AIDS epidemic [10], and by 1990 constituted 20-25% of all Zambian pediatric malignancies [12]. Given the high prevalence of HIV infection in the population, this may have been associated with either an increased risk of acquisition of HHV-8 infection or an increased risk of HHV-8 transmission to young children. Hence, there is a need for a better understanding of the frequency of HHV-8 transmission during early childhood.

In both Western and developing countries, the risk for HHV-8 infection is likely associated with horizontal transmission through saliva however; in children there is a paucity of data regarding when and how transmission occurs and, in particular, which behavioral factors are associated with an increased risk of transmission. Several studies have shown that horizontal transmission occurs within families, most likely through saliva exchange [15-17]. Our laboratory has previously reported the isolation of HHV-8 viral DNA in saliva samples from seropositive mothers in Zambia. Results from these studies strongly suggest that child rearing behaviors associated with saliva exposure could be a risk factor for increased HHV-8 transmission [18]. Results from a recent study conducted in rural Uganda lend credence to this theory when it reported a possible, albeit weak association between variables associated with saliva exposure such as sharing of food and/or sauce plates and increased risk for infection with HHV-8 [19].

Our earlier longitudinal cohort study demonstrated that children in Zambia acquire HHV-8 infection early in life, with up to 40% of children being infected by 48 months of age [20]. These results provide compelling evidence that horizontal transmission of HHV-8 infection during early childhood is associated with the high incidence of infection in children. Therefore, we hypothesized that behaviors associated with saliva exchange, such as sharing food, would increase the risk for HHV-8 seropositivity. To date, no large epidemiological study has been conducted in an endemic area such as Zambia to investigate horizontal transmission of HHV-8 infection from within a household to children. In the present analysis we evaluated behavioral risk factors associated with household saliva exposure to delineate risk factors for HHV-8 infection in young children in Zambia.

MATERIALS AND METHODS

Study design and population

The present study is a part of a larger longitudinal cohort study recruited for the purpose of determining of risk factors for early childhood infection with HHV8 in Zambia. This is a collaborative study among investigators at the University of Nebraska-Lincoln and the University Teaching Hospital (UTH) of University of Zambia School of Medicine in Lusaka, Zambia to study HHV-8 and HIV infections in Zambia. Details of the study design, rationale and recruitment process for this study have been previously described in detail [21]. In brief, participant recruitment was conducted from August of 2004 to April of 2007 at the KS/HHV8 Study Clinic situated within UTH. Community workers were hired from eligible participants in our previous mother to child HHV-8 cohort transmission study specifically to inform community members about the study and to help with the screening and enrollment process [21].

Inclusion criteria for enrollment were as follows; families with a child less than 2 years of age (the index child), a resident of Lusaka, all family members willing to participate in annual follow-up visits and the primary caregiver and index child should commit to 4-month follow-up visits. The primary caregiver was the family member self-described as the individual with the most individual contact with and providing care for the index child. Of the enrolled children, 75 HHV-8 positive children chosen at random were also enrolled for the present analysis to understand the risk factors associated with HHV-8 prevalence in this cohort. These children were randomly selected if the caregiver expressed the willingness to participate.

Written consent was obtained from the study participants after describing the purpose of the study. Informed consent was obtained from all adult participants, and primary caregivers provided consent for children in the household. Study approval was granted by the Institutional Review Boards of the University of Zambia and the University of Nebraska and all suggestions and modifications were incorporated in the study protocols.

Data collection and measures

The primary caregiver in each household was interviewed by trained study nurses using structured questionnaires that included questions on socio-demographic variables (gender, age, education of the primary caregiver, household size, number of playmates), household living conditions (electricity, water source, toilet type, number of rooms/sleeping areas, household density), behaviors involving food and drink (pre-mastication of food, sharing sweets and/or drinks), and healthcare and personal care practices (bathing habits, use of traditional medicine, and use of saliva to clean children's faces, soothe injuries or insect bites). The questionnaires were written, designed and initially tested on focus groups in 2004 before study enrollment was initiated [22].

Laboratory testing

Sample collection. Blood samples were collected by venipuncture from all members of the household within eight months (mean 2.3 months) of each other. Family size is often large in Zambian households and everyone in the family is often unable to attend the clinic at the same time. HIV-1 testing was performed at the UTH clinic in Lusaka, Zambia and PBMC's were isolated for PCR analysis for HIV-1 positive individuals.

Plasma samples were shipped to University of Nebraska-Lincoln, Nebraska for HHV-8 serological analysis.

HIV-1 Serology. Plasma was screened for HIV-1 antibodies using both Capillus (Cambridge Biotech), and Determine (Abbott Laboratories) according to the manufacturer's instructions. A result was considered positive if one or both of these rapid screening assays revealed a positive result. For children under 18 months of age, early infant diagnosis using dried blood spot (DBS) was conducted. If children under 18 months of age were found to be seropositive but no confirmatory DBS data was available, serostatus was based on serology results that were obtained at subsequent follow-up visits (>18 months of age). If follow-up visits were not completed, the HIV-1 result was considered indeterminant.

HHV-8 Serology. HHV-8 serological testing was conducted using a monoclonal antibody-enhanced immunofluorescence assay (mIFA). Plasma samples were diluted 1:40 in phosphate buffered saline, and screening was performed on BC-3 cells (an Epstein-Barr virus negative and HHV-8 positive cell line) (American Type Culture Collection, Manassas, Virginia). BC-3 cells were grown in RPMI media supplemented with 20% heat-inactivated fetal calf serum, and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin). Cells were stimulated to promote HHV-8 lytic cycle with 20 ng/ml of tetradecanoyl phorbol ester acetate. At 48 hours post stimulation, cells were fixed using 4% paraformaldehyde, permeablized using 0.1% Triton X-100, and then spotted on 12-well teflon coated slides. The mIFA was performed with mouse monoclonal anti-human IgG antibody (CRL 1786) (American Type Culture Collection, Manassas, Virginia) as secondary antibody, and DyLight 488-conjugated donkey anti-mouse IgG

(Thermo Scientific) as tertiary antibody. A plasma sample was considered to be positive if two readers independently determined the sample to be positive on two independent mIFA tests. Positive samples were tested on BJAB cells (an Epstein-Barr virus negative and HHV-8 negative B cell line) which performed the role of negative controls to rule out any non-specific binding of antibodies to cells.

Data analysis

Dataset for the present analysis was built and statistical analysis was conducted using SAS (v9.2) (Cary, NC, USA). Logistic regression model with manual forward stepwise selection was conducted to explore the strength and significant association between HHV-8 seroprevalence (outcome) and a range of household behavioral habits (covariates). Associations between covariates and outcome were also evaluated to identify potential confounders. Odds ratios (ORs), 95% confidence intervals (CI) and *P* values were calculated to identify risk factors for HHV-8 infection in children under 2 years of age. Variables with a *P* value <0.05 in univariable analysis were included in a multivariable logistic regression model utilizing a manual forward selection process, to control for possible confounders and identify independent associations. Anthropometric Z scores were calculated using the NUTSTAT anthropometric software package (Epi Info, version 3.2.2; Centers for Disease Control and Prevention). Three households had multiple index children in one household. To account for a clustering effect, the data was also analyzed utilizing GEE logit link type 3 analyses though we did not observe any influence of clustering on the final results.

RESULTS

We screened index children under the age of 24 months from 1,600 households within the city of Lusaka, Zambia. Eligibility criteria were met by participants of 1,023 households. Of these, 622 primary caregivers along with their index children returned for enrollment and 368 of these returned with the complete household. There were 117 households that had incomplete data and record completion at enrollment, thus information from 251 households was available for analysis. Total enrollment in the study included 251 households with 254 index children, and 877 family members as outlined in Figure 1. Of the 254 index children enrolled, 75 (29.5%) were HHV-8 positive. Table 1 summarizes the characteristics of the HHV8 positive and negative children, their household sizes, primary caregivers, and the HHV-8 status of the household members. Comparison of behavioral habits related to the care of the index child that could be potential risk factors for HHV-8 transmission to children was conducted in the group of HHV-8 positive children as compared to HHV-8 negative children. On univariate analysis, we found significant associations between the HHV-8 serostatus of the primary caregiver including mother and father, youth and children in the household and risk for HHV-8 infection in the index child (Table 2). Specifically, an increased risk for HHV-8 infection was associated with a child having a primary caregiver who was HHV-8 positive [OR = 2.5 (95% CI: 1.44, 4.32) $P < 0.01$]. There was a slightly stronger association if the mother was HHV-8 positive as opposed to other caregivers [OR = 2.7 (95% CI: 1.48, 4.77) $P < 0.01$]. However, an increased risk for HHV-8 prevalence was associated with having any HHV-8 positive adult household member [OR = 2.1 (95% CI: 1.11, 3.92) $P = 0.03$], and there was an increased odds of

1.8 [(95% CI: 1.22, 2.51) $P < 0.01$], for every additional adult household member who was HHV-8 positive. The increasing total number of HHV-8 positive household members, including youth, was associated with an even higher odds of 2.5 (95% CI: 1.91, 3.21) $P < 0.01$.

We found no associations between child anthropometrics (weight and height percentiles), and risk for HHV-8 prevalence (Table 3). Additionally, there was no association between CD4 and CD8 cell count, hematocrit and hemoglobin levels and risk for HHV-8 prevalence in children (Table 3). The association between HHV-8 prevalence and socio-demographic variables was also examined, including age, gender, number of household members categorized by age, relationship of primary caregiver to index child, parental age, and primary caregiver education. There was an increased risk associated with having a greater number of adults in the household (Number of household members > 15 years of age) [OR = 1.5 (95% CI: 0.99, 2.18) $P = 0.05$] (Table 3), however we found no association between the other variables including child age, gender, adult age, education level and increased risk of infection.

We also examined for an association between HHV-8 seroprevalence in children and household and community specific living condition covariates including crowding, water source, toilet availability, number of rooms and sleeping areas in the home, crowding of persons in sleeping areas, number of playmates and number of playmates under the age of 5, and number of times weekly the index child spends overnight with friends. No associations were found with any of these covariates and child HHV-8 serostatus (Table 4). Similarly, we found no association between HHV-8 seroprevalence in children and health and hygiene exposures including number of full-body baths, daily

face cleanings, use of traditional medicine, and use of saliva to clean child's face, soothe childhood injuries, or insect bites (Table 4) although having a toilet within the household was marginally significant for protecting against HHV-8 infection [OR = 0.1 (95% CI: 0.01, 1.34) $P=0.09$].

We also investigated covariates related to feeding practices to investigate the association with HHV-8 seroprevalence and observed that there was decreased odds of HHV-8 seropositivity when the children was currently being breastfed [OR = 0.5 (95% CI: 0.27, 0.88) $P = 0.01$] or if the child had ever been breastfed [OR = 0.4 (95% CI: 0.21, 0.74) $P < 0.01$] (Table 5). A number of feeding practices that involved exchange of saliva were evaluated to assess for association with HHV-8 prevalence including premastication and sharing of foods. Having a HHV-8 positive caregiver premasticate the food prior to feeding the child did not demonstrate significantly increased odds for HHV-8 prevalence, although a small number of caregivers indicated that this practice was performed limiting our overall sample size (data not shown).

Upon multivariable logistic regression analysis, an independent association for HHV-8 prevalence included total number of HHV-8 positive household members [OR = 2.5 (95% CI: 1.93, 3.30) $P < 0.01$] or having the primary caregiver taste the temperature of the food prior to feeding [OR = 2.4 (95% CI: 1.19, 4.73) $P = 0.01$] (Table 6). Current or past breastfeeding [OR = 0.3 (95% CI: 0.16, 0.72) $P < 0.01$] was protective against infection with HHV-8.

DISCUSSION

In sub-Saharan Africa children become infected with HHV-8 at an early age. Our cohort studies have reported that by 13.4% of Zambian children were infected 12 months of age [23]. An additional Ugandan study found comparable levels of infection by 2 years of age [24]. Zambia is a part of the “KS belt”, an area with endemic KS that has been severely impacted subsequently by the HIV epidemic, underscoring the need for epidemiologic studies to better understand patterns of HHV-8 transmission in young children. Although sexual transmission in adults and mucosal shedding of HHV-8 along with salivary exposure has been implicated in the transmission of HHV-8 in adults, there is little information about routes of transmission in children, and behavioral habits that could be potential risk factors and sources of transmission within the family.

Although other studies have examined childhood HHV-8 infection as a result of transmission between mother and child or between siblings [17, 25, 26], few have examined specific household behaviors that could contribute to virus transmission to a susceptible child within a household. The strengths of our study include utilization of a detailed questionnaire designed to assess behavioral habits not only engaged by the mother, but by household members and even the child’s interaction with neighborhood children. Ours is one of the first studies demonstrating an association between behavioral factors associated with saliva exchange, specifically feeding practices such as testing food prior to feeding, and infection with HHV-8 in early childhood. Butler et al. previously demonstrated a significant association with HHV-8 in children <14 years of age and sharing a sauce plate with other household members [19]. Our study did not demonstrate a significant association in any of our household food sharing variables in

the univariable analysis, most importantly, an absence of any association with premastication, however our sample size was small in contrast with other studies [19]. However, we did observe that in the multivariable analysis a specific behavior i.e. the primary caregiver testing temperature of food prior to feeding was associated with HHV-8 prevalence, which potentially supports Butler et al's association with food sharing behaviors [19].

In the present study, we observed that presence and number of HHV-8 positive household members was consistently associated with childhood HHV-8 infection. This suggests person-to-person contact with HHV-8 positive members is likely to play a key role in transmission. These results have been further confirmed by a study completed in our lab utilizing molecular analysis of the K1 gene sequence data of KSHV-positive individuals from nine households in our cohort [27]. Olp et al found that in six of the nine households, the child had 100% sequence identity to all household members, supporting that intra-household transmission occurs [27].

Our study tested multiple behavioral habits of caregivers and household members in which saliva exchange could occur with the child. We also investigated whether these behaviors were practiced by the primary caregiver, or if the primary caregiver observed other household members practicing the behavior to test the hypothesis that HHV-8 was spread through child-rearing behaviors associated with saliva exposure. We observed that both the primary caregiver's and the mother's HHV-8 status were the most significant factors, most likely due to more frequent and close person-to-person contact. This contact likely occurs during food exchange behaviors, when possible saliva exchange occurs. But in this study, none of the child rearing behaviors individually were associated

with HHV-8 prevalence with the exception of the primary caregiver testing the temperature of the food before feeding the child. It is likely that for a HHV-8 transmission event to occur the amount of saliva and the amount of viral shedding at the time of the behavior may be key factors. Seroreversion, the fluctuation in antibody titers to undetectable levels, in adults is well documented, and could correlate to viral shedding and likelihood of transmission. Also, the frequency of occurrence of each behavior over time could be critical but this was not fully explored in this study. It is also likely that the behavioral habits analyzed in this study such as sharing drinks and foods, involve exposure to only minimal amount of saliva. Similarly, Butler et al's (2011) paper, reporting only a marginal association of sharing food and/or sauce plates with HHV-8 infection in children could support the fact that the amount or frequency of saliva sharing could be a factor [19].

Breastfeeding as a protection against HHV-8 infection is a novel finding among herpesviruses. Breast milk has been implicated in mother to child transmission of several viruses, including cytomegalovirus and HIV-1 [28, 29]. However, to our knowledge, this is the first report of breastfeeding as protection against childhood infection of HHV-8 or any herpesvirus. It is likely that there may be non-specific immune factors responsible for breast milk protection, including lactoferrin, complement components, or even commensal organisms. Overall, our finding of the decreased risk of HHV-8 infection associated with breastfeeding is intriguing and warrants further studies to identify the protective factors that may be responsible.

HIV-1 infection has been documented to be a risk factor associated with HHV-8 infection. However, we did not analyze the association of HIV-1 with HHV-8 infection in

the current study, because our subjects were selected to be HHV-8 negative as part of a larger longitudinal study with an aim to study HHV-8 incidence in early childhood. Our earlier cohort studies have clearly demonstrated that HIV infected children are at a significantly higher risk for acquiring HHV-8 infection [23]. Therefore, during the enrollment of this cohort, in which HHV-8 negative children were being recruited, it is likely that HIV-1 positive children were under-represented because they were already HHV-8 infected.

A limitation of our study is that the behavioral questions were self-reported which could potentially result in recall bias. HIV-1 status of the household members was reported to the study subjects at study enrollment and counseling was done regarding transmission of HIV-1 and preventative health measures. However, since HHV-8 status was not known by the caregivers at the time of the study, we do not believe that this potential for bias was a significant factor.

In summary, we have demonstrated that the presence of HHV-8 in the household plays a key role in transmission of the virus to a susceptible child. This likely occurs through saliva sharing during behaviors such as testing the temperature before sharing to the child. This finding has implications for public health risk, and the need for education of families of potential transmission of the virus when sharing food with young children, and needed behavior changes. Although the current analysis supports the view that the primary caregiver poses the most significant risk of transmitting HHV-8 infection to a child, other results from the same cohort also demonstrate that HHV-8 transmission to a child can also occur from other household members and even from others outside of the household [27]. Whether similar factors are also associated with HHV-8 incident

infection in “at-risk” children is the current focus of this study. Longitudinally followed children in this cohort up to 48 months after enrollment will improve our understanding of the nature of interpersonal contact with a child and its role in HHV-8 transmission.

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TABLES AND FIGURES

Table 1. Demographics and HHV-8 Characteristics of HHV-8 Positive and HHV-8 Negative Children in Lusaka, Zambia, 2004-2007.

Characteristic	HHV8 + (n=179)	HHV8 - (n=75)
<i>Demographics</i>		
Age of index child (months):		
Range	4-25	2-28
Mean	13.4	13.5
Sex of index child:		
Males	28	93
Females	47	86
Number of household members:		
Range	2-8	2-8
Mean	4.7	4.5
Number of adults:		
Range	1-4	1-4
Mean	1.8	1.7
Age of primary caregiver (years):		
Range	17-49	14-58
Mean	28.9	27.3
Education of primary caregiver:		
None	7	18
Primary school	47	105
Secondary school	21	56
<i>Household HHV-8</i>		
Primary caregiver HHV-8+	52/75 (69.3%)	82/179 (45.8%)
Mother HHV-8+	51/72 (70.8%)	79/175 (45.1%)
Father HHV-8+	23/36 (63.9%)	44/74 (59.5%)
≥ 1 other household member HHV-8+	68/75 (90.7%)	143/179 (79.9%)

Table 2. Univariable Analysis to Investigate the Association of HHV-8 Serological Status of Household Members with Child HHV-8 Seropositivity in Lusaka, Zambia, 2004-2007.

Characteristic	Odds Ratio	95% Confidence interval	P value
<i>Adult and Household HHV-8 Risk Factors</i>			
≥ 1 household member HHV-8+	2.7	1.23, 5.78	0.01 ^a
Increasing number of HHV-8+ household members	2.5	1.91, 3.21	<0.01 ^a
Primary caregiver HHV-8+	2.5	1.44, 4.32	<0.01 ^a
Mother HHV-8+	2.7	1.48, 4.77	<0.01 ^a
Father HHV-8+	1.2	0.49, 2.67	0.66
≥ 1 adult household member HHV-8+	2.1	1.11, 3.92	0.03 ^a
Increasing number HHV-8+ adult household members	1.8	1.22, 2.51	<0.01 ^a
<i>Youth HHV-8 Risk Factors</i>			
≥ 1 household youth HHV-8+	1.4	0.70, 2.84	0.30
Increasing number of household youth HHV-8+	1.3	0.90, 1.99	0.16
Any other household child HHV8 +	1.3	0.66, 2.74	0.52

^a Significant at α level of 0.05 ^bTotal numbers vary reflecting family members enrolled in study

Table 3. Univariable Analysis to Investigate the Association of Socio-Demographic Factors and Nutritional and Immunological Factors with Child HHV-8 Seropositivity in Lusaka, Zambia, 2004-2007.

Characteristic	Odds Ratio	95% Confidence interval	P value
<i>Child specific characteristics</i>			
Age of index child (months)	1.0	0.96, 1.04	0.99
Gender of index child (Reference: male)	0.6	0.37, 1.19	0.12
<i>Household specific characteristics</i>			
Number of household members	1.1	0.94, 1.36	0.18
Number of household adults > 15 yrs	1.5	0.99, 2.18	0.05 ^a
Number of household youth (age > 5, ≤ 15yrs)	1.1	0.85, 1.37	0.51
Number of household children (age ≤ 5 yrs)	1.0	0.68, 1.52	0.93
Two parents vs. single parent household	1.6	0.86, 2.93	0.45
Extended family parenting vs. single parent household	1.6	0.75, 3.58	0.45
<i>Caregiver and adult specific characteristics</i>			
Relationship of primary caregiver to index child:			
- Aunt vs. Mother	5.0	0.44, 56.0	0.99
- Grandmother vs. Mother	0.7	0.27, 10.2	0.98
Maternal age (years)	1.0	0.99, 1.08	0.13
Paternal age (years)	1.0	0.98, 1.06	0.42
Primary caregiver age (years)	1.0	0.99, 1.08	0.10
Primary caregiver education:			
- No school vs. junior primary	1.5	0.48, 4.53	0.30
- No school vs. upper primary	1.1	0.41, 2.79	0.93
- No school vs. junior secondary	1.1	0.38, 3.19	0.87
- No school vs. upper secondary	0.7	0.21, 2.59	0.37
<i>Nutritional status indicators</i>			
Length-for-age or stature-for-age percentile	1.0	0.99, 1.01	0.70
Weight-for-length or weight-for-stature percentile	1.0	0.99, 1.00	0.42
Weight-for-age percentile	1.0	0.99, 1.00	0.13
<i>Laboratory values</i>			
CD4 count	1.0	0.99, 1.00	0.27
CD8 count	1.0	0.99, 1.00	0.18
Hematocrit	1.1	0.86, 1.48	0.39
Hematocrit (normal)	1.2	0.44, 3.11	0.76
Hemoglobin	1.3	0.76, 2.30	0.33

^a Significant at α level of 0.05

Table 4. Univariable Analysis to Investigate the Association of Household and Personal Care Factors with Child HHV-8 Seropositivity in Lusaka, Zambia, 2004-2007.

Characteristic	Odds Ratio	95% Confidence interval	P value
<i>Household and Community Characteristics</i>			
Water source (Community vs. within household)	0.9	0.41, 2.12	0.85
Toilet location (Community vs. within household)	0.1	0.01, 1.34	0.09
Number of rooms in the household	1.2	1.00, 1.54	0.12
Number of sleeping areas in the household	1.4	0.94, 2.19	0.10
Persons per sleeping area	1.0	0.79, 1.32	0.90
Number of playmates	1.0	0.91, 1.23	0.99
Number of playmates < age 5	1.0	0.84, 1.29	0.61
<i>Hygiene practices</i>			
Number of daily full-body baths for index child	1.2	0.59, 2.39	0.58
Number of daily face cleanings for index child	1.1	0.51, 2.12	0.87
Saliva used to clean child's face	1.1	0.42, 2.89	0.81
Biting the child's nails to shorten them	0.6	0.34, 1.30	0.21
<i>Health practices</i>			
Saliva used to soothe insect bites	1.3	0.41, 4.22	0.60
Caregiver sucks mucus from child's nose	1.7	0.56, 4.83	0.28
Traditional teething remedies used	1.0	0.72, 1.48	0.88
Use of Traditional Medicine	0.2	0.03, 1.60	0.13
Lifebuoy soap for constipation	1.0	0.54, 2.26	0.92

Table 5. Univariable Analysis to Investigate the Association of Child Feeding Behavioral Habits of Household with Child HHV-8 Seropositivity in Lusaka, Zambia, 2004-2007.

Characteristic	Odds Ratio	95% Confidence interval	P value
<i>Breastfeeding practices</i>			
Child has ever been breastfed	0.4	0.21, 0.74	<0.01 ^a
Child is currently being breastfed	0.5	0.27, 0.88	0.01 ^a
Primary caregiver moistens nipples with saliva prior to breastfeeding	1.0	0.38, 2.21	0.96
<i>Premastication</i>			
Adult premasticates food prior to sharing with children	1.2	0.60, 2.72	0.61
Primary caregiver performs premastication	1.1	0.54, 2.47	0.86
Other household members perform the premastication	1.8	0.51, 5.68	0.35
<i>Adult feeding variables</i>			
Primary caregiver testing temp of food with tongue prior to sharing w/ child	1.6	0.91, 2.89	0.09
Blowing on food prior to sharing with child	1.1	0.67, 1.90	0.69
Sucking on sweets prior to sharing with child	1.1	0.61, 1.89	0.80
<i>Child and household food sharing variables</i>			
Child shares sweets with other neighborhood children	0.9	0.44, 2.02	0.71
Child shares sweets with other household members	1.1	0.64, 1.89	0.87
Child shares drinks with other children	0.5	0.24, 1.37	0.19
Household members share common utensils for meals	0.8	0.34, 1.88	0.58
Household members share drinks with child	1.0	0.50, 1.84	0.89

^a Significant at α level of 0.05

Table 6. Multivariable Analysis to Investigate Independently Associated Risk Factors Associated with HHV-8 Seropositivity in Children in Lusaka, Zambia, 2004-2007.

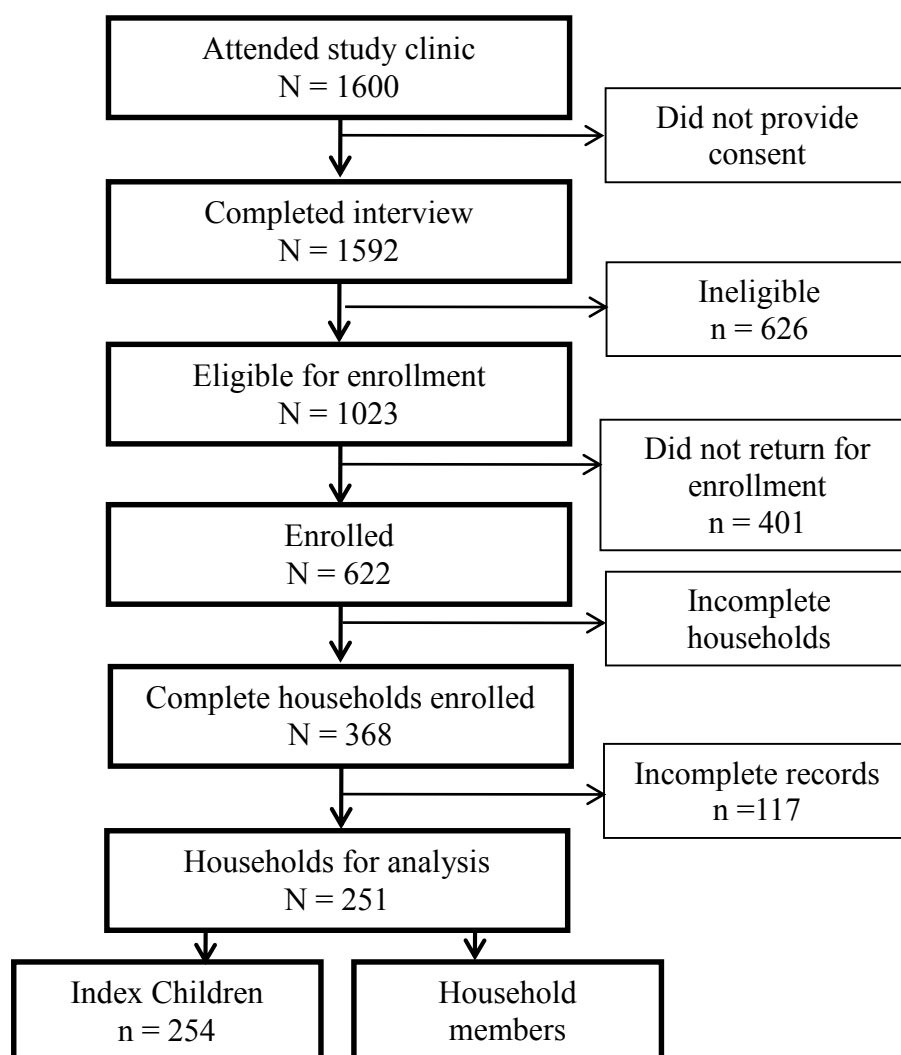
Characteristic	Odds Ratio	95% Confidence interval	P value
Age of index child (in months)	1.0	0.94, 1.10	0.75
Total number of HHV-8+ household members	2.5	1.93, 3.30	<0.01 ^a
Primary caregiver testing temperature of food prior to feeding	2.4	1.19, 4.73	0.01 ^a
Child breastfed currently or in the past	0.3	0.16, 0.72	<0.01 ^a

^a Significant at α level of 0.05

FIGURE LEGEND

Figure 1. Flow Chart outlining the screening and recruitment of study cohort in Lusaka, Zambia, 2004-2007.

Figure 1.



CHAPTER 4: CHILDHOOD FEEDING PRACTICES AS A RISK FOR CHILDHOOD HHV-8 ACQUISITION

ABSTRACT

Background: Human herpesvirus-8 (HHV-8) infection occurs at an early age in endemic areas such as sub-Saharan Africa. Although saliva is commonly implicated as the culprit in viral transmission to young children, specific behavioral practices as sources of transmission have not been clearly delineated.

Methods: To further investigate specific characteristics and household behaviors that are associated with the risk of transmission of HHV-8 within households to children in Lusaka, Zambia, we enrolled and followed 270 children, age two and under. For all children, plasma was screened for HHV-8 and HIV-1 and health and behavioral questionnaires were completed. Multi-level Cox proportional hazard analysis was conducted to assess independent factors for HHV-8 infection in children.

Results: Most significant independent risk factors for HHV-8 seroconversion included age at seroconversion ($P < 0.0001$), age at enrollment [HR 2.62 (95% CI: 1.64, 4.21) $P < 0.001$], child currently being breastfed [HR = 2.13 (95% CI:1.31, 3.44) $P = 0.002$], use of pacifier [HR = 9.00 (95% CI:1.65, 49.19) $P = 0.01$], child HIV-1 status [HR = 2.26 (95% CI:1.19, 4.30) $P = 0.013$], and other individuals blowing on food prior to sharing with child [HR = 2.79 (95% CI:1.01, 7.76) $P = 0.05$].

Conclusions: These results demonstrate behaviors in the household that potentially expose the young child to HHV-8 through exposure to saliva increases risk for early childhood infection.

INTRODUCTION

Human Herpesvirus-8 (HHV-8) or Kaposi's sarcoma associated herpesvirus (KSHV) is the eighth known human herpesvirus to be discovered. HHV-8 is known to be associated with the development of Kaposi's sarcoma (KS), primary effusion lymphoma, and multicentric Castleman disease. Seroprevalence of HHV-8 varies according to geographic location. In the United States and Western Europe, HHV-8 infection is uncommon in the general population, with higher prevalence in men who have sex with men (MSM) [1, 2]. In sub-Saharan Africa and Mediterranean regions, HHV-8 is considered to be endemic, however much is yet to be elucidated regarding HHV-8 risk factors and transmission. Our lab has previously shown that Zambian children acquired HHV-8 infection early in life, with up to 40% of children becoming HHV8 positive by 48 months of age [3]. Transmission via breastmilk is a possibility, as cytomegalovirus, another herpesvirus is transmissible via breastmilk, however HHV-8 DNA is not readily isolated from breast milk [4-6]. In HHV-8 seropositive mothers high viral titers of HHV-8 is frequently isolated in saliva [5-7]. In a previous study from a South African population, children of mothers with high viral load (>50,000 copies/mL) in saliva, were nearly three times more likely to be HHV-8 positive, than if the mother did not shed HHV-8 in saliva [5]. Several studies have shown that transmission of HHV-8 occurs within households, most likely via saliva exchange [8-10]. Taken together, these findings implicate salivary transmission as the likely culprit for early childhood infection, however, the mode by which saliva exchange could occur is unclear. A rural Ugandan study found food and/or sauce plate sharing within households as an associated risk for HHV-8 in children under age 14 [11]. These studies support our hypothesis that

behaviors within the household that expose children to saliva at an early age increase the risk of childhood acquisition of HHV-8. To investigate horizontal transmission of HHV-8 infection from within a household to children, we conducted an epidemiologic study in collaboration among investigators at the University of Nebraska-Lincoln and the University Teaching Hospital (UTH) of University of Zambia School of Medicine in Lusaka, Zambia. In this present study, we report findings from a longitudinal cohort in order to assess childhood incidence rates of HHV-8 infection and the associated socio-demographic, child feeding and rearing behaviors and health care practices that could be associated with acquisition of HHV-8 infection.

METHODS

Study setting

Details of the study design, rationale, and recruitment process for this study have been described in detail previously [12]. In brief, participants were recruited from August, 2004, to April, 2007 at the study clinic in Lusaka, Zambia. Community workers were hired to participate in informing community members about the study, recruitment of eligible study participants, and assist in maintaining follow-up with study participants [12].

Inclusion criteria for enrollment were; family with a child less than 2 years of age (the index child), family should be a resident of Lusaka, all family members should be willing to participate in annual follow-up visits and the primary caregiver and index child should commit to 4-month follow-up visits. The primary caregiver was the family member self-described as the individual with the most individual contact with and provided care for the index child. Children and members of their households who meet study eligibility criteria, signed informed consent before enrolling into the study. Written informed consent was obtained from all adult participants, and primary caregivers provided consent for children in the household.

Once study participants were enrolled, the primary caregiver and index children returned for follow-up visits every four to six months to reassess their serological status for HHV-8 and/or HIV, and to complete a risk assessment questionnaire for acquisition of HHV-8 infection. At each visit, a thorough physical examination was performed, new blood specimens and saliva specimen collected and tested for HHV-8 and HIV-1

infection. All family members were assessed at enrollment, with specimen collection and annually thereafter.

As part of the study incentives, each participant was provided multivitamins and other medications when indicated and a chance for their infant to be followed-up by a reputable pediatrician. At all visits, all subjects were provided with counseling about risk reduction for HIV. Study approval was granted by the Institutional Review Boards of the University of Zambia and the University of Nebraska.

Data collection and measures

Questionnaires were designed as described in detail in chapter 2. In brief, questionnaires specific for risk assessment were completed by primary caregivers and evaluated upon seroconversion of the child. These questionnaires address potential sources of blood or body fluids from household members to the index child, with detailed questions surrounding incidents that could possibly account for salivary exposure. Data collected included: socio-demographics (age, gender, occupation, place of residence, marital status, number-gender-age of children, number-gender-age of siblings); medical conditions (height, weight, blood pressure, immunization history, breastfeeding history including duration, frequency and type of any illness-related signs and symptoms, major disease history, current medication use); laboratory testing (HIV, HHV-8); household living conditions (number of people living in the house, number and type of rooms in the house, nature of the water supply, access to toilet, etc.); family structure (two, one or no parents, number and type of child's caretakers: mother, extended family, siblings); household life-style risk factors (sharing dental cleaning materials, sharing pacifiers, eating with fingers from a common bowl, sharing drinks); and child care giving behaviors (frequency

and type of feeding, bathing, face washing, etc.). The questionnaires were written and designed and initially tested on focus groups in 2004 before study enrollment was initiated [13].

Laboratory testing

Sample collection. Blood samples were collected by venipuncture from all members of the household. Family size is often large in Zambian households and everyone in the family is often unable to attend the clinic at the same time. HIV-1 testing was performed at the UTH clinic in Lusaka, Zambia and PBMC's were isolated for PCR analysis for HIV-1 positive individuals. Plasma samples were shipped to University of Nebraska-Lincoln, Nebraska for HHV-8 serological analysis.

HHV-8 Serology: Antibodies against HHV-8 latency-associated nuclear antigen (LANA) and HHV-8 lytic antigens was determined by immunofluorescence assays (IFAs), at a dilution of 1:40, using a primary effusion lymphoma (BC-3) cell line, negative for Epstein-Barr virus. The viral lytic cycle was induced by incubating BC-3 cells with 20 ng/mL 12-Otetradecanoylphorbol-13-acetate (TPA) (Sigma) for 48 hours. Fixing of cells was completed with 4% paraformaldehyde and permeabilized using 0.1% Triton X-100. Post-fixing, the cells were spotted on 12-well Teflon coated slides and allowed to air dry. The mIFA signal was enhanced using a monoclonal mouse anti-human IgG antibody (CRL 1786, ATCC) [14] as the secondary antibody, and DyLight™ 488-conjugated donkey anti-mouse antibody (Jackson Immuno Research) as the tertiary and detection antibody. A plasma sample was considered to be positive if two readers independently determined the sample to be positive on two independent mIFA tests. Positive samples were tested on BJAB cells (an Epstein-Barr virus negative and HHV-8 negative B cell

line) which performed the role of negative controls to rule out any non-specific binding of antibodies to cells.

HIV-1 Serology. Plasma was screened for HIV-1 antibodies using two rapid detection kits; Capillus (Cambridge Biotech), and Determine (Abbott Laboratories) according to the manufacturer's instructions. A result was considered positive if one or both of these rapid screening assays revealed a positive result. All children born to HIV positive mothers were also screened for HIV infection. For children under 18 months of age, early infant diagnosis using dried blood spot (DBS) was conducted. If children under 18 months of age were found to be seropositive but no confirmatory DBS data was available, serostatus was based on serology results that were obtained at subsequent follow-up visits (>18 months of age). If follow-up visits were not completed, the HIV-1 result was considered indeterminant.

Data analysis

Dataset was built and statistical analysis was conducted using SAS (v9.2) (Cary, NC, USA). The crude incidence rate per 100 child-years was calculated by dividing the number of new HHV-8 seroconverters by the total number of child-years at risk and multiplying by 100. Seroconverting children contributed HHV-8 free child-years at risk until testing positive for HHV-8. All data was right censored at 48 months. Cox proportional hazards modeling was conducted to explore the strength and significant association between HHV-8 infection (outcome) and each individual characteristic (covariates). Associations between covariates and outcome were also evaluated to identify potential confounders. Hazard ratios (HRs), 95% confidence intervals (CI) and *P* values were calculated to identify risk factors for HHV-8 infection. All comparisons were

considered statistically significant at $P \leq 0.05$. Variables with a P value ≤ 0.05 in univariable analysis were included in a multivariable model utilizing a manual forward stepwise selection process, to control for possible confounders and identify independent associations.

RESULTS

Index children and members of 1,600 households were screened for study eligibility criteria as described elsewhere [12]. As outlined in Figure 1, a total of 270 index children were enrolled in the current study. Of the 1023 initial households eligible for enrollment, 622 returned for enrollment. There were 254 families unable to return with all members of their household, these were considered incomplete and were dropped from the study. The total enrolled were 464 children, comprising 368 households. Of these children, 75 were tested to be HHV-8 positive at the time of enrollment. Data was collected separately from these children, and a cross-sectional analysis was performed as described in chapter 3. Insufficient risk assessments were completed for 119 children, excluding them from the study. Of the remaining 270 children followed, 137 (50.7%) children seroconverted to be HHV-8 positive within the four year follow-up time of the study, and 133 (49.3%) remained negative.

Table 1 summarizes the demographics and characteristics of the HHV-8 seroconverting children, and non-seroconverting children. Of the seroconverting children, at enrollment 11/137 (8.0%) were HIV-1 positive, and 12/133 (9.0%) were HIV-1 positive.

The incidence rate of HHV-8 seroconversion in the total cohort of 270 children was 29.94 per 100 child-years, based on a total of 457.57 child-years of follow-up (see Table 2). We examined crude incidence rates by primary caregiver and child HIV-1 status, finding for HIV-1 positive children, incidence rates of 29.19 per 100 child-years, and for HIV-1 negative children, incidence rates of 30.01 per 100 child-years. If the caregiver was HIV-1 positive, incidence rates were slightly lower, at 25.28 per 100 child-

years, as compared to if the caregiver was HIV-1 negative, at 34.32 per 100 child-years. When only HIV-1 negative children were examined, a similar finding was also noted, with incidence rates for HIV-1 positive caregivers at 24.48 per 100 child-years, and for HIV-1 negative caregivers at 34.32 per 100 child-years.

Figure 2 presents the probability curves as obtained from Kaplan-Meier survival analysis. Panel A represents the total cohort, showing the probability of annual HHV-8 infection occurring over the 4 year time span. This rate of seroconversion is also reflected in Table 3, note the age of seroconversion was highly significant when comparing age groups of >24M to \leq 36M to the reference age of <24M [HR = 0.23 (95% CI: 0.14, 0.37) $P < 0.001$] and >36M to the reference age of <24M [HR = 0.02 (95% CI: 0.007, 0.03) $P < 0.001$]. These findings demonstrate a higher risk associated with an age of <24M with HHV-8 seroconversion, and decreasing with age. Figure 2, panel B shows that the probability of males being HHV-8 free is nearing significance (Log Rank $P = 0.08$).

Comparison of HIV status of the child, primary caregiver, or any household member was conducted in the group of HHV-8 seroconverting children to non-seroconverting children. HIV-1 positive status of the mother is nearing significance [HR = 0.74 (95% CI: 0.53, 1.05) $P = 0.09$] (Table 3). The probability curve as seen in Figure 2 panel C displays the slightly less probability of a child of an HIV-1 positive caregiver to become HHV-8 positive (Log Rank P -value = 0.09). HIV-1 status of any other household member was not significant (Table 3). An HHV-8 positive caregiver showed a nearly significant risk [HR = 1.38 (95% CI: 0.98, 1.94) $P = 0.06$], but most notably significant was the increased risk of the child to seroconvert to HHV-8 for every

additional household member that is HHV-8 positive [HR = 1.20 (95% CI: 1.06, 1.39) $P = 0.005$] (Table 3).

No significant associations were found when examining the associations of household and living conditions with child HHV-8 seroconversion. This included variables such as, electricity to the house, water source of the house (well water, vs. community tap, vs. private water source), number of rooms and sleeping areas in the household, number of persons in the household, and number of persons per sleeping area (data not shown).

We found a significant association of the child currently being breastfed with child HHV-8 seroconversion [HR = 6.26 (95% CI: 4.02, 9.75) $P < 0.0001$] (Table 4). Also significant was caregiver pre-masticating food prior to sharing with children [HR = 3.15 (95% CI: 1.00, 9.98) $P = 0.05$]. Of interest, all of the adult feeding variables assessed, including testing food with tongue to assess the temperature prior to feeding to the child, blowing on the food to cool the temperature of the food prior to feeding to the child, eating of food with hands from a common bowl at mealtimes, and sharing of eating utensils with the child were all significant (Table 4). Most significant findings included other household members testing temperature of food with tongue prior to sharing with child [HR = 5.52 (95% CI: 2.00, 15.20) $P = 0.0009$], primary caregiver blowing on food prior to sharing with child [HR = 4.13 (95% CI: 2.37, 7.19) $P < 0.0001$], and primary caregiver sharing of eating utensils with child [HR = 4.28 (95% CI: 2.07, 8.85) $P < 0.0001$] (Table 4).

Table 5 shows several behavioral habits related to sharing sweets and packaged drinks that could be potential risk factors for sharing of saliva. All of the behaviors were

highly significant, demonstrating a risk for the child to seroconvert to HHV-8. The exception of the primary caregiver sucking on sweets prior to sharing with the child nears significance [HR = 2.06 (95% CI: 0.90, 4.74) $P = 0.09$] (Table 4). The sharing of food, candy, or other sweets was most significant when done with other household children [HR = 2.97 (95% CI: 1.57, 5.61) $P = 0.0008$] (Table 4). Sharing of packaged drinks such as bottled soda was most significant when done with household members other than the primary caregiver [HR = 4.87 (95% CI: 2.32, 10.21) $P < 0.0001$] (Table 4). Interestingly, an association was found with childhood use of a pacifier [HR = 17.62 (95% CI: 4.18, 74.35) $P < 0.0001$] (Table 4).

We observed significant associations with twice per day full-body baths and face cleanings for the index child [HR = 4.35 (95% CI: 2.88, 6.57) $P \leq 0.0001$], [HR = 3.95 (95% CI: 2.47, 6.31) $P = 0.0001$] respectively, but no significance was found if bathing was performed more than twice daily (Table 6). No significance was found in health practice behaviors such as sucking mucus from child's nose, using saliva to clean child's face, use of Ashton Powder to relieve teething pain, tooth brushing, biting of nails to shorten them, or use of Lifebuoy soap for constipation (Table 6).

Significantly higher hazard was found upon investigating the association of childhood interaction variables and child HHV-8 seroconversion, including number of playmates ($P < 0.0001$), and if the number of playmates were under the age of 5 ($P < 0.0001$). Index child spending the night at someone else's house within the last 6 months, and the index child has visiting children spending the night at their house in the last six months [HR = 4.35 (95% CI: 2.88, 6.57) $P \leq 0.0001$], [HR = 3.95 (95% CI: 2.47, 6.31) $P = 0.0001$] respectively (Table 7) had significantly higher hazard of seroconversion.

However, none of these variables continued to be significant in the multivariable analysis.

In multivariable analysis, independent associations for childhood HHV-8 acquisition included child age at seroconversion ($P < 0.0001$), child age at enrollment [HR = 2.62 (95% CI:1.64, 4.21) $P < 0.0001$], child currently being breastfed [HR = 2.13 (95% CI:1.31, 3.44) $P = 0.002$], use of pacifier [HR = 9.00 (95% CI:1.65, 49.19) $P = 0.01$], number of daily full-body baths for index child ($P = 0.0006$), primary caregiver using saliva to clean child's face, [HR = 38.69 (95% CI:4.50, 332.75) $P = 0.0009$], and other individuals blowing on food prior to sharing with child [HR = 2.79 (95% CI:1.01, 7.76) $P = 0.05$]. Interestingly, although child HIV-1 positive status was not found to be significant in the univariable analysis, it became significant when building the multivariable model [HR = 2.26 (95% CI:1.19, 4.30) $P = 0.013$].

DISCUSSION

Childhood acquisition of HHV-8 in sub-Saharan Africa occurs at an early age. We had previously reported in an earlier study of Zambian mother and infant pairs, incidence rates of 13.8 per 100 child-years in children by 48 months of age [3]. Our current findings of 29.94 per 100 child-years are more than twice as high as our earlier study findings. This may be explained by the change in HHV-8 serodiagnostic assay used. For this study we performed IFAs with BC-3 cells, a cell line naturally infected with HHV-8. Our earlier study utilized a second confirmatory antibody detection assay developed from Sf9 insect cells, expressing 3 key immunogenic recombinant proteins [15]. The increased repertoire of antigenic proteins expressed by naturally infected cell lines increases the sensitivity of the assay, reportedly between 97% to 100% [16] explaining the differences between our findings. The increased sensitivity can result in a loss of specificity, however the overall seroprevalence of our current cohort by the end of the study is 50.7%, which is comparable to 20-60% HHV-8 seroprevalence rates in young children as reported by other African population studies [10, 17, 18].

Key significant findings in this study include age at seroconversion, significant in the univariable analysis ($P < 0.00001$), and the most significant variable in the multivariable analysis ($P < 0.00001$). A decreased risk as the child becomes older was noted when comparing $>24M$ to $\leq 36M$ and $<24M$ [HR = 0.016 (95% CI: 0.09, 0.28) $P < 0.001$], and an even lower risk when comparing $>36M$ [HR = 0.006 (95% CI: 0.002, 0.016) $P < 0.001$] (Table 8). These findings along with our total cohort incidence rates support that child seroconversion happens very early in age, often before 24 months of age.

Findings of current breastfeeding as a risk for HHV-8 seroconversion were unexpected, given previous study findings of a lack of HHV-8 DNA in breastmilk [6]. However, the well documented transmission of herpesvirus family member cytomegalovirus in breastmilk, and the reports of other labs isolation of HHV-8 DNA in breastmilk makes transmission a possibility [5]. These findings are in contrast to our findings reported in chapter 3, in which breastfeeding offered a protection against childhood HHV-8 infection at cohort enrollment. Several factors could affect these results, such as duration of breastfeeding, whether saliva was used to moisten the nipples, and age of starting solid foods that this study did not thoroughly investigate. These conflicting results warrant further studies, including further behavioral assessments and possibly the investigation of neutralizing antibodies in breastmilk.

Childhood use of a pacifier was significant in univariable analysis, and remained significant in the multivariable analysis. It is unlikely that the use of the pacifier itself provides a risk for childhood HHV-8 acquisition, rather the care and cleaning of the pacifier, and the sharing of the pacifier between children. This is supported by the strong significance of sharing of food, candy, sweets and drinks behaviors with child HHV-8 seroconversion (Table 5). Interestingly, although behaviors performed by the primary caregiver were significant contributors to risk of childhood HHV-8 seroconversion, several variables associated with risk were performed by other household members, including other household children. Although interactions with other young children did not remain significant in the multivariable analysis, the importance of other HHV-8 positive household members' contribution to HHV-8 transmission may be influenced by frequency of sharing, and possibly with other young children due to the

nature of close personal interactions among children. The significance of transmission among household members has been confirmed by a study completed in our lab utilizing molecular analysis of the K1 gene sequence data of HHV-8 positive individuals from nine households in our cohort [19]. Olp et al found that in six of the nine households, the child had 100% sequence identify to all household members, supporting that intra-household transmission occurs [19].

The most significant adult feeding behavior remaining in the multivariable model was other household members blowing on food prior to sharing with the child. It is likely that this variable is not the only behavior contributing to the risk of childhood HHV-8 seroconversion, however confounding among feeding variables and sharing of food, eliminated many of the significant variables from the univariable analysis in the multivariable model. A wide confidence interval was noted when analyzing using saliva to clean child's face, which likely reflects the very small numbers of primary caregivers that responded to participating in this behavior.

In the multivariable model as well is the number of daily full-body baths for the index child. These results suggest that the risk is greater than 2x more for a child that has baths more than twice daily [HR = 2.18 (95% CI: 1.40, 3.38) $P = 0.005$]. This result could reflect bathing water quality, or shared water. Since a majority of these households do not have water sources on their property, water must be hauled into their households on a regular basis from a community water source. Bathing water is likely shared among family members, with the possibility of saliva from family members contaminating the water. However, there is no noted significance when the full body bathing occurs twice or more times daily [HR = 0.94 (95% CI: 0.54, 1.62) $P = 0.8102$]. These findings lend

doubt to the overall significance of the practice of full-body bathing, most likely making the significance a spurious finding.

The strengths of this study were the size of the cohort and the prospective nature of the study. To our knowledge, this is the first prospective study to examine children during such early childhood years for the acquisition of HHV-8. A similar study by Butler et al. demonstrated a significant association with HHV-8 children <14 years of age and the sharing of a sauce plate with other household members [11]. Our detailed questionnaire enabled us to evaluate whether potential exposures were coming from the primary caregivers, other family members within the household, or outside of the household, as well as to assess other food sharing variables such as sweets and drinks. Butler et al. did not find significance in premastication of food to children and HHV-8 [11]. However their cohort evaluated children of an older age, up to age 14. Although in our study premastication did not remain significant in multivariable analysis, we did find a 3x greater risk for a child to seroconvert to be HHV-8 positive if the caregiver pre-masticates food prior to sharing with the child when performing the univariable analysis. Our unique findings could be due to our investigation of young children. Premastication is an uncommon practice in which caregivers chew up food to a consistency soft enough for a child just starting to eat solid foods can swallow likely practiced between the ages of 6-12 months. Although premastication was not found to be frequently performed in our cohort, the amount of saliva that would be needed to soften food could pose a risk to the very young child.

The weakness of the study was the self-reported nature of the data from the behavioral questions. We believe that the potential for recall bias was limited because

the caregivers were not made aware of their HHV-8 status at the time of enrollment and counseling and treatment was done regarding transmission of HIV-1 to all study participants.

In summary, we have demonstrated that behaviors performed in the household that could potentially share saliva play a key role in transmission of HHV-8 virus to a susceptible child. Of particular interest are feeding behaviors practiced by adults while feeding the index child, such as testing the temperature of food with the tongue prior to feeding to a child. This finding supports a public health risk and the need for education of families in endemic areas such as Zambia risks involved with sharing food with young children, and needed behavior changes.

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TABLES AND FIGURES

Table 1. Demographics, HIV-1 and HHV-8 Characteristics of HHV-8 Seroconverters and Non-seroconverting Children in Lusaka, Zambia, 2004-2009.

Characteristic	HHV-8 seroconverter (n=137)	Non- seroconverter (n=133)
Demographics		
Enrollment age of index child (months):		
Range	2-25	2-29
Mean	12.4	14.2
Sex of index child:		
Males	65	76
Females	72	57
Number of household members:		
Range	2-8	2-10
Mean	5.4	4.9
Education of primary caregiver:		
None	15	11
Primary school	73	78
Secondary school	49	44
HIV-1 serology at enrollment		
Index child HIV-1+	11/137 (8.0%)	12/133 (9.0%)
Primary caregiver HIV-1+	56/137 (40.9%)	65/133 (48.9%)
≥ 1 other household member HIV-1+	69/137 (50.4%)	71/133 (53.4%)
HHV-8 serology at enrollment		
Primary caregiver HHV-8+	66/135 ^a (48.9%)	47/129 ^a (36.4%)
≥ 1 other household member HHV-8+ (other than primary caregiver)	94/137 (70.8%)	83/133 (62.4%)
Number of HHV-8+ household members:		
Range	0-7	0-4
Mean	1.8	1.4

^a Numbers do not match total due to discrepant or unavailable serology at enrollment

Table 2. Seroincidence rates Child HHV-8 Seroconversion in Lusaka, Zambia, 2004-2009.

Characteristic	Number of Children	Number Seroconverted	KSHV free child-years	Incidence (per 100 child-years)
Total	270	137	457.57	29.94
Gender				
Male	141	65	255.50	25.44
Female	129	72	202.06	35.63
Child HIV Status				
Positive	23	11	37.69	29.19
Negative	247	126	419.88	30.01
Caregiver HIV Status (total cohort)				
Positive	121	56	221.54	25.28
Negative	149	81	236.03	34.32
Caregiver HIV Status (HIV negative child)				
Positive	98	45	183.85	24.48
Negative	149	81	236.03	34.32

Table 3. Univariable Analysis to Investigate the Association of Gender, Age, Caregiver and Household Characteristics with Child HHV-8 Seroconversion in Lusaka, Zambia, 2004-2009.

Characteristic	Hazard Ratio (95% CI)	P-value
<i>Child specific characteristics</i>		
Gender of Index Child (Reference: Females)	0.75 (0.53 - 1.04)	0.09
Age at enrollment (Ref: <12M)	0.65 (0.46 - 0.92)	0.01*
Age at Seroconversion:		<0.0001*
<24M	Reference	
>24M to ≤36M	0.23 (0.14 - 0.37)	<0.001
>36M	0.02 (0.007 - 0.03)	<0.001
<i>Caregiver and household member specific characteristics</i>		
Primary caregiver education:		0.95
No education	Reference	
Junior Primary	1.10 (0.55 - 2.26)	0.78
Upper Primary	1.06 (0.62 - 1.81)	0.85
Junior Secondary	0.90 (0.51 - 1.61)	0.73
Upper Secondary	1.09 (0.52 - 2.28)	0.82
Caregiver HIV status(enrollment) (Ref: Negative)	0.74 (0.53 - 1.05)	0.09
Child HIV status (Ref: Negative)	0.97 (0.52 - 1.79)	0.91
Any household member HIV status (Ref: Negative)	0.81 (0.58 - 1.14)	0.23
Caregiver HHV-8 status(enrollment) (Ref: Negative)	1.38 (0.98 - 1.94)	0.06
Any other household member HHV-8 status (Ref: Negative)	1.09 (0.76 – 1.57)	0.63
Increasing number of HHV-8 positive household members	1.2 (1.06 – 1.39)	0.005*

^a Significant at α level of 0.05

Table 4. Univariable Analysis to Investigate the Association of Breastfeeding Practices, Premastication, and Adult Feeding Behavioral Habits with Child HHV-8 Seroconversion in Lusaka, Zambia, 2004-2009.

Characteristic	Hazard Ratio (95% CI)	P-value
<i>Breastfeeding practices</i>		
Child breastfed in the past, currently weaned	1.24 (0.79 - 1.95)	0.34
Child is currently being breastfed	6.26 (4.02 - 9.75)	<0.0001*
<i>Premastication</i>		
Caregiver premasticates food prior to sharing with children	3.15 (1.00 - 9.98)	0.05*
Other household members perform the premastication	3.15 (0.44 - 22.71)	0.25
<i>Adult feeding variables</i>		
Primary caregiver testing temp of food with tongue prior to sharing w/ child	3.39 (1.75 - 6.60)	0.0003*
Other household members testing temp of food with tongue	5.52 (2.00 - 15.20)	0.0009*
Primary caregiver blowing on food prior to sharing w/ child	4.13 (2.37 - 7.19)	<0.0001*
Other individuals blowing on food prior to sharing w/ child	4.45 (1.92 - 10.29)	0.0005*
Eating of food with hands from a common bowl	4.44 (2.05 - 9.60)	0.0002*
Primary caregiver sharing of eating utensils w/ child	4.28 (2.07 - 8.85)	<0.0001*
Other household members sharing of utensils w/ child	3.53 (1.54 - 8.12)	0.003*

^a Significant at α level of 0.05

Table 5. Univariable Analysis to Investigate the Association of Child Behaviors of Sharing Sweets and Drinks with Child HHV-8 Seroconversion in Lusaka, Zambia, 2004-2009.

Characteristic	Hazard Ratio (95% CI)	P-value
<i>Child sharing variables</i>		
Primary caregiver sucking on sweets prior to sharing w/ child	2.06 (0.90 - 4.74)	0.09
Other household members sucking on sweets prior to sharing w/ child	2.87 (1.38 - 5.97)	0.005*
Other non-household members sucking on sweets prior to sharing w/ child	3.54 (1.43 - 8.74)	0.006*
Index child shares food, candy or other sweets with other household children	2.97 (1.57 - 5.61)	0.0008*
Index child shares food, candy or other sweets with other neighborhood children	3.06 (1.12 - 8.37)	0.029*
Exchange of food, candy or other sweets from another child's mouth to Index child	2.54 (1.27 - 5.10)	0.008*
Primary caregiver sharing of packaged drinks w/ child	3.36 (1.54 - 7.33)	0.002*
Other household members sharing of packaged drinks w/ child	4.87 (2.32 - 10.21)	<0.0001*
Other non-household members sharing of packaged drinks w/ child	7.44 (2.68 - 20.69)	0.0001*
Neighborhood children sharing of packaged drinks w/ child	5.36 (1.95 - 14.75)	0.001*
Use of pacifier	17.62 (4.18 - 74.35)	<0.0001*

^a Significant at α level of 0.05

Table 7. Univariable Analysis to Investigate the Association of Hygiene and Health Practices with Child HHV-8 Seroconversion in Lusaka, Zambia, 2004-2009.

Characteristic	Hazard Ratio (95% CI)	P-value
<i>Hygiene practices</i>		
Number of daily full-body baths for index child:		<0.0001*
Once per day	Reference	
Twice per day	4.35 (2.88 - 6.57)	<0.0001*
Twice or more daily	0.83 (0.50 - 1.39)	0.48
Number of daily face cleanings for index child:		<0.0001*
Once per day	Reference	
Twice per day	3.95 (2.47 - 6.31)	<0.0001*
Twice or more daily	0.54 (0.32 - 0.90)	0.02
<i>Health practices</i>		
Primary caregiver sucking mucus from child's nose	3.95 (2.47 - 6.31)	0.58
Primary caregiver used saliva to clean child's face	0.54 (0.32 - 0.90)	0.41
Use of Ashton Powder to relieve teething pain	1.72 (0.24 - 12.39)	0.06
Use of toothbrushing to clean teeth	2.30 (0.32 - 16.50)	0.37
Primary caregiver biting the child's nails to shorten them	3.91 (0.94 - 16.18)	0.45
Other household members biting the child's nails to shorten them	0.63 (0.23 - 1.71)	0.16
Lifebuoy soap for constipation	1.72 (0.42 - 6.98)	0.20

^a Significant at α level of 0.05

Table 8. Univariable Analysis to Investigate the Association of Childhood Interaction Practices with Child HHV-8 Seroconversion in Lusaka, Zambia, 2004-2009.

Characteristic	Hazard Ratio (95% CI)	P-value
<i>Childhood interaction variables</i>		
Number of playmates:		<0.0001*
None	2.50 (0.61 - 10.20)	
2-3	1.41 (0.75 - 2.68)	0.29
>3	0.61 (0.33 - 1.12)	0.11
Don't know	8.33 (1.05 - 65.93)	0.04
Number of playmates under the age of 5		<0.0001*
None	Reference	
2-3	1.16 (0.64 - 2.11)	0.63
>3	0.52 (0.29 - 0.92)	0.02
Don't know	7.14 (0.91 - 55.81)	0.06
Index child has spent the night at someone else's house within the last 6 months	4.25 (1.55 - 11.63)	0.005*
Visiting child has spent the night at Index child's house within the last 6 months	7.18 (1.74 - 29.58)	0.006*

^a Significant at α level of 0.05

Table 9. Multivariable Analysis to Investigate Independently Associated Risk Factors Associated with HHV-8 Seroconversion in Children in Lusaka, Zambia, 2004-2009.

Characteristic	Hazard Ratio (95% CI)	P-value
Age at Seroconversion:		<0.0001
<24M	Reference	
>24M to ≤36M	0.16 (0.09 - 0.28)	<0.001
>36M	0.006 (0.002 - 0.016)	<0.001
Age at enrollment (Ref: <12M)	2.62 (1.64 - 4.21)	<0.001
Child is currently being breastfed	2.13 (1.31 - 3.44)	0.002
Use of pacifier	9.00 (1.65 - 49.19)	0.01
Number of daily full-body baths for index child:		0.0006
Once per day	Reference	
Twice per day	2.18 (1.40 - 3.38)	0.0005
Twice or more daily	0.94 (0.54 - 1.62)	0.8102
Primary caregiver used saliva to clean child's face	38.69 (4.50 - 332.75)	0.0009
Child HIV status (Ref: Negative)	2.26 (1.19 - 4.30)	0.013
Other individuals blowing on food prior to sharing w/child	2.79 (1.01 - 7.76)	0.05

^a Significant at α level of 0.05

FIGURE LEGEND

Figure 1.

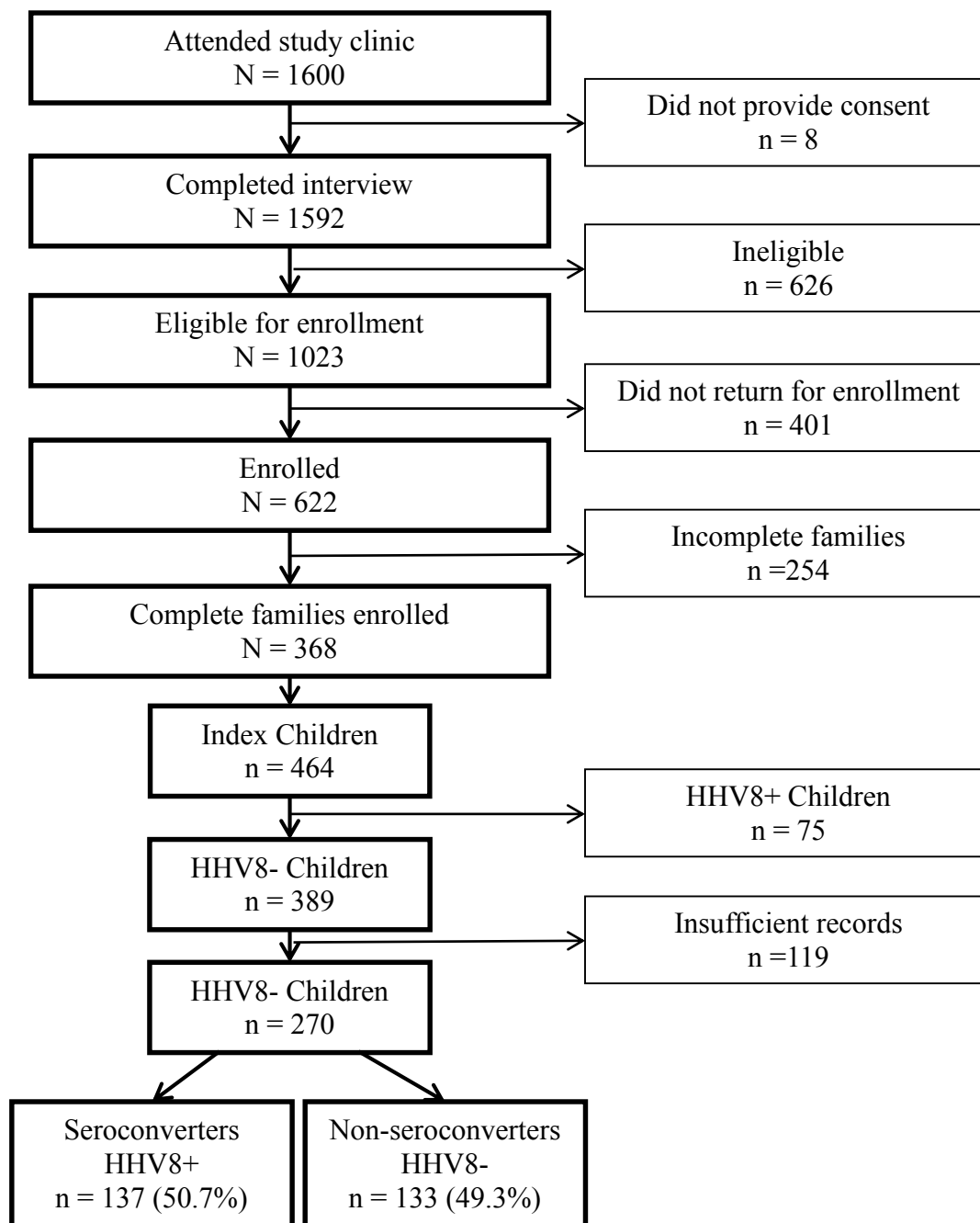


Figure 1. Flow Chart outlining the screening and recruitment of study cohort in Lusaka, Zambia, 2004-2009.

Figure 2.

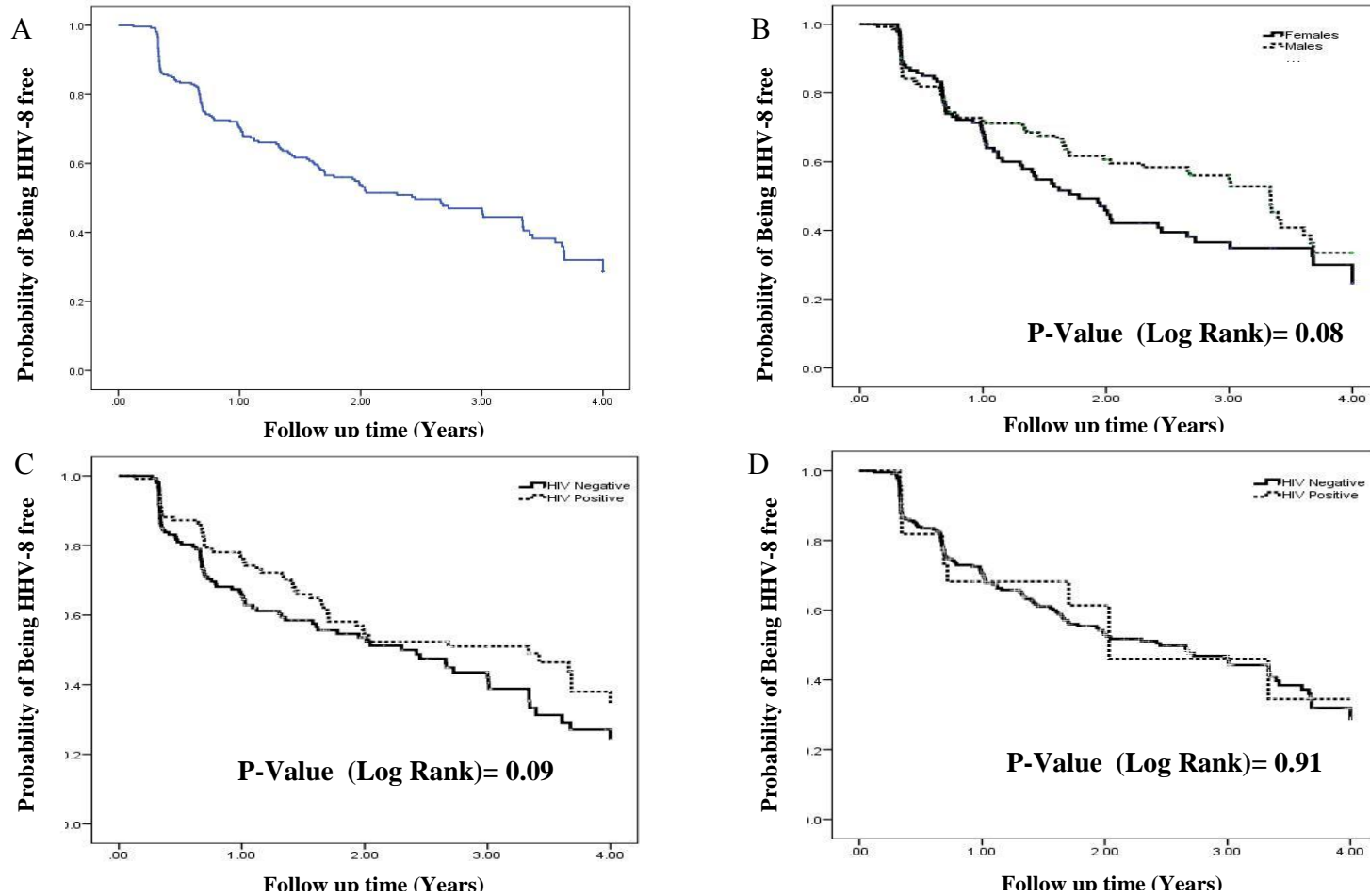


Figure 2. Results from Kaplan-Meier survival analysis estimating the probability of a child seroconverting to become positive for HHV-8 in Lusaka, Zambia, 2004-2009. Results are represented by the total cohort (n=270) (panel A); males vs. females (panel B); human immunodeficiency virus type 1 (HIV-1) infection status of the mother at enrollment (panel C); HIV-1 infection status of the child at time of seroconversion (panel D).

**CHAPTER 5: DEVELOPMENT OF AN IMMUNOFLUORESCENT
ASSAY USING RECOMBINANT PROTEINS EXPRESSED IN
INSECT CELLS FOR THE SCREENING AND CONFIRMATION OF
HUMAN HERPESVIRUS 8 ANTIBODIES**

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ABSTRACT

Human herpesvirus-8 (HHV-8) or Kaposi's sarcoma-associated herpesvirus (KSHV) has been linked to all forms of Kaposi's sarcoma (KS). Most current serological assays to detect HHV-8 antibodies have low concordance amongst themselves. To establish a sensitive and specific testing strategy to screen for HHV-8 antibodies, three HHV-8 proteins, ORF65, ORF73 and K8.1A, were expressed using baculoviral vectors in insect cells and incorporated into an monoclonal-enhanced immunofluorescence assay (mIFA) termed Sf9 3-antigen mIFA. The results with this monoclonal-enhanced mIFA were compared to those obtained with a standard mIFA utilizing a HHV-8 infected B-cell line (BC3 mIFA). Test sera were obtained from patients with diagnosed KS, HIV-1 infected patients at high risk for HHV-8 infection, and healthy controls from a local blood bank. Combined use of both assays together had a sensitivity of 94% and a specificity of 96%. The performance of these two assays when used together indicates that they may be useful for reliable detection of HHV-8 IgG antibodies in a population.

INTRODUCTION

Human herpesvirus 8 (HHV-8), also known as Kaposi's sarcoma-associated herpesvirus (KSHV), is the latest human herpesvirus identified. It has been associated with all four clinical presentations of KS (classic, endemic, AIDS related and the iatrogenic form) Kaposi's sarcoma (KS) [6, 15]. HHV-8 has also been detected in primary effusion B-cell lymphomas (PEL) and in multicentric Castleman's disease (MCD) [4, 30].

In the general population, HHV-8 seroprevalence shows marked geographical variations. HHV-8 infection is endemic in Africa and the Mediterranean region and in non-endemic areas is higher in homosexual men and immunosuppressed individuals [8, 9, 13, 24]. Routes of transmission are still not well understood but both horizontal and vertical transmission are possible [2, 10, 21]. Horizontal transmission can occur by sexual and non-sexual routes. HHV-8 seroconversion is observed during adulthood in most developed countries, most likely due to sexual transmission, and occurs in childhood in endemic areas, most likely due to non-sexual horizontal transmission. HHV-8 DNA has been detected in saliva making it to be a potential source of transmission via close interpersonal contact [1-3, 9, 18, 22, 31]. HHV-8 DNA cannot be detected in all infected individuals therefore; serology is the method of choice in epidemiological studies to screen for infected individuals.

Development of high performance serologic tests has been achieved to a limited degree only due to an incomplete understanding of the known immunodominant proteins, lack of well characterized uninfected and infected individuals that serve as controls and reported wide variations in antibody titers among infected individuals. While various

serological assays have been shown to have variable performance and concordance, immunofluorescence assays have been considered as one of the most sensitive assays for detecting antibodies against HHV-8 [11, 23, 27]. IFA was one of the first assays to be used for the detection of HHV-8 antibodies [20]. Cell lines derived from PEL and chronically infected with HHV-8, expressing mainly latent and a low level of lytic antigens have been used for latent or lytic IFAs. The level of lytic antigens can be increased by induction with tetradecanoyl phorbol acetate (TPA). Using sera of KS patients, several proteins have been identified as highly reactive antigens. These include open reading frames (ORF) 6, 8, 9, 25, 26, 39, 59, 65, 68, 73, K8.1A and K8.1B [5]. Of these proteins, ORF 59, K8.1A, ORF65, and ORF73 have been used in the development of various enzyme immunoassays (EIA) and reported to be good candidate antigens [5, 14, 16, 19, 28, 32]. There are now two commercially available EIAs using whole virus lysate and synthetic peptides.

Here we report the use of a screening strategy for detecting HHV-8 antibodies in plasma samples. IFA utilizing Sf9 cells expressing predominant proteins encoded by HHV-8 (ORF65, ORF73, K8.1) was used in conjunction with IFA utilizing stimulated BC3 cells to obtain a sensitive and specific testing strategy.

MATERIALS AND METHODS

Cell culture: BC-3 cells (ATCC) were grown in RPMI 1640 supplemented with 20% fetal calf serum, L-Glutamine, sodium pyruvate, HEPES and D-glucose. Sf9 insect cells were maintained as suspension culture in SF 900 II medium (Invitrogen, CA) supplemented with 20% fetal calf serum and 1% gentamicin.

Patient sera: A total of 219 samples were used in this study. Of these, 108 samples were collected from patients visiting the Adult Oncology unit at the University of Miami Miller School of Medicine. Two KS plasma samples were collected from the University Teaching Hospital, Lusaka, Zambia as a part of an ongoing study to investigate HHV-8 transmission within families. Blood banks at Lincoln, Nebraska and Kansas City, Kansas contributed 109 plasma samples. Ethics committee of the Institutional Review Board at the University of Nebraska approved the study. All samples were coded and screened without knowledge of identity of the patient or the diagnosis. Subsequently, patient serum samples were divided into 3 groups. Group A (positive group) consisted of a total of 33 samples collected from histologically identified KS patients. This group also included samples from one PEL and one MCD patient. Group B (high risk group) consisted of 77 samples collected from HIV-1 positive patients with other cancers (not KS and PEL). Group C (negative group) consisted of 109 samples collected from healthy blood bank donors with low risk life style behaviors.

Preparation of BC-3 slides: BC3 cells at a concentration of 7×10^5 per ml were treated with tetradecanoyl phorbol acetate (TPA) at a final concentration of 20 ng/ml for 72 hrs. The cells were fixed in 4% paraformaldehyde for 20 min at room temperature, washed with PBS and permeabilized with 0.1% Triton X 100 for 15 minutes at room temperature.

The cells were washed and resuspended in PBS. Approximately 10^4 cells were spotted per well onto 12 well teflon coated slides (Electron Microscopy sciences, PA) and stored at $-80\text{ }^{\circ}\text{C}$.

Preparation of Sf9 cell slides expressing HHV-8 antigens: Recombinant baculoviruses expressing glutathione S-transferase (GST) tagged lytic proteins, ORF65 and K8.1A, and latent protein, ORF73 (provided by Dr Bala Chandran, Rosalind Franklin University of Medicine and Science, Chicago), were used to develop the Sf9-mIFAs. Baculovirus-infected Sf9 cells expressing GST alone were used as a negative control to detect background and nonspecific fluorescence. All baculovirus stocks were titrated and infections were initiated separately with the three baculovirus stocks each of which expressing one recombinant HHV-8 protein at a MOI of 10. Infected cells were monitored daily for viability and cell diameter using Vi-Cell counter (Beckman-Coulter, CA). The expression of each protein was evaluated by Western blot analysis with anti-GST antibody (Santa Cruz Biotechnology, CA) following SDS-PAGE (Figure 1). At 72 hours post-infection (hpi) cells were harvested, mixed in equal ratio (1:1:1) of viable cells and subsequently fixed using the BC3 cell method. This was called as the ‘Sf9 3-antigen’ test. All fixed slides were stored at $-80\text{ }^{\circ}\text{C}$.

A modification of the method was also used. At 72 hpi, Sf9 cells infected with baculovirus expressing ORF73, ORF65 or K8.1 antigens were harvested, fixed and spotted individually on separate slides. This method was used to screen patients for the presence of latent or lytic antibodies and is referred to as ‘single-antigen Sf9’ assay

Monoclonal-enhanced immunofluorescence assay (mIFAs): All serum samples were diluted 1:40 and centrifuged at high speed for one minute immediately before being used.

All slides were warmed to room temperature, individual serum samples were applied to each well and the slides were incubated at 37 °C for 30 minutes in a humidified chamber. The slides were washed (6 times) with PBS and then incubated with mouse anti-human IgG monoclonal antibody at 37 °C for 30 minutes. The slides were washed with PBS again and then incubated with goat anti-mouse Cy2 conjugated antibody (Jackson Laboratories, City) at 37 °C for 30 minutes. After washing, the cells were stained with 0.004% Evan's blue for 5 minutes, washed and mounted. The procedure of mIFA was the same for BC3 and Sf9 slides.

Criteria for being HHV-8 seropositive: All slides were read by two independent readers without knowledge of patient identity, clinical diagnosis, HIV-1 status or the other reader's results. To reduce subjectivity in observing specific fluorescence, slides were read independently by two experienced laboratory workers. A sample was considered positive if specific fluorescence was observed by both readers. In case of discordant results the assay was repeated. On repetition if discordant results were again obtained then these patients were considered seronegative. All samples determined to be positive by BC-3 mIFA and the Sf9 3-antigen assay were considered positive. If a patient was positive on just one assay or negative by both assays, it was considered as HHV-8 negative. This testing scheme is summarized in Figure 2.

Statistical analysis: All data was entered and analyzed using SPSS (v15). Kappa statistic was computed to determine the concordance between the standard BC3 mIFA and the Sf9 mIFA. Sensitivity of detection of HHV-8 antibodies was calculated as: True positives / (True positives + False Negatives). All serum samples collected from reliably diagnosed cases of KS, PEL and MCD and found serologically positive by both assays were

considered as true positives. Results were considered false negative if serum samples collected from these patients were found to be negative by our criteria.

Specificity was calculated as $\text{True Negatives}/(\text{True Negatives} + \text{False Positives})$. All serum samples collected from healthy blood donors and found serologically negative by either assay were considered as true negatives. Results were considered false positive if samples collected from these patients were found to be positive.

RESULTS

HHV-8 seroprevalence

All serum samples were assigned a unique identification number and were screened blinded using both mIFAs. Representative mIFA images of a positive and negative patient are shown in Figure 3. After serological screening was completed, the specimens were divided into 3 groups based on their clinical diagnosis as described in Materials and Methods section (Table 1). Using the strategy described in Figure 2, in Group A (KS/PEL/MCD) the overall seroprevalence was 93.9%. Two samples were considered seronegative, including one sample which tested positive by BC3 mIFA but negative by Sf9 3-antigen mIFA and a second sample that was not positive by either of the two assays. In Group B, (high risk group) the seroprevalence was 58.4 % with 45 out of 77 samples being positive by both assays. The remaining 32 samples were considered seronegative. Fifteen out of these 32 seronegative samples (19.5%) were negative by both assays. There were 9 (11.7%) and 8 (10.4%) samples that were positive by BC3 and Sf9 3-antigen assay alone, respectively. In Group C (blood bank donors) both assays detected HHV-8 antibodies in only 4 (3.6%) of the patients. The remaining 105 samples were considered seronegative. Of these 105 seronegative samples, 2 (1.8%) and 7 (6.4%) were positive by BC3 and Sf9 3-antigen assay alone, respectively.

Latent and lytic antibody profiles

All samples that were positive on Sf9 3-antigen assay were screened using the single-antigen Sf9 method to ascertain the latent and lytic antibody profiles present in different groups. Also, we compared the antibody profiles of those samples that were

positive with both BC3 mIFA and Sf9 3-antigen assays to samples that were BC3 mIFA negative and Sf9 3-antigen positive (Table 2).

BC3 and 3 antigen positive samples - When mIFAs were performed using ORF65, K8.1 and ORF73 antigens separately, 74% (23/31) of Sf9 3-antigen and BC3 mIFA positive samples in Group A reacted to both latent (ORF73) and lytic (ORF65 or K8.1) antigens. Two samples had detectable antibodies against latent antigen only and 6 samples reacted to at least one lytic antigen. In Group B, 64% (29/45) of seropositive samples reacted to both latent and lytic antigens. There were 8 samples each that reacted only to either latent or lytic antigen. In Group C, there was only 1 out of 4 seropositive samples that was positive for both latent and lytic antigens. There were 3 samples that were positive for lytic antigens only.

BC3 negative and 3 antigen positive samples - We also wanted to compare the above antibody profiles of patients who were negative by BC3 mIFA. We did not find any such sample in group A but there were 6 of those samples in Group B. All these 6 samples were negative by BC3 mIFA and had detectable titers to both latent and lytic antigens as observed by using the 3-antigen assay. In the same Group there was one sample each that was positive for latent or lytic antigens only. In Group C individuals, we observed that there was only one sample that was positive for both latent and lytic antigens, but 6 samples that reacted positively to lytic antigens.

Concordance, assay sensitivity and specificity

Kappa value (κ -value) denoting the concordance of BC3 mIFA and Sf-9 3-antigen assay was calculated. The overall κ -value of all the 219 samples was 0.75. The sensitivity of the screening strategy was then evaluated by using group A (positive group) samples

and calculated as described in the Materials and Methods section. Sensitivity of detecting positive samples by this combined strategy was 93.9%. The specificity was calculated by using group D (blood bank donors) samples. This yielded a specificity of 96.3%.

DISCUSSION

A large proportion of infected but asymptomatic individuals do not have detectable viral DNA in peripheral blood therefore, thus serology is a more reliable method to identify infection. But a major hurdle in obtaining clear seroprevalence data in a population, understanding the route of transmission and routine monitoring of ‘at risk’ individuals, is the lack of a reliable assay that can detect antibodies in human sera.

Most laboratories use ‘in house’ assays with varying levels of sensitivity and specificity and concordance for screening of HHV-8 antibodies [23, 26, 27]. Currently no assay is clearly superior in terms of sensitivity and specificity. Our goal was to develop a sensitive and specific Sf9 3-antigen IFA that is cost effective and can be used as a confirmatory test to validate IFA results using HHV-8 infected cells. In this report both BC3 assay and Sf9 3-antigen assay were used in parallel to investigate the concordance of the two assays. Given the findings in this report, the Sf9 3-antigen assay can be used in tandem as a confirmatory assay with BC3 assay, for screening for HHV-8 antibodies. To evaluate the sensitivity and specificity of this testing strategy, we used samples from well characterized patients. Both BC3 and Sf9 3-antigen mIFAs are designed to detect HHV-8 specific IgG antibodies against latent and lytic antigens. ORF73 is the major latent protein and encodes for latency associated nuclear antigen (LANA). ORF65 is a lytic phase protein and encodes for minor capsid antigen. ORF65 has been reported to be one of the immunodominant antigens that can be used for a sensitive serological assay [17]. Sf9 3-antigen assay is flexible because it can be expanded to incorporate more antigens if needed. Overexpression of the immunodominant proteins also helps to easily identify patients having very low titer of HHV-8 antibodies. In Sf9 cells, each antigen can be

expressed individually to monitor antibody responses to latent and lytic antigens separately. More importantly, the use of a GST-expressing Sf9 cells served as bonafide negative controls that were used to check for non-specific binding and fluorescence. Lack of matched negative control cells is one of the major drawbacks of assays based on the use of BC3 cells. Background or non-specific fluorescence which is commonly found in immunofluorescence assays cannot be ruled out when using BC3 or other HHV-8 harboring cell lines. Populations which have been shown to produce this nonspecific reactivity include serum samples from individuals with a high number of sexual partners, patients with parasitic infections and among people exposed to a high number of pathogens. Residue, sediments or high lipid content can also cause high background and was controlled in our assay by centrifugation of each sample immediately before being used [7].

Our criterion to consider a patient as positive in our study was based on two immunofluorescence assays. A patient was considered positive if the plasma sample gave specific fluorescence at a dilution of 1:40 by both IFAs. While this strategy increases the specificity of detecting HHV-8 antibodies it may decrease the sensitivity of detection. By using this testing strategy we could detect HHV-8 antibodies in 94% of clinically diagnosed cases of KS, PEL and MCD. In the positive patients with KS there was one patient that was negative by both BC3 and Sf9 IFAs. This patient has undergone antiretroviral treatment after the development of KS, which could have suppressed the viral load and led to complete seroreversion for HHV-8 antibodies by then. Seroreversion has in fact been documented in patients after treatment and regression of symptoms [25]. CD4+ cell count data was not available to help gauge the level of immunosuppression

which may also help explain the unexpected HHV-8 seronegativity. It is possible that the sensitivity of both the assays was too low to detect the very low antibody titer in this patient. One sample in the positive group was positive by BC3 mIFA but negative by Sf9 mIFA. This could be non-specific fluorescence shown by BC3 mIFA and cannot be reliably confirmed because of lack of matched negative cell line. It is also likely that for this patient none of the three antigens that were expressed in Sf9 cells were eliciting an immune response and further underscores the importance of using multiple antigens for screening. This suggests that there is a need for identification and incorporation of more antigens for routine screening of patients.

We had chosen the blood bank donor group as our truly negative controls because they are selected for minimal behavioral risk. Using this testing scheme, we found a seroprevalence of 3.7% in this group. This is not surprising because a low seroprevalence of about 3% has been reported in United States blood donors [23]. We observed a similar seroprevalence in the blood bank donors which we had assumed to be negative. In our case it reduced the specificity (96%) of this testing strategy. The lack of a “gold standard” serological assay that can reliably identify patients that are truly infected versus those that are uninfected makes it difficult to test the accuracy of the assays currently in use.

Group B patients were included as a test group. The seroprevalence in Group B samples was within the expected range. HIV-1 infected patients from North America have been reported to have HHV-8 seroprevalence in the range of 20-50% [12, 20, 29]. We observed a similar seroprevalence rate of 58.4% in this group. The antibody profile showed that 82% of the seropositive samples had lytic antibodies which is indicative of active replication and has been shown to be a risk factor for development of KS. No

follow-up was available for these patients and we do not know if any of these developed KS.

This assay could have some other drawbacks. It is more labor intensive than EIA and reading of slides can be subjective. We tried to reduce the subjectivity effect by employing two readers who read all the slides independently. EIA's have frequently employed KS patients to obtain cutoff values for optical density. This approach might exclude asymptomatic individuals who are seropositive and frequently have very low titer of HHV-8 specific antibodies. This method has proved to be adequately sensitive in detecting HHV-8 specific antibodies from asymptomatic children and adults in Zambia; an endemic region for HHV-8 infection (manuscript submitted). In our experience most of the asymptomatic individuals in this cohort in Zambia have very low antibody titer and EIA's utilizing high cutoff values are not suitable for conducting such epidemiological studies. We believe that our stringent criteria of detection may still be underestimating the number of seropositive cases and reducing the level of sensitivity. But this scheme increases the specificity of detection of HHV-8 antibodies by excluding false positives. For this study we did not test these patient samples to other known ubiquitous herpesviruses. Our observations from other adult patient samples from similar locations have shown a very high seroprevalence.

Reliable serological assay could be a useful tool in the accurate monitoring and diagnosis of HHV-8 infection. In the absence of a gold standard this strategy has proven helpful in conducting seroepidemiological studies in an endemic area. A better understanding of HHV-8 antibody response is required to perfect the current serological testing strategy. In conclusion, we describe a new serological approach to screen patients

for the presence of HHV-8 antibodies that is sensitive and specific and reduces the chances of detecting false positives. Finally, further refinement of this approach to incorporate more antigens is ongoing.

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TABLES AND FIGURES

Table 1: Seroprevalence of HHV-8 antibodies in positive control, high risk and blood bank donors.

	Positive group	High risk group	Negative group
	n (%)	n (%)	n (%)
HHV-8 Positive			
BC3+/3-antigen+	31 (94.0)	45 (58.4)	4 (3.7)
HHV-8 Negative			
BC3+/3-antigen-	1 (3.0)	9 (11.7)	2 (1.8)
BC3-/3-antigen+	0	8 (10.4)	7 (6.4)
BC3-/3-antigen-	1 (3.0)	15 (19.5)	96 (88.1)

Note: Positive sign (+) refers to patients who tested positive and negative sign (-) refers to patients who tested negative by that assay.

Table 2: Antibody profiles of all Sf9 3-antigen positive samples, against latent (ORF73) and lytic (ORF65 or K8.1) antigens.

Latent	Lytic	Positive group		High risk group		Negative group	
		BC3+	BC3-	BC3+	BC3-	BC3+	BC3-
+	+	23	.	29	6	1	1
+	-	2	.	8	1	.	.
-	+	6	.	8	1	3	6
Total		31	0	48	8	4	7

Note: Positive sign (+) refers to patients who tested positive and negative sign (-) refers to patients who tested negative

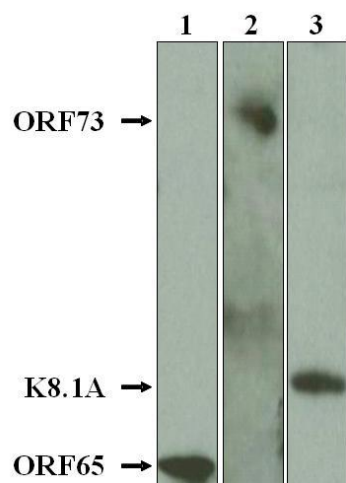
FIGURE LEGENDS**Figure 1.**

Figure 1. Western blot analyses of ORF65, ORF73 and K8.1A proteins expressed in Sf9 insect cells. Infected cells were harvested at 72 hours post-infection and lysed by sonication. Specific proteins were detected by using anti-GST antibodies: Lanes 1-3, ORF65 (48 kda), ORF73 (>150 kda) and K8.1A (52 kda), respectively. Arrows indicate the expressed proteins.

Figure 2.

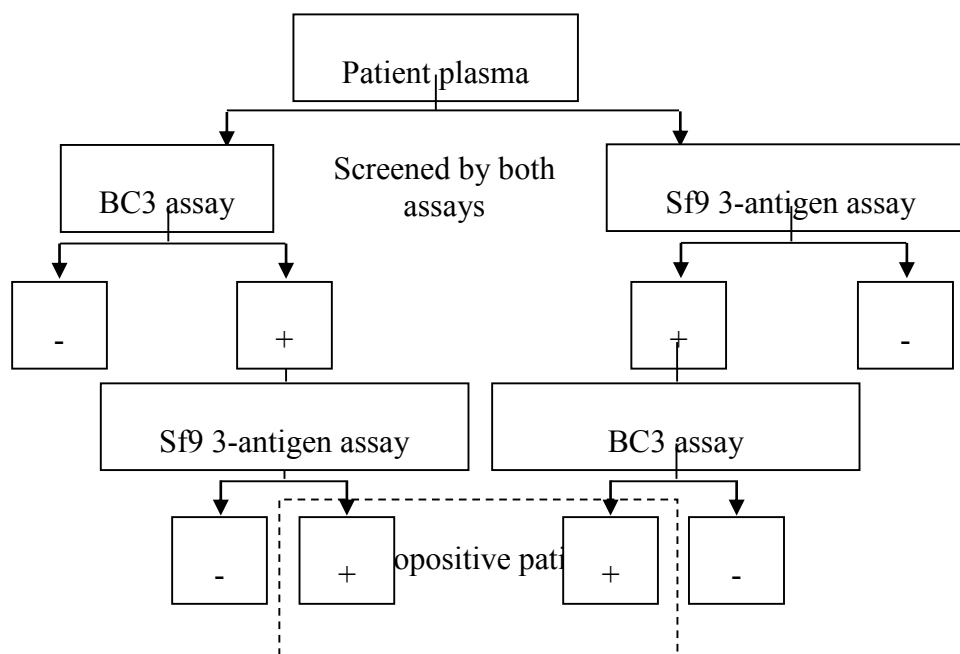


Figure 2: Scheme followed to screen patients for HHV-8 antibodies and determining their serostatus.

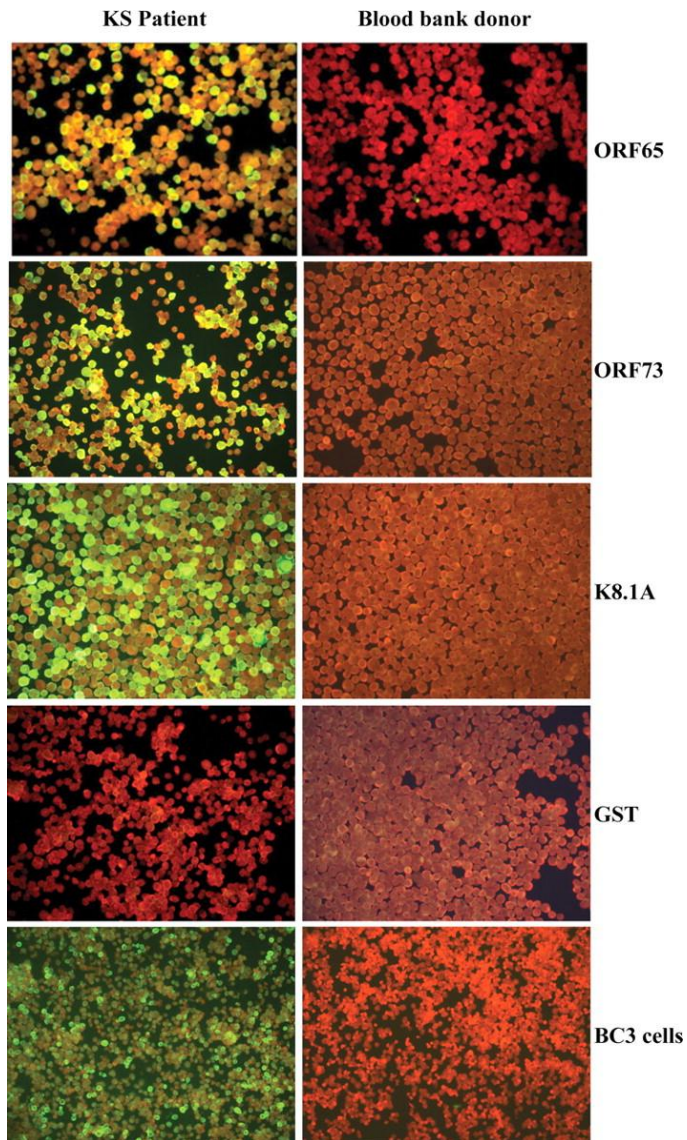
Figure 3.

Figure 3. Representative staining patterns of mIFAs of ORF65, ORF73 and K8.1A proteins expressed in Sf9 insect cells and of BC3 cells using a positive (KS) (left column) and a negative (blood bank donor) (right column) patient sera. by that assay.

CHAPTER 6: CONCLUDING REMARKS

Human herpesvirus-8 is the etiologic agent for several malignant pathologies, including body cavity-based B-cell lymphoma (BCBL), primary effusion lymphoma (PEL), multicentric Castleman's disease, and Kaposi's sarcoma (KS). The onset of the HIV epidemic in sub-Saharan Africa where HHV-8 is endemic has led to an increase in KS incidence from 3.2% to 19%, making KS the most common tumor in children in sub-Saharan Africa [1-3]. Although several epidemiologic studies have examined transmission risk factors in adults, there is a paucity of data about the transmission of HHV-8 to susceptible children.

Several studies have implicated saliva as the mode of perinatal transmission, as HHV-8 DNA is readily isolated from buccal swabs and whole saliva [4, 5]. In early childhood, close contact with caregivers, household feeding practices, health care practices, and child-rearing behaviors all are possible sources of saliva exposure. In Zambia, additional traditional and cultural practices also can increase saliva sharing [6]. Our overall hypothesis is that household behaviors that expose the child to saliva increase the risk of transmission of HHV-8 to the child. To test our hypothesis, we launched the Zambia KS-HHV-8 Study, a large prospective cohort study in Lusaka, Zambia, to follow young children and their households, investigating incidence and associated risk factors in early childhood HHV-8 infection.

In the undertaking of any large epidemiologic investigation, careful planning of study design is essential to attain reliable results. In chapter 2, the study design, the methodology in cohort development, the recruitment, screening and enrollment are

described in detail. Loss to follow-up is a great concern in large studies and was a challenge in our earlier Zambian study of mother and infant pairs [7]. To minimize this, we utilized community workers to assist in recruitment process, follow-up and participant retention. In total, 1,600 households were screened and 368 households comprised of 464 children were enrolled.

Chapter 3 examines the seropositivity of HHV-8 in children at enrollment of the cohort, and associated behavioral risks. This is a cross-sectional study of 75 children found to be HHV-8 positive and their households. Key findings were the risk for HHV-8 infection included increasing number of HHV-8 positive household members [OR 2.5 (95% CI: 1.9, 3.3) $P < 0.01$] and having a primary caregiver who tested the temperature of food with their tongue prior to feeding the child [OR 2.4 (95% CI: 1.93, 3.30) $P = 0.01$]. Breastfeeding was protective against infection with HHV-8 for children [OR 0.3 (95% CI: 0.16, 0.72) $P < 0.01$].

In chapter 4 we looked longitudinally at HHV-8 negative children, examining incidence of HHV-8 infection, and the household and behavioral risks associated. Most significant independent risk factors for HHV-8 seroconversion included age at seroconversion ($P < 0.0001$), age at enrollment [HR 2.62 (95% CI: 1.64, 4.21) $P < 0.001$], child currently being breastfed [HR = 2.13 (95% CI: 1.31, 3.44) $P = 0.002$], use of pacifier [HR = 9.00 (95% CI: 1.65, 49.19) $P = 0.01$], child HIV-1 status [HR = 2.26 (95% CI: 1.19, 4.30) $P = 0.013$], and other individuals blowing on food prior to sharing with child [HR = 2.79 (95% CI: 1.01, 7.76) $P = 0.05$].

This dissertation also included a study on developing an analytical assay to detect anti-HHV-8 antibodies. There is no gold standard for diagnostic assays for HHV-8.

Wide variations reported in population prevalence studies for HHV-8 are often attributed to the different diagnostic assays used [8]. As a part of launching the study in Zambia we have to adapt the current assays to the study setting. This study describes the development of a monoclonal immunofluorescent assay protocol using an HHV-8 infected B cell line (BC3), and a recombinant protein expressed in Sf9 insect cells to screen patient plasma for HHV-8 antibody detection. A sensitivity of 94% and a specificity of 96% is reported.

Overall, this dissertation describes the development of an epidemiologic study to examine HHV-8 transmission within households, and the association of transmission of HHV-8 to a susceptible child with behaviors that expose a child to saliva. Taken together, the studies in this report suggest that associations between feeding habits that expose a child to saliva are associated with the risk of early childhood acquisition of HHV-8. Although our work supports that the most significant risk of HHV-8 transmission to a susceptible child lies with the primary caregiver, we know that transmission can also occur with other household members and even outside of the household [9].

Although other studies have looked at HHV-8 transmission in families, the studies reported here are unique and have several strengths. The importance of longitudinal studies in HHV-8 epidemiologic studies cannot be understated. It is well documented that antibody titers can vary significantly across time, making antibody levels drop below detection limits of most antibody detection assays [7]. This affects the validity of point prevalence studies in HHV-8. In our study, trends across time could be monitored to help insure accuracy of diagnostics.

Another strength of our study is the cohort design. We enrolled very young children, as young as 2 months of age to ensure enrollment of HHV-8 negative children. Children become HHV-8 positive at a very young age, and investigating the influence of behaviors as they occur is important in avoiding recall bias. Another important component of our study is that our cohort was comprised of complete households. In resource-poor counties, households often move frequently, and family members may leave and reenter households due to work situations. Moreover, Zambian families continue to be affected by the high mortality rates secondary to AIDs. Households are often comprised of extended family as caregivers, or children from extended families which may move in and out of the household on a frequent basis. Maintaining our cohort and following complete households for 48 months allowed us to collect valuable information for epidemiologic analysis. The final strength of our study is the detailed questionnaire, allowing us to assess not only how HHV-8 transmission may occur, but if the source is from the primary caregiver, other household members, or from neighborhood children.

A surprising but interesting finding in our studies is the seemingly contradictory effect of breastfeeding. In chapter 3, we report that breastfeeding was protective; however in chapter 4 in looking at the longitudinal cohort, breastfeeding became a risk for transmission. We believe that this is an important question that should be explored further. It warrants exploring breast milk further for viral presence, and also investigating for maternal antibodies that may be neutralizing. Future behavioral studies are warranted, such as use of saliva on the nipples prior to feeding the child, or presence of a confounding factor such as initiation of solid foods while breastfeeding.

In summary, we have demonstrated that HHV-8 presence in the household plays a key role in transmission of the virus to a susceptible child. This likely occurs through saliva sharing during behaviors such as testing the temperature or blowing on food before sharing to children.

The significance of this project is that understanding the possible source and route of transmission of HHV-8 virus should lead to developing strategies to facilitate behavioral changes to decrease the incidence of infection in children and the development of KS. This supports the need for increased public awareness and education regarding risks of viral transmission with child-feeding behaviors that expose children to saliva. These findings have potential implications in public health, in identifying populations at highest risk for evaluation, and potentially for possible future vaccine studies.

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