SEROEPIDEMIOLOGICAL STUDIES ON HUMAN GAMMA-HERPESVIRUS AND HUMAN IMMUNODEFICIENCY VIRUS INFECTION IN A MOTHER-INFANT COHORT IN ZAMBIA

by

Veenu Minhas

A DISSERTATION

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Biological Sciences

Under the Supervision of Professor Charles Wood

Lincoln, Nebraska

February, 2008

UMI Number: 3297662

UMI Microform 3297662

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Veenu Minhas, Ph.D.

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Human herpesvirus-8 and Epstein-Barr virus are oncogenic gamma-herpesviruses that have been associated with Kaposi's sarcoma and AIDS-related non-Hodgkin lymphomas respectively. Therefore, it is of interest to understand the natural history of infection of these two gamma-herpesviruses in an endemic area like Zambia which is experiencing a human immunodeficiency virus (HIV) epidemic, and a dramatic rise in AIDS-associated cancers. Here, we report results from a prospective study to investigate the epidemiology and transmission of human gamma-herpesviruses in a mother-infant cohort, conducted between 1998 and 2004, in Lusaka, Zambia.

For the reliable serological diagnosis of HHV-8 infection a novel insect cell-based monoclonal-enhanced immunofluorescence assay (mIFA) was developed as a part of an algorithm to identify the true positive cases. Results from children in this cohort, who were followed from birth through 48 months of age show that HHV-8 seroconversion occurs early in life and the incidence of HHV-8 seroconversion is 13.8 infections per 100 child-years. Seroconversion in adult women was comparatively lower, indicating that primary infection occurred during childhood. HIV-1 infection was a major risk factor in acquiring HHV-8 infection in both children and in adults. Maternal HIV-1 and HHV-8

infection status were not independently associated with risk of HHV-8 seroconversion in the child.

HHV-8 antibody titers measured by following children and adults at all consecutive time-points revealed that seroreversion occurred frequently. This may lead to underestimation of HHV-8 seroprevalence in cross-sectional studies. Thus, HHV-8 prevalence studies based on analysis at just one time point may not give a true representation of the HHV-8 infection rates in a population.

While HHV-8 and EBV have similar modes of transmission, we observed a significantly higher seroprevalence of EBV (60% at 12 months) in children. Thus the transmission for EBV is either more effective or occurring at a much higher rate as compared to HHV-8. Also, HIV-1 infection of the mother and not of the child was a risk factor in acquiring EBV infection probably due to increased shedding by the mother.

ACKNOWLEDGMENTS

The long journey to compile this dissertation would not have come to fruition without the assistance of many people. I am deeply indebted to my advisor, Dr. Charles Wood, for giving me the help, support and direction to complete this research project. I also applaud his research efforts in Zambia of which I realized the impact only after my visit to Lusaka. This visit also helped to bring this project near completion. I would like to thank my committee members, Dr. Jones, Dr. Angeletti, Dr. Zhang and Dr. Atkin, for the thoughtful discussions and the helpful comments when this work was in progress. I would like to thank Dr. Marc Bulterys and Dr. Ann Chao (associated with CDC, Zambia during the time of this study) for their immense help. I also thank our collaborators, Dr. Mitchell (Univ of Miami) and Dr. Wojcicki (UCSF) for their help and critical comments.

I acknowledge the support of Dr Kankasa, Dr M'soka and the late Dr Bhat as well as all the staff in Zambia, who have kept the study going for the past decade. I thank the hundreds of mothers for bringing their infants to participate in our study. Their contributions have made it possible to draw meaningful conclusions from this study. I have learned a lot from all the Zambians in Lincoln who have patiently answered my countless questions.

I appreciate the help I have received from Kay and Lynsey, who understand what it takes to spot 200 slides, run IFAs on a 100 samples and then spend hours on the microscope in the dark. I thank Hui-Ju and Nithal for all the help, support and honest feedback. I am also grateful for all the support I received from Jun, Hong, Danielle, Zhilong, Serena and all the past members of the lab. Traveling to Zambia with Danielle

was an unforgettable experience. I would also like to thank Dianna for being the unique person that she is. Marzena and Costa will remain my friends for the rest of my life.

My group of friends: Tareq, Fadia, Farida, Samira, Sucheta, Nagamani, Dr Ali Naushad and Farhana; you will always remain close to my heart.

No matter how hard I try, I cannot adequately express my gratitude towards my family for always believing in me and my decisions. Their endless love and support makes me one of the luckiest people alive. I thank my sisters, Deepti and Meha, and my brother-in-law for all that they have done for me. My cute nieces, Sonali and Serena, have kept me optimistic and sane during this period and I hope that some day they will realize how much their love meant to me.

Finally, my parents are my reason for everything I have achieved or hope to achieve in life. I have always received their unwavering love, support, encouragement and good wishes from across the seas. They have provided me with an environment that shaped me into the person that I am today. A simple thank you won't suffice for them, for their sacrifices have been great. I dedicate this dissertation to them.

ABBREVIATIONS

AIDS - Acquired immune deficiency syndrome

CI - Confidence interval

CMV – Cytomegalovirus

DNA - Deoxyribonucleic acid

EBV - Epstein-Barr virus

EIA - Enzyme immunoassay

ELISA - Enzyme linked immunosorbent assay

GST - Glutathione-S-transferase

HAART - Highly active antiretroviral therapy

HHV-6 - Human herpesvirus-6

HHV-7 - Human herpesvirus-7

HHV-8 - Human herpesvirus8

HIV - Human Immunodeficiency Virus

HR - Hazard ratio

HSV-1 - Herpes simplex virus-1

HPI - Hours post infection

IRR - Incidence rate ratio

KS - Kaposi's sarcoma

KSHV - Kaposi’s Sarcoma-associated herpesvirus

LANA - Latency associated nuclear antigen

M - Months

MCD - Multicentric Castleman’s disease

mIFA - Monoclonal-enhanced immunofluorescence assay

MIP - Mother-infant pair

MOI - Multiplicity of infection

NHL - Non-Hodgkins lymphoma

ORF - Open reading frame

PBMC - Peripheral blood mononuclear cells

PBS - Phosphate buffered saline

PCNSL - Primary central nervous system lymphoma

PCR - Polymerase chain reaction

PEL - Primary effusion lymphoma

PPH - Primary pulmonary hypertension

Ref - Reference group

STD - Sexually transmitted disease

TPA - Tetradecanoyl phorbol acetate

UTH - University Teaching Hospital

VCA – Viral capsid antigen

VCT - Voluntary counseling and testing

κ-value - Kappa value

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REVIEW OF LITERATURE

Africa is home to two thirds of the HIV infected population worldwide and therefore there is an urgent need to study the incidence and risk of AIDS-associated diseases. During the early stages of the AIDS epidemic, Kaposi's sarcoma (KS) was the most common AIDS defining illness. It was the sudden appearance of KS, now known to be caused by human herpesvirus 8 (HHV-8) or Kaposi's sarcoma-associated herpesvirus (KSHV), in young homosexual men that signaled the start of a new AIDS epidemic (141). The following review describes the worldwide epidemiology and public health impact of HHV-8 infection. This research describes a new algorithm for defining HHV-8 infection using established and novel serological assays which was used to screen for HHV-8 antibodies among children and adults in Zambia in the background of the HIV epidemic. The potential impact of this research has also been addressed with specific attention being paid to public health and epidemiology.

Human Herpesviruses

Herpesviruses are large double stranded DNA viruses that can infect humans and other mammals, birds, reptiles, amphibians, fish and even oysters (117). Infection by human herpesviruses is ubiquitous and over 80% of the human population is infected. There are currently eight known human herpesviruses belonging to three subfamilies: alpha, beta and gamma herpesvirinae. They were sub-divided based on morphology, biological properties, genome structure and sequence homology (117). This diversity of hosts indicates that these viruses have developed successful and efficient ways to infect different kinds of cells (neurons for alpha herpesvirus, lymphocytes for gamma

herpesvirus). A distinct feature of herpesviruses is their ability to establish a latent, life long infection which is interrupted by frequent reactivation leading to lytic replication. The latent phase provides a relatively stable and immunologically silent mode of persistence, whereas the lytic phase allows virions to be shed and transmitted to new hosts. Asymptomatic shedding of infectious virus in saliva is common in infected individuals. Saliva transmission is responsible for horizontal primary transmission usually from mother to child and therefore the initial infection occurs early in life.

Clinical Manifestations associated with HHV-8

HHV-8 is now known to be associated with three conditions: Kaposi's sarcoma (KS), multicentric Castleman's disease (MCD) and primary effusion lymphoma (PEL) (29, 31, 126). During the early stages of the AIDS epidemic, KS was the most common AIDS defining illness. In fact, it was the sudden appearance of Kaposi's sarcoma (KS) and shortly thereafter, the appearance of high-grade non-Hodgkin's lymphoma (NHL) in a handful of young homosexual men who otherwise were in good health signaled the start of a new AIDS epidemic (141). For most immunocompetent individuals, infection remains subclinical as shown by the low incidence rates.

i) Kaposi's sarcoma

The most important clinical manifestation of HHV-8 infection is KS. KS was originally described by Moritz Kaposi in 1872 as an idiopathic, multipigmented sarcoma of the skin (78). Since that time four epidemiologic forms of KS have been defined; classic, endemic, iatrogenic and HIV-associated or epidemic form. HHV-8 is an etiologic co-factor in common to all forms of KS, and the histological features of KS are

indistinguishable between the clinical variants. KS is a proliferative condition with lesions comprised of spindle cells surrounding vascular slits (1). There are three distinct histologic stages of KS and include the patch, plaque, and nodular forms. Though KS is most commonly localized to the skin, organ involvement can occur and is not uncommon in HIV-associated KS (137). It is thought that increased replication of HHV-8 results in an increased detection rate or an increase in viral load in peripheral blood. It is also associated with an increased risk for developing KS and with higher titers of antibodies to lytic HHV-8 antigens (122). Higher HHV-8 antibody titers are themselves associated with the development of KS although very high titers might be protective against KS lesions. Furthermore, titers of neutralizing antibodies to HHV-8 are lower in patients with AIDS-KS than in HIV infected HHV-8 seropositive patients without KS (83).

Classic KS

Classic KS, the syndrome described by Moritz Kaposi, was considered a rare disease in the 19th century. Geographic location, ethnicity, time interval, age and gender heavily influence the incidence rate (75). It presents as a disease of the limbs and occurs primarily among older men of Mediterranean and Jewish descent. The incidence of classic KS in North America, Northern Europe and Asia is low (48, 65, 74-76). In a retrospective analysis performed by Biggar et al, the annual KS incidence prior to the AIDS epidemic was estimated to be 0.29 cases per 100,000 per year among men and 0.07 cases per 100,000 per year among women (15). The male to female ratio has been reported to be 15:1 with age of onset usually greater than 50 years of age and rarely (4%-8%) prior to 30 years of age (15, 47, 61). This is the least aggressive form of KS and is a slow or non progressing disease that when even left untreated rarely invades the visceral

compartments leading to death (75, 115). The mean survival of patients with classic KS is 10 to 15 years, although patients have been known to live for 50 years with cause of death generally attributed to an unrelated condition associated with age (75, 115).

Endemic Kaposi's Sarcoma

Even before the AIDS epidemic, endemic KS was common in central and southern Africa and comprised what was called the 'KS belt' (36). The highest incidence of KS was observed in a belt-like path stretching from Cameroon, the former Zaire, to Uganda and downward through Tanzania, north and eastern Zambia, Zimbabwe and northern South Africa. KS in Africa was first reported in Nigeria in the early 1900s. Recognition of KS as a common cancer in parts of Africa became apparent from a report in the 1950s showing high prevalence of KS among Bantu tribesmen of South Africa (102). KS was soon recognized to be one of the most common neoplastic diseases in equatorial Africa (88, 102, 104). Prior to the AIDS epidemic, KS was not uncommon and represented 9-13% of all malignancies in Zaire and 3-9% in Uganda (88, 104). The prevalence of KS in Zambia has been discussed in a separate section. Although the other African subvariants of KS have a male to female ratio similar to that of classic KS and a mean age of onset of 48 years, the lymphadenopathic form is seen mostly in prepubertal children with no sex predilection. These children commonly present with localized or generalized lymphadenopathy. Rapid visceral involvement can cause early death. Skin lesions are sparse (57). Studies of endemic KS are now difficult to perform in the setting of the African AIDS epidemic.

Iatrogenic form

The third form of KS appeared in 1960s and 1970s following advances in transplant medicine. With the introduction of immunosuppressive regimens used to prevent graft rejection, post-transplant patients developed disseminated KS as early as 2 months and up to 8 years after initiation of therapy (57). This form often resolves when immunosuppressive therapy is stopped, calling attention to immune deficiency as an etiologic cofactor. Among transplant recipients, KS develops mostly in those with pre-existing HHV-8 infection, and seropositive recipients have a 28-75 fold higher KS risk than seronegative recipients (105). A recent epidemiological study in the United States observed that though KS is rare in the general population, transplant recipients are at a 54 fold higher risk of developing KS compared to the general population (95).

AIDS-associated Kaposi's Sarcoma

KS and primary central nervous system lymphoma (PCNSL) were recognized as AIDS-defining conditions when they were first reported (56). In early 1980s, large numbers of homosexual and bisexual men in New York and California, without any recognized risk factor, began presenting to hospitals with the distinctive skin lesions of KS. It soon became apparent that a number of rare opportunistic infections were suddenly of epidemic proportions, hence is also referred to as 'epidemic KS'.

Compared to classic KS, epidemic form is a very aggressive form and not only involves skin, but also the lymph nodes and often disseminates to lungs, gastrointestinal tract, liver and spleen (13). The development of KS may precede or occur simultaneously with other HIV-related symptoms. However, the tumor can appear late in the course of disease, from several months to years after the occurrence of one or more opportunistic infections (57). AIDS-KS due to its rapid dissemination multiple organ involvement and

difficulty with treatment can be a painful and debilitating disease. AIDS-KS incidence decreased with a decline in sexual promiscuity and HIV incidence, as well as after HAART introduction in 1996 probably due in part to enhanced immune reconstitution and perhaps a direct effect of protease inhibitors as antiangiogenic factors. HIV infection has been suggested to contribute to the pathogenesis of KS through immunosuppression, perturbation in the expression of certain cytokines and the effect of HIV Tat protein (3). Other factors have also been suggested to influence KS development, such as degree of immunosuppression, temporal order of HIV-1 and HHV-8 infection, HIV-1 or HHV-8 viral load and homosexual behavior. The incidence rate of KS among HIV-infected individuals has been reported to be significantly higher than the general population (75). Among homosexual men with HIV infection at the beginning of the AIDS epidemic the risk of developing KS approached 50%, and approximately 40% of patients who received a diagnosis of AIDS presented with KS (16). More recently, it has been noted that among HHV-8/HIV coinfected males the 10 year risk of developing KS is 39%-50% (92, 101).

ii) Primary Effusion Lymphoma (PEL)

In 1995, Cesarman et al identified HHV-8 DNA sequences within a distinct subgroup of AIDS-related NHL localized in body cavities and presenting as pleural, peritoneal, and pericardial lymphomatous effusions (29). PEL remains a rare lymphoma, comprising approximately 3% of AIDS-related lymphomas (59). The prognosis of PEL is poor with a median survival of less than 6 months (25). These lymphomas usually contain large amounts of viral DNA, ranging between 40 and 80 copies per cell. Most PELs are thought to originate from post-germinal center B cells, since they have hypermutation of the immunoglobulin genes. The cytologic morphology bridges large

cell immunoblastic lymphoma and anaplastic large-cell lymphoma (1). Since PELs are so uncommon, even in populations in which the seroprevalence of HHV-8 is relatively high, it is evident that infection by this virus represents only one of several genetic events involved in their development.

iii) Multicentric Castleman's disease (MCD)

Fifty years ago, Dr Benjamin Castleman first described the unusual lymphoproliferative disorder that now bears his name. MCD is a poorly understood atypical lymphoproliferative disorder thought to be related to immune dysregulation. The population prevalence rate of MCD is unknown. A large retrospective study from a cancer center estimated in 1996 that the total number of cases in the United States ranges from 30,000 to 100,000 (26). MCD presents in two distinct histopathologic subtypes with different clinical characteristics. The first and more common is the hyaline vascular type, which presents as a solitary mass that is usually curable surgically. The second is plasma cell type, which is associated with more generalized lymphadenopathy and immunological abnormalities (1). Patients with MCD frequently develop malignancies, most commonly KS and non-Hodgkin's lymphoma (126).

iv) Other conditions

Recently a few studies have suggested the association of HHV-8 with other neoplastic and non-neoplastic conditions but worldwide epidemiological surveys are required to test the strength of these associations. Primary infection with HHV-8 has been reported to cause bone marrow failure in patients undergoing peripheral blood stem cell transplantation (38, 90). A case report of transient angiolymphoid hyperplasia leading to Kaposi's sarcoma has also been described (103). Spurious associations between HHV-8

and multiple myeloma (14, 114) have been reported but these results could not be confirmed in later studies (130). Similarly, associations between other skin cancers and sarcoidosis have been reported but again are considered unreliable (45, 112). Recently, HHV-8 infection in lung tissue samples of patients with primary pulmonary hypertension (PPH) was reported (22, 37). Plexiform lesions in PPH patients resembled cutaneous KS lesions. This study has since been questioned by several other reports who have found no evidence of HHV-8 infection in patients with PPH or with secondary pulmonary hypertension (39, 69, 70, 79, 86).

Human Herpesvirus-8 and its control by the host

HHV-8, also known as Kaposi's sarcoma-associated herpesvirus (KSHV), is the most recently described human herpesvirus (117). HHV-8 was co-discovered by Chang and Moore in 1994, from lesions of a Kaposi's sarcoma (KS) patient by a subtractive PCR technique called representational differential analysis (31). This technique allows for the preferential amplification of DNA sequences representative of a diseased tissue, which is absent in the healthy tissue from the same individual. Hence, the HHV-8 DNA sequences in the KS tissue, which are not found in normal skin tissue, were amplified. HHV-8 is a non-ubiquitous herpesvirus which often causes clinical manifestations among immunocompromised hosts.

HHV-8 particles are about 100-150 nm in size with a lipid envelope and an electron dense core (113). The genome of HHV-8 contains approximately 140-kb of long unique DNA flanked by 2 terminal repeat regions, 25-35 kb each (119). It encodes over 80 open reading frames and has significant homology to the Rhadinovirus genus of the

gamma-herpesvirus sub-family, all of which are known to infect lymphocytes (99). HHV-8 is the only member of the genus Rhadinovirus known to infect humans, but is closely related to another human gamma-herpesvirus, Epstein-Barr virus (EBV), which belongs to genus Lymphocryptovirus. EBV is well known to transform human B-cells in culture and is also etiologically linked to human cancers, including Burkitt's lymphoma and nasopharyngeal carcinoma.

Although it is difficult to obtain infectious virus from culture explants of KS tissue, cell lines derived from primary effusion lymphomas, such as BC3, KS-1, BCBL-1, BC1 have proved very valuable for studies on HHV-8 (1).

After primary infection, HHV-8 establishes a life-long infection and exhibits periodic latent and lytic phases. In latency, HHV-8 persists predominantly in B cells and is characterized by a restricted pattern of viral gene expression that facilitates the avoidance of immune surveillance (128, 140). While the host immune response generally prevents disease, it does not prevent viral persistence. The periodic reactivation of the virus to undergo lytic reactivation possibly represents a loss of immunogenic control. This leads to the spread of the virus from the lymphoid reservoir to other cells types contributing to pathogenesis (133). A number of proteins expressed in the lytic phase that have been identified to elicit antibody responses in infected individuals and have been discussed in the section regarding the diagnosis of HHV-8 infection. Preliminary reports have indicated that specific neutralizing antibodies against HHV-8 virion proteins may be able to limit the spread of HHV-8 in an infected individual or help in control of viral titers (46). The virion proteins that elicit a neutralizing antibody response have still not been identified. During this phase several viral-encoded cellular homologues with known

cytokine or cytokine-receptor signal-transduction potential are also up-regulated. These include, IL-6, IL-8 receptor, GPCR and interferon regulatory factors that are important for HHV-8 to evade innate and adaptive immune responses (55).

HHV-8 Epidemiology

After the identification of HHV-8, several epidemiological studies have been conducted to understand the extent of infection in different human populations. Clear evidence now prevails that geographical differences in HHV-8 seroprevalence exist. The highest seroprevalences are found in sub-Saharan Africa, where seroprevalence up to 80% has been reported in adults. In Africa, HHV-8 infection is common in countries such as Uganda (14-86%) (32, 40), Cameroon (28-62%) (62, 109, 116), Botswana (76-87%) (54) and The Gambia (29-84%) (87). There is no evidence that the prevalence of HHV-8 has changed in Africa since the spread of HIV (42). Primary infection seems to occur during childhood and seroprevalence increases with age (44, 93, 109). In countries surrounding the Mediterranean Sea, the seroprevalence averages 10%, although there are regions where higher prevalence can be found such as Sardinia and Sicily (50) where classic KS is diagnosed more often (6, 7, 33). HHV-8 infection is uncommon in the general population in North America, UK and Western Europe. In countries with low prevalence of HHV-8, higher seroprevalence is found in high risk groups such as homosexual men, HIV-1 infected immunocompromised patients and immunosuppressed transplant patients. In the United States, prevalence was found to be between 5 to 20% range and in HIV-1 infected patients, prevalence can be between 20-50% (60, 87, 125). Prevalence in Asia has not been studied extensively but appears to be low. In South

America, studies involving blood donors from Chile (3%), Brazil (3-9%) and Argentina (4%) have demonstrated low HHV-8 seroprevalence in the general population (28, 108, 138). The only exception is a study of Peruvian blood donors with very high seroprevalence of 56% (98). Also, studies involving Amerindians in Brazil, Eucador, Peru and French Guiana have demonstrated high HHV-8 seroprevalence and have identified a new subtype of HHV-8 in this population (subtype E) (17, 82, 135).

HHV-8 genomes are now classified into five major subtypes (A-E) that reflect sequence heterogeneity in the highly variable ORF K1 (67). K1 gene encodes for a 46 KDa type 1 glycoprotein and has been used to study the evolution and worldwide distribution of HHV-8. HHV-8, subtype A is found in Northern Europe and America, subtype B (thought to be the most ancient) is from Africa, and subtype C, often associated with classical Kaposi's sarcoma, is found in Mediterranean countries (67, 129). HHV-8 subtype D (mainly from South America and the Pacific Islands) and more recently, subtype E (South America) have also been described (82).

Currently, the exact risk factors that predispose a person to HHV-8 infection are not clear. An uneven geographic distribution of HHV-8 and its familial clustering raises the question as to the existence of environmental or host genetic factors that predispose to HHV-8 infection, increased replication, and/or development of KS (73, 124). A study performed in the US indicated that the incidence of classic KS in Jews born in Eastern European and Mediterranean countries was higher than second generation American Jews, suggesting that differences in transmission or environmental factors are important (118). Recently, an 'oncoweed hypothesis' has been proposed to implicate environmental cofactors present in KS endemic regions, which cause frequent reactivation of HHV-8

leading to increased viral shedding and transmission. This leads to increased prevalence on HHV-8 in a region as well as increased viral load levels and higher antibody titers (134). This study has identified 184 plant extracts from 38 countries that can cause HHV-8 lytic replication *in vitro*. This is a new hypothesis and further studies are needed to fully understand the role of environmental cofactors.

Transmission of HHV-8

The modes of transmission of HHV-8 may be different depending on the endemicity of that region, and are still being investigated but both horizontal and vertical transmission has been reported. HHV-8 can be found in the PBMCs, saliva, oropharyngeal mucosa, semen and prostate glands (34).

i) Vertical transmission

Vertical transmission of HHV-8 has been documented but is believed to occur infrequently. Reactivation of herpes virus infections occurs during pregnancy but very little is known about HHV-8 infection and pregnancy. Rare cases of KS in newborns have been described (64, 97). Our laboratory has also reported that HHV-8 DNA can be detected in a small percentage of infants born to HHV-8 seropositive mothers in Zambia (91). Together, these findings indicate that in utero or intrapartum HHV-8 infection might, albeit rarely, occur in countries where HHV-8 is endemic. HHV-8 is often found in cervicovaginal secretions of HIV-1 and HHV-8 co-infected women, suggesting that HHV-8 viral load in the female genital tract might influence vertical transmission. Reported data also shows that the rates of detection are higher among African women from areas of non-endemicity or sub-endemicity (19, 23, 85, 132, 136).

ii) Horizontal transmission

Horizontal transmission is mostly believed to occur in childhood in endemic countries and during adulthood in non-endemic countries. Recent reports indicate that in Africa, where the prevalence of the virus is also high among children, transmission from mother to child and between siblings accounts for a substantial proportion of infections in childhood. Saliva and breast milk are now emerging as the likely candidates that mediate this transmission. Breast milk of mothers has been reported to contain herpesviruses such as CMV, EBV and HHV-7 (2, 51, 58, 66, 77). A study conducted in South Africa has reported the presence of HHV-8 DNA in breast milk of seropositive mothers with high titer antibodies (43). But our studies in Zambia have failed to detect any viral DNA in breast milk and it does not seem to be an important route of transmission to young children (20). Not much is known about transmission of HHV-8 in children in developed countries including United States and the results of these reports are conflicting (93).

Vieira et al have reported that HHV-8 DNA recovered from saliva was similar to HHV-8 DNA recovered from the cultured 293 cells that had been infected with saliva fluid (131). Transmission of HHV-8 from mother to child and between siblings most likely occurs via saliva, given that HHV-8 DNA has been detected in saliva in high copy number (43, 94, 110). It has been reported that there is an increased prevalence of HHV-8 antibodies in children of mothers shedding high number of viral DNA copies/ml of saliva, suggesting that large amounts of HHV-8 in maternal saliva may be associated with transmission to the child (5). All these studies suggest that HHV-8 can be transmitted via the saliva like other human herpesviruses (EBV, cytomegalovirus, HHV-6, HHV-7 and HSV-1) (89).

In addition, transmission from contaminated blood products has also been suggested (49, 139). However, the risk for such transmission events appears to be very low. But a recent study has shown conclusively that transmission of HHV-8 is possible by blood transfusion but this risk diminishes as the period of blood storage increases (71). Transmission of HHV-8 via this route is not efficient because of the cell associated nature of the virus. Also, low and/or intermittent viremia among seropositive donors and low level of circulating virus in asymptomatic donors may contribute to the low level of transmissibility.

Sexual transmission is believed to be the most common route of HHV-8 transmission in adult immunocompetent populations in countries of low seroprevalence, even though viral loads found in vaginal, seminal and prostatic secretions are much lower than in saliva (52). Early epidemiologic studies suggested that since the development of KS was more frequent in HIV-1 seropositive homosexual men, HHV-8 might be a sexually transmissible agent. HHV-8 can be detected in semen samples in HHV-8 endemic areas (18). Specific intimate behaviors which increase the risk of transmission are still not clear. Varying detection rates have been reported in cervical or vaginal samples (19, 23, 85, 132, 136). Sexual risk factors associated with HHV-8 seropositivity include HIV-1 infection, increasing numbers of homosexual partners, history of STDs, orogenital insertive and receptive sexual practices, use of amyl nitrates, and unprotected sex with commercial sex workers. In endemic areas, HHV-8 seropositivity was also associated with increasing age, history of STDs, not using condoms, and having a seropositive spouse (9, 84, 96, 116).