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Seroprevalence and Viral Quantification of Kaposi Sarcoma-associated Herpes Virus (KSHV) in a Human Immunodeficiency Virus (HIV) infected adult South African cohort

By

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Seroprevalence and Viral Quantification of Kaposi Sarcoma-associated Herpes Virus (KSHV) in a Human Immunodeficiency Virus (HIV) infected adult South African cohort

By

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Declaration

I, Shoohana Singh, hereby declare that this thesis entitled, ***Seroprevalence and viral quantification of Kaposi Sarcoma-associated Herpes Virus (KSHV) in a Human Immunodeficiency Virus (HIV) infected adult South African cohort***, is the result of my own investigation and research and has not been submitted in part or full for any other degree or to any other university.

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Ethical Approval

This study was approved by the Nelson R. Mandela School of Medicine Institutional Review Board, Durban, South Africa – Ethics No. **BE 194/010**

Presentations

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- Awarded Scholarship from IAS to attend the conference.

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AIDS - Acquired Immunodeficiency Syndrome

ARV - Anti-retro viral

CAPRISA - Center for AIDS Program of Research in South Africa

DNA - Deoxyribonucleic acid

ELISA – Enzyme-Linked Immunosorbent Assay

HAART – Highly active anti-retroviral therapy

HHV8 - Human herpes virus 8

HIV - Human Immunodeficiency Virus

KSHV – Kaposi's sarcoma-associated herpes virus

KS – Kaposi's sarcoma

KZN – KwaZulu-Natal

OD - Optical density

PCR - Polymerase chain reaction

PI - Principal investigator

SA - South Africa

SANAS - South African National Accreditation System

USA - United States of America

WHO - World Health Organization

ABSTRACT

Background

Kaposi sarcoma-associated herpes virus (KSHV), also known as human herpes virus 8 (HHV8), is aetiologically implicated in Kaposi's sarcoma (KS). Although HIV associated KS has increased in incidence and is a public health problem in South Africa, serological studies of KSHV have not been extensively documented in this population. This cross-sectional study investigates the seroprevalence and viral load of KSHV in an adult South African cohort.

Method

Cross-sectional data of 140 participants attending an urban research HIV counseling and testing (HCT) clinic site in Durban, KwaZulu-Natal, between July and October 2013 was analyzed. Detection of antibodies against latent (Orf73) and lytic (K8.1) KSHV antigens was performed on 70 HIV-seropositive and 70 HIV-seronegative participants. Subjects reactive to either antigen were considered KSHV seropositive and analyzed for salivary KSHV DNA, which was quantified using primers for the K6 gene region.

Results

The demographic characteristics of the two groups were similar, with 36% males (median age, 35yrs.) and 64% females (med. age, 34yrs.) in the HIV-positive group, and 31% males (med. age, 36.5yrs.) and 69% females (med. age, 36.5yrs.) in the HIV-negative group. Of 70 HIV-positive participants, 100% were black Africans, as was 97% of the HIV-negative group, with the remaining 3% being Indian/Asian and Mixed race. Only 24% of HIV-positive patients were on Anti-retro viral treatment.

Fifty-four percent of all participants tested positive for KSHV, with 33% reactive to lytic K8.1, 37% to latent Orf73 and 21% to both. Of those HIV-positive, 50% were seropositive for K8.1 and 46% for Orf73. In those HIV-negative, 16% were seropositive for K8.1 and 29% for Orf73. The HIV-positive group demonstrated a significantly higher percentage KSHV seropositivity (70% vs. 37%, p=0.0001). Amongst the KSHV seropositive participants, KSHV DNA was detected in 41 % HIV-positive and 23% HIV-negative participants**.**

Conclusion

KSHV seroprevalence was high in South African adults attending an urban HCT clinic. HIV positive status was associated with a higher KSHV seropositivity and a greater KSHV salivary shedding. HIV positive individuals should be tested for KSHV infection and those found infected, be monitored aggressively for development of KS.

CHAPTER 1. INTRODUCTION

1.1 Introduction

South Africa has the highest incidence of HIV/AIDS globally, with approximately seven million people infected, or 19.2% of the population, specifically those between the ages of 15 and 49. A number of conditions are known to be associated with those who are immune-compromised, specifically tuberculosis and other infectious conditions. The incidence of various cancers is also reported to be above the norm in this population, notably Kaposi's sarcoma (KS) an AIDS-defining cancer. Of concern is the increased prevalence in reported cases of KS and it has been documented as a public health concern. Kaposi's Sarcoma-associated herpes virus (KSHV) also known as Human Herpesvirus type 8 (HHV 8) is the virus known to be aetiologically linked to KS. It is highly infectious and not treatable. With the limited research on the seroprevalence and viral quantification of this condition, it is difficult to evaluate the risk that the South African population is exposed to.

This chapter presents the background to the study, and provides an overview of HIV prevalence and HIV related KS incidence. The literature review gives insight into the history of KS and its associated causative virus, KSHV, along with the cyclic phases and replication. In order to understand the ingenious survival of this otherwise dormant virus, it was important to study the transmission pathways documented from previous studies. Since endemic and epidemic KS exists, populations with high KSHV seropositivity globally, in Africa and South Africa are described. This led to a more focused evaluation of KSHV/HIV co-infected population studies.

1.2 Background

Viral-associated cancers are common amongst HIV-positive individuals, especially in sub-Saharan Africa (SSA), which carries the highest burden of disease[1]. In 2014, UNAIDS estimated that 66% of those infected by the global HIV epidemic (25.8 million people) were in SSA, with 1.4 million new infections in that year.[1] According to the South African National HIV Prevalence, Incidence, Behaviour and Communication Survey of 2012, there were 1.2 million more people living with HIV compared to 2008.

The overall HIV prevalence in the age group of 15-49 years in 2008 was 18.8%, with females having a higher infection rate than males in all age groups.[2] In 2015, it was estimated that approximately 7 million individuals in South Africa were infected with HIV, with 380 000 being newly infected in that year.

The province of KwaZulu-Natal (KZN) is the most affected, with an estimated 40% of its population being infected, and an HIV prevalence of 16.9%. Within this province the metropolitan eThekwini Municipality (Durban) has a prevalence of 14.5 %. [2, 3] KSHV, also known as Human Herpes Virus8 (HHV8), is implicated in the aetiology of KS [4, 5] a marker for advanced HIV/AIDS, and a WHO clinical stage 4 AIDS-defining illnesses.[6] The HIV epidemic in SSA has been responsible for the increase in incidence of KS, with the incidence in endemic areas, having increased by 20-fold, making it the commonest cancer in males and second most common in females.[7] The age-specific incidence of KS has also changed from being 59.7 year olds in 1983 to 36.5 year in 2006. It also mimics the trend of HIV incidence, with a sharp increase from 15 years, peaking at 33-39 years of age.[7, 8]

A cross-sectional study of KSHV in Johannesburg, South Africa, demonstrated a high prevalence among HIV-infected adults initiating antiretroviral therapy (ART), with almost half of the 404 participants being positive for the virus.[9] The relationship between KSHV and HIV infection in Durban is however unknown, although previous studies suggest that uncontrolled HIV and lower CD4 counts may be associated with salivary shedding of KSHV, and thus potential infectivity.[8, 10, 11] KZN continues to display a steadily increasing incidence of HIV [2], with little documentation on its co-infection with KSHV. Improving the knowledge of KSHV seroprevalence is central to the understanding, preventing and effectively managing KS in KZN.

1.3 Literature Review

Kaposi's sarcoma is a malignant vascular neoplasm which was first described by Dr. Moritz Kaposi in 1872. It affects the skin, mucous membranes, internal organs and lymph nodes.[12] The lesions associated with KS are histologically intricate and consists mainly of spindle-shaped cells, possibly of endothelial origin.[13] In addition, KSHV has been implicated in the aetiology of KS. [14, 15]

Based on epidemiology and clinical characteristics, KS can be classified into four subtypes:

- Classic KS: commonly occurs in elderly men of Mediterranean and Jewish descent who are HIV negative.[16]
- Iatrogenic KS: occurs in patients who received solid organ transplants and those using prolonged immunosuppressive therapy.[17]
- African endemic KS: described in younger individuals mainly from Sub-Saharan Africa.[18]
- Epidemic/ AIDS-related KS: an aggressive form of KS first described in a group of young HIV positive homosexual men in the United States of America (USA).[19]

Since 1981, KS has emerged as an AIDS defining disease rather than just a previously known, uncommon malignancy. In its early stages, KS was the most frequent malignant tumour in HIV/AIDS patients, specifically amongst homosexual men.[20] Not all HIV patients presented with KS. The rapid rise in KS incidence prompted the search for a sexually transmitted causative agent. [21] Chang, Moore and their collaborators provided some answers in 1994 with their discovery of short DNA sequences of a unique human herpes virus that was associated with KS in the dermis of a patient with AIDS.[14] This gamma 2 herpes virus was called KSHV, and is considered to be the principal cause of KS, being very similar to Epstein-Barr virus (EBV). Since KSHV was discovered, its full genome has been cloned and its DNA sequence determined.[22] Besides being the causal agent in the most common AIDS-defining malignancy, KSHV was subsequently determined to be a cause of other malignancies in patients with AIDS, including primary effusion lymphoma (PEL) and multicentric Castleman's Disease.[23, 24]

In the quest to uncover how KSHV is transmitted, scientists had to develop serologic assays to detect host antibody reactivity to the virus's lytic and latent phases, and associated replication with polymerase chain reaction (PCR) assays.[25]

1.3.1 Lytic and latent phases of KSHV

Epidemiological studies for KSHV infection became possible with the development of serological assays, which enabled the detection of host antibody reactivity to KSHV. However, the antibody response to a KSHV infection was not fully understood, as the virus expresses proteins differentially. This differential protein expression is dependent on whether the virus is in a latent or lytic phase of infection, hence the development of the first immunofluorescence assay (IFA) that could quantify both antibodies. This test was found to be 90% sensitive. Unfortunately, the results for IFA did not correspond with other assays in HIV-positive patients with KS, and yielded a lower sensitivity of 75% for KSHV infected patients.[26, 27] The IFA was followed by the development of the enzyme immunoassays (EIAs), which were 100% sensitive and 95.8% specific, [28] making them a more reliable method of testing. Many studies show the EIAs improved specificity with differing antigenic targets, including latent and lytic proteins, as well as whole viral lysates. Serological assays are based on expression of genes from the viral episomes and during latent KSHV infection phase, a limited number of genes are expressed: including viral cyclin D (orf 72), latency-associated nuclear antigens (LANA-1 or orf 73) and viral Fas-ligand interleukin-1B-converting enzyme inhibitory protein (K13 or vFLIP).[29] These genes serve several functions and when infected host cells are undergoing mitosis, LANA-1 facilitates the proliferation of viral episomes by assuring transcription through the attachment of KSHV DNA to the H1 histone on host chromatin.[30] In combination, the 3 genes: orf73, LANA-1 and vFLIP control the host cell cycle, triggering tumourigenesis. Furthermore, LANA-1 represses apoptosis by blocking the transcriptional activity of p53.[31] The Fas death receptor pathway is hindered by *v*FLIP as it protects cells latently infected with KSHV from apoptosis. The counteraction of the host cell cycle growth arrest controlled by cyclin-dependent kinases and pRb is executed by *orf 72*.[32]

The initiation of the lytic phase is dependent on the gene product *orf 50* (Rta), which is similar to the lytic phase of other human herpes virus, where gene products are manufactured sequentially, i.e. the initial genes articulate subsequent gene expression. This is followed by DNA replication genes, and finally those responsible for virion production and genes encoding homologues of cellular proteins.[33] Once in this phase, the viral titre increased in the host and quantifying KSHV viral loads was possible using quantitative PCR assays.

1.3.2 KSHV Viral Load

Viral quantification of KSHV, an important indicator for KS progression, can be performed on DNA extracted from peripheral blood mononuclear cells (PBMC), a biopsy of KS lesions and salivary samples.[34-36] KSHV viral load quantification has been used in many studies for different reasons. In a Spanish study viral loads were performed to understand the routes of transmission, where 200 women (100 female prostitutes and 100 random females from the general population) were evaluated. KSHV DNA was detected in 3% of oral cavity samples, in 2% of cervical samples of prostitutes and 1% of the general female population. This showed that oral shedding and heterosexual contacts are potential pathways of transmission.[37]

In some studies KSHV viral loads have been used to show associated increased KS risk, with those who were K8.1 seropositive, having a tenfold higher increase in KSHV viremia.[7, 38] An important HIV co-infection study in Uganda showed that among 74 HIV-positive patients, KSHV DNA was detected in the PBMCs of 93% of patients and quantified in 77% thereof. The KSHV viral load was higher in men than women and those with faster (>20 lesions per year) rate of KS lesion eruption. It also showed that KSHV load was unrelated to CD4 lymphocyte count.[39] In a Kenyan study, high KSHV seroprevalence and quantities of oral KSHV shedding were observed in a cohort of woman. Their findings supported the observation that oral replication is an important factor for KSHV infection, with likely implications of KSHV transmission and KS pathogenesis.[11]

1.3.3 KSHV Transmission

Many studies have been conducted in an attempt to understand AIDS-associated KS and its transmission by trying to detect for KSHV in PBMCs, plasma, genital and oral secretions.[36, 40] It appears that the mode of transmission is still a highly debatable issue, with saliva most commonly harbouring KSHV, thus being considered an important channel for transmission.[41-47]

In the USA, a study conducted by the National Health and Nutrition Examination Survey III sampled 13,894 individuals from the general population with an enzyme assay to measure KSHV antibodies. KSHV transmission amongst men occurred through sexual activity, especially in men having sex with other men (MSM), with no conclusive evidence being found for heterosexual transmission to women.[48] A study in Nigeria indicated risky sexual behaviour as enabling KSHV transmission amongst men and women.[49]

Horizontal non-sexual transmission has been increasingly supported and accepted, with qualitative analysis in a KZN study finding 14 possible practices that expose children to saliva, and therefore at risk of KSHV transmission. Of the 14, no single method was indicated as more important. However, the positive aspect of this study is that it can be used to educate female caregivers in preventing transmission of KSHV to children.[50]

Further investigation of KSHV seroprevalence was carried out in high risk population like those infected with HIV in order to find other links in viral transmission.

1.3.4 Global KSHV Seroprevalence and HIV co-infection

The incidence of KS and KSHV seroprevalence has geographical and sub-population variation. KSHV seroprevalence in the general population of HIV-negative individuals in northern Europe, Asia and the USA is less than 10%, is 10-30% in the Mediterranean, and greater than 50% in Sub-Saharan Africa.[51] A study from various regions in Italy of 747 healthy blood donors displayed 13.8% KSHV infections.[52] A seroprevalence study of KSHV in Southeast Asia, the USA, the Caribbean, and Africa reported it to be low in healthy and HIV-positive individuals in the first two, which correlated with low

reports of AIDS-related KS.[53] A study in the Shandong area of China showed that 16.3% (14/86) of HIV-positive patients were KSHV positive compared to the 5.7% in 230 healthy blood donors who were negative.[54] This highlights the importance of HIV as a cofactor for the development of KS in the setting of KSHV infection. The KS incidence in the general population is 1 in 100 000, but with HIV-infection it is 1 in 20 [55] and higher in MSM with almost 1 in 3.[56]

1.3.5 KSHV and HIV co-infection in MSM

A study in San Francisco, USA, in a population of 593 MSM in an area with a high incidence of HIV reported that 37.6% were KSHV-positive.[20] A retrospective study in the USA also supported the observation that KSHV and manifestations of KS are most frequently seen in HIV-positive homosexual men.[57] A 30-month follow-up was also performed in 75 men and showed KS development in two.[57] In a Danish study initiated in 1981, 21.1% (52/246) of homosexual men were KSHV seropositive.[48] An Amsterdam cohort study of 1458 homosexual men indicated the incidence of KSHV to be 20.9% (305/1458) being highest in the HIV-positive group.[58] For many years it was observed that KSHV infections were more prominent in homosexual men, however, with the development of the HIV/AIDS pandemic, the rates of KSHV infection have increased in heterosexuals as well.[49, 59]

1.3.6 KSHV and HIV co-infection in Africa

In Ghana, West Africa, a cohort of 3275 HIV-seronegative healthy blood donors displayed 23.7% positivity for KSHV, and 65.6% from 250 HIV/AIDS patients.[60] African countries such as Ghana, Uganda, and Zambia showed increased seroprevalence of KSHV in both healthy and HIV-positive individuals, with Uganda at 38.7% (24/62) in HIV-negative and 45.7% (16/35) in HIV-positive patients.[53] In contrast to Uganda, South African children had a seroprevalence of 7.5% - 9% and the KSHV seroprevalence did not increase with the age of the children.[61] It was also evident in a KSHV transmission study that the risk of South African children acquiring KSHV was higher for those who had KSHV seropositive mothers and the HIV status of the mothers was marginally associated with an elevated risk of KSHV seropositivity in their children.[62]

Determining the mode of transmission from mother to child has been a standing question as studies to support vertical transmission (during birth and breastfeeding) have been limited.[63]

1.3.7 KSHV Seroprevalence and HIV co-infection in South Africa

Studies conducted thus far have focused on areas of Africa where KS is endemic.[64] The HIV epidemic has highlighted KS in South Africa, where an increased number of cases have been reported. A Durban cancer prevalence study of 152 patients with HIV-associated KS showed an equal female to male ratio, with more severe disease in younger females.[65] A retrospective study in KZN showed that age-standardized incidence increased from 1:100 000 in 1990 to 15:100 000 in 2006.[8]

Studies in KZN of KSHV seroprevalence have focused mainly on mother-child pairs in trying to understand the transmission of the disease. In a cohort of 427 children, 7.5-9.0% were KSHV positive by testing for antibodies.[61] In an earlier study in the Hlabisa District, north of Durban, 34.6% of 136 patients who were randomly tested for antibodies against KSHV tested positive.[66] In a later study in the Hlabisa district, 2546 mother-child pairs were tested for latent and lytic antigens to KSHV. Mother-child lytic antigens were higher (40%-12%) than the latent antigens (14%-7%) as was their HIV prevalence at 28% and 22%, respectively.[67]

Further north in the Gauteng Province, in the areas of Johannesburg and Soweto, a cancer prevalence study showed that 36.3% (1196/3293) of patients were KSHV positive via the immunofluorescence assay. This study also showed that the prevalence of KSHV antibodies increased with increasing age and sexual partners, and was not strongly related to HIV infection.[68] Another South African study aimed at understanding the transmission of KSHV as well as other co-infections and lifestyle factors, recruited pregnant women from 37 clinics in Gauteng. In that cohort of 1740 woman, 44.6% were KSHV seropositive. These women were four times more likely of being HIV-positive as well. [69] Another South African study recruited pregnant women from 37 clinics in Gauteng, to understand transmission of KSHV, as well as other co-infections and lifestyle

factors. In that cohort of 1740 woman, 44.6% were KSHV seropositive, with those being four times more likely of being HIV-positive as well.[69]

One of the later studies in Johannesburg looked only at HIV-positive patients who had not initiated ART. Of the 404 patients screened for lytic and latent KSHV antibodies, 43% (193/404) were positive.[9] There are no studies comparing the seroprevalence of KSHV in an HIV-positive versus HIV-negative population in KwaZulu-Natal, South Africa. The province is the epicenter of the HIV epidemic in the country making it important to investigate the extent of the co-infection with KSHV.

1.4 Problem Statement

The combination of KSHV/KS and HIV/AIDS has detrimental outcomes for the dually infected patients. South Africa is one of the highest HIV burden countries, with KZN being the worst affected. The prevalence of HIV/KSHV co-infection in KZN adults has not been established and determining this will assist us in earlier identification of those at risk of developing KS.

CHAPTER 2. METHODOLOGY

2.1 Aim and Objectives

The study aimed to establish the seroprevalence of KSHV in an adult population in Durban, KwaZulu-Natal Province.

The study had the following objectives:

1. To establish participant HIV status and medical history.
2. To quantify the antibodies against lytic (K8.1) and latent (LANA) KSHV encoded proteins of the HIV-positive and HIV-negative participants.
3. To evaluate KSHV salivary shedding through a cell associated KSHV viral loads in subjects who were KSHV-seropositive by quantitative real-time PCR.

2.2 Study design

This was a cross-sectional seroprevalence study conducted between July and October 2013. The site HIV Counselling and Testing (HCT) counsellors explained the study with reference to an information sheet (Appendix I). Where necessary a verbal translation in the native language of the participant was used to explain along with translated information sheets (Appendix II). The counsellor then referred patients to research nurses who obtained the signed informed consent (Appendix III) with native language translation where necessary (Appendix IV). The nurse interviewed the patient in order to evaluate eligibility criteria and completed the data sheet (see Appendix V). The eligible candidates were screened for HIV with dual HIV rapid tests. Specimens were collected with a tracking laboratory form (Appendix VI). A single 5ml venous blood draw in a serum separating tube was obtained as well as a non-invasive mouthwash for saliva specimen collection.

2.3 Study Site and population

This study took place at an HIV counselling and testing (HCT) clinic in a public sector in Durban, the largest city in KZN province. The clinic serviced local community with

regard to voluntary counselling and testing. ART and palliative care were offered and those individuals who were HIV-positive and not at a critical stage to receive treatment would have returned for repeat CD4 counts and HIV viral load quantification. The clinic was involved in AIDS treatment program. These participants were not in any clinical trial. Two independent proportion (null case) power analysis was done using the two-sided Fishers exact test to calculate sample size. Based on the assumption that 40% of the HIV-positive group will be KSHV-positive and 10 % HIV-negative group will be KSHV-positive, a sample size of 36 per group would achieve an 80% power to detect a difference between the two groups. The sample size was doubled and included 70 HIV-positive and 70 HIV-negative adult individuals.

The following inclusion criteria applied:

1. Male/ female participants of any race
2. 18 years and older
3. Screened HIV status available
4. Agreed to provide whole blood and salivary specimens
5. Voluntary agreement to participate and from whom signed informed consent was obtained.

The following exclusion criteria applied:

1. Participants under 18 years of age
2. Refusal to provide whole blood and salivary specimens
3. Participants with known clinical KS

2.4 Data and Clinical specimen collection:

Participants were screened for HIV by a dual rapid HIV testing system to determine HIV prevalence in this population. The two preferred HIV rapid testing kits used were Abon^{TM} HIV Tri-Line Rapid test and First Response HIV 1-2.0 Card Test of which detected for HIV-1 and HIV-2. Both HIV-positive and HIV-negative patients were secured for the study. They were asked to provide a sample of blood and saliva as part of the study. A single 5ml venous blood draw was collected in serum separator tubes (SST) to quantify the antibodies against lytic (K8.1) and latent (LANA) KSHV encoded proteins, and a non-invasive mouth wash for saliva specimen collection to quantify KSHV salivary shedding through a cell associated KSHV viral loads in subjects who were KSHV-seropositive. The SST's were centrifuged and serum transferred into labelled cryovials where they were stored in an ultra-freezer until testing. Saliva samples were centrifuged to obtain a pellet of oral cells. The pellet and supernatant were stored in labelled cryovials, in an ultra-freezer. The HIV-positive patients were requested to provide their last known CD4 count and HIV viral load results, as well as information regarding their ART.

2.4.1.

Objective 1: To establish HIV status and medical history of participants.

The HIV screening was performed at the clinic site and results were recorded to evaluate the HIV prevalence. Whole blood from a finger prick was used on the rapid testing kits, Abon^{TM} HIV Tri-Line Rapid test and First Response HIV 1-2.0 Card Test in accordance to the package insert instructions. Abon^{TM} HIV Tri-Line Rapid test is 99.9% sensitive and 99.8% specific in detecting anti-HIV-1 including subtype O and anti-HIV-2 antibodies. The reading of a control line and either one or both of the test lines was regarded as a reactive result whilst just a control line was non-reactive. An invalid test was that without a control line irrespective of appearance of any of the test lines.

The First Response HIV 1-2.0 Card Test is 100% sensitive and 99.9% specific in detecting anti-HIV-1 including subtype O and anti-HIV-2 antibodies. The reading of a control line and either one or both of the test lines was regarded as a reactive result whilst

just a control line was non-reactive. An invalid test was that without a control line irrespective of appearance of any of the test lines. These results were recorded and attached onto data sheets, which not only provided demographic information, but also other illnesses that participants may have been experiencing as well as treatments they were receiving. Characteristics of age, gender, ethnic groups and employment status were documented. HIV-positive participants were required to provide last known, if any CD4 counts, HIV viral loads, and ART for their data sheet entries.

2.4.2

Objective 2: To quantify the antibodies against lytic (K8.1) and latent (LANA) KSHV encoded proteins of the HIV-positive and HIV-negative participants.

Serum was used to detect dual antibodies to KSHV by serologic assays. Optical density (OD) measurements to latent nuclear antigen (LANA) encoded by Orf73 as well as the lytic phase glycoprotein K8.1. A method adapted from previously described literature.[28] Positive and negative controls were triplicates in a 96-well plate along with triplicates of each participant's serum samples. The serum samples for the Orf73 assay were initially diluted 1:100 in assay buffer. The OD was measured at 630nm with the BMG Spectrostar Nano (Orthanberg, Germany). The results for the Orf73 and K8.1 assay were analyzed, adhering to assay cut off, titration and quality controls. The OD values falling below the negative cut-off line were regarded as seronegative whilst those falling above the positive cut-off line were regarded as seropositive. The OD values falling between the positive and negative cut off lines were indeterminate. Subjects who were K8.1 and/or LANA seropositive were regarded KSHV seropositive. GraphPad Prism 5 statistical software package was used thus to determine significance of results between HIV-positive and HIV-negative groups.

2.4.3

Objective 3: To quantify KSHV salivary shedding through a cell associated KSHV viral loads in subjects who were KSHV-seropositive.

Saliva samples were pelleted to obtain oral endothelial cells. The DNA was extracted using a QiagenQiAamp DNA mini extraction kit. The DNA quality and cell quantitation

were determined using real-time PCR for endogenous retrovirus 3 (ERV3) whilst the KSHV DNA was quantified using probes and primer that amplified the K6 region (viral macrophage inflammatory protein α). The KSHV Probe (p-K6-10) 5'-[FAM] CACCCACCGCCCGTCCAATTC [TAMRA]-3', forward primer (K6-10F) 5'-CGCCTAATAGCTGCTGCTACGG-3' and reverse Primer (K6-10R) 5'TGCATCAGCTGCCTAACCCAG-3' was used on the Roche LightCycler 480 PCR platform. The ERV3 and K6 standard curves were optimized. KSHV viral load was extrapolated, calculated and reported as KSHV copies per million (10^6) cells.

2.5 Data Analysis –to interpret the resulting data collected from data sheets and laboratory assays

The participants' demographics were extracted from the data sheets and stratified by HIV status and summarized as simple proportions or medians. GraphPad Prism 5 statistical software package was used to determine any significance between the HIV-positive and HIV-negative groups. Unpaired t test was used to determine the prevalence odds ratio (OR) for KSHV infection and 95% confidence intervals (CI). Statistical analysis of the lytic and latent assays was performed using unpaired t test with software GraphPad Prism 5 to show significant difference between the HIV-positive and negative groups.

For KSHV salivary shedding, the ERV3 (endogenous retrovirus 3) and K6 (viral macrophage inflammatory protein α) standard curves were optimized, shown in Figure S1 and Figure S2, respectively (Supplementary Figures). KSHV viral load was extrapolated, calculated and reported as KSHV viral copies per million (10^6) cells. Subjects were categorized as "non-shedders" (KSHV not detectable in saliva), "low shedders" (KSHV detectable at <3,000 copies/10^6 cells), or "high shedders" (KSHV >3000 copies/10^6 cells) for comparisons between groups. Additionally, the KSHV viral loads for each group was averaged and variance between the two groups (HIV-positive vs. HIV-negative) was analyzed using the F test for comparison of variance with 95% CI.

2.6 Data Management

Information from all data sheets for each participant was captured by the PI onto Microsoft Excel Spreadsheet on a password protected PC. The actual data sheets and HIV screening results are filed and locked away.

ELISA results for both lytic and latent assays were exported in Microsoft Excel format from the BMG Spectrostar Nano and stored with patient data sheet information.

Quantitative real time PCR results for KSHV viral quantification were exported from the Roche LightCycler and stored with patient data sheet information.

All biological specimens remaining after testing are stored in a secured ultra-freezer until completion of the study.

2.7 Ethical considerations

This study was only conducted at the specified site and ethical clearance was obtained before commencement of any work.

Ethical clearance reference: **BE 194/010**

Participants were reimbursed for their traveling costs in accordance with:

1) Guidelines for Good Practice in the Conduct of Clinical Trials in Human Participants in South Africa – Department of Health (2006), and

2) Ethics in Health Research: Principles, Structures, and Processes – (2004).

Potential risks (none in this study) and discomforts (small blood draws) were explained clearly.

The onsite designated study personnel explained the study to the subject, who had the opportunity to review it and ask any questions. All subjects signed the informed consent. A copy was given to the subject and a copy filed for official purposes.

To maintain patient confidentiality, patients' identity was not on the data sheet but instead assigned a random study number. All documents and results are safely locked away.

CHAPTER 3. RESULTS

3.1 Introduction

This chapter presents the results of the study with respect to the study objectives. The participants HIV status and demographics are followed by medical history, with some insight into any treatments received, especially ART for HIV-positive patients. The KSHV seroprevalence was established by laboratory testing the serum, processed from whole blood specimens collected at the clinic, and the differences and variances seen within and between the HIV-positive and HIV-negative individuals is indicated. KSHV salivary shedding was established through cell associated KSHV viral loads in subjects who were KSHV-seropositive. KSHV DNA was amplified from processed saliva specimens collected at the clinic.

3.2 Objective 1: To establish participants HIV status, medical history and demographic data

At the end of enrolment, 140 participants were enrolled for the study by adhering to the inclusion/exclusion criteria. After establishing HIV status with the rapid test results, participants were divided into HIV-positive and HIV-negative groups. Their demographic data and medical history are presented in Table 3.1. Of the 70 HIV-positive participants, 25 (36%) were males, with ages ranging from 26 to 53 years old, and a median age of 35years. Females accounted for 45 (64%), with ages ranging from 22 to 56 years old, and a median age 34 years. In the HIV-positive group, 49% were employed, 40% were unemployed, and 11% did not state employment. All 70 of the HIV-positive were Black Africans, of whom 24% were on ART, which included first line therapy of either regimen 1 or 3TC (Lamivudine: a nucleoside reverse transcriptase inhibitor), TDF (Tenofovir Disoproxil Fumarate: a non-nucleoside reverse transcriptase inhibitor) and EFV (Efavirenz: a non-nucleoside reverse transcriptase inhibitor). Two male participants documented a history of a sexually transmitted infection (STI) and a female of TB.

Table 3.1: Participants HIV status, demographics and medical history

Variable	Characteristics	HIV positive N (%) (n=70)	HIV negative N (%) (n=70)
Gender	Male	25 (36%)	22 (31%)
	Female	45 (64%)	48 (69%)
Age	Male	35yrs (26-53)	36.5yrs (23-58)
Median (range)	Female	34yrs (26-56)	36.5yrs (18-77)
Employment status	Employed	34 (49%)	44 (63%)
N (%)	Unemployed	28 (40%)	8 (11%)
	Not stated	8 (11%)	18 (26%)
Race	Black African	70	68
	Coloured	-	1
	Indian	-	1
Medical history			
Anti-retro viral treatment	Male	5 (7%)	-
	Female	12 (17%)	-
Sexually transmitted	Male	2	1
Infection	Female	-	-
Tuberculosis	Male	-	-
	Female	1	-
Asthma	Male	-	-
	Female	-	1

- *denotes no applicable information*

Of the 70 HIV-negative participants, 22 (31%) were males, with ages ranging from 23 to 58 years old, and a median age of 36.5years. The number of females was 48 (69%), with ages ranging from 18 to 77 years old, and a median age 36.5 years. In the HIV-negative group, 63% were employed, 11% were unemployed and 26% did not declare their employment status. Sixty-eight participants in this group were Black Africans, 1 mixed race and 1 was Indian/Asian. One male had documented history on an STI and a female of asthma.

3.3 Objective 2. To quantify the antibodies against lytic (K8.1) and latent (LANA) KSHV encoded proteins of the HIV-positive and HIV-negative participants

K8.1 and LANA seroreactivity were evaluated both individually and together. Participants reactive to either or both viral proteins were considered KSHV seropositive. Regarding the Lytic (K8.1) assay, the OD values were plotted and graphs extrapolated in Figure 3.1 for HIV-positive versus HIV-negative participants.

Lytic K8.1 ELISA Assay

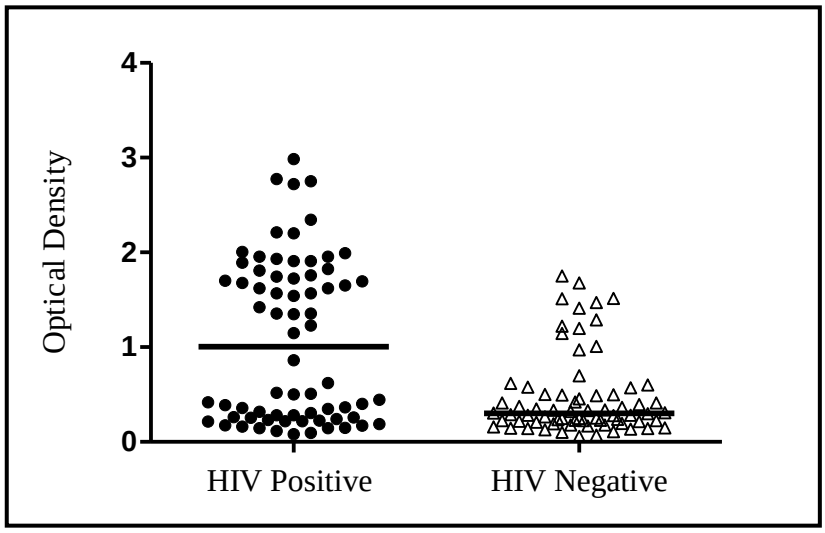

Figure 3.1: Lytic K8.1 ELISA assay for HIV-positive vs. HIV-negative participants showing optical density values per participant.

Of the 70 participants in the HIV-positive group, 50% (35/70) were seropositive for K8.1 ELISA, 49% (34/70) were negative and 1% fell into the indeterminate category. Of the 70 participants in the HIV-negative group, 16% (11/70) were seropositive for K8.1 ELISA, 83% (58/70) were negative and 1% fell into the indeterminate category.

A comparison of the lytic assay between the two groups in Figure 3.2 indicated that 50% (35/70) of HIV-positive patients were KSHV seropositive whilst only 16% (11/70) HIV-negative patients were KSHV seropositive. The HIV-positive group showed 49% (34/70) seronegativity and the HIV-negative group 83% (58/70), with a 1% indeterminate in both groups. HIV-positive group reported a significantly higher percent of participants with KSHV lytic antibody as compared to the HIV-negative group (p-value <0.0001, 95%CI, 0.378 to 0.825).

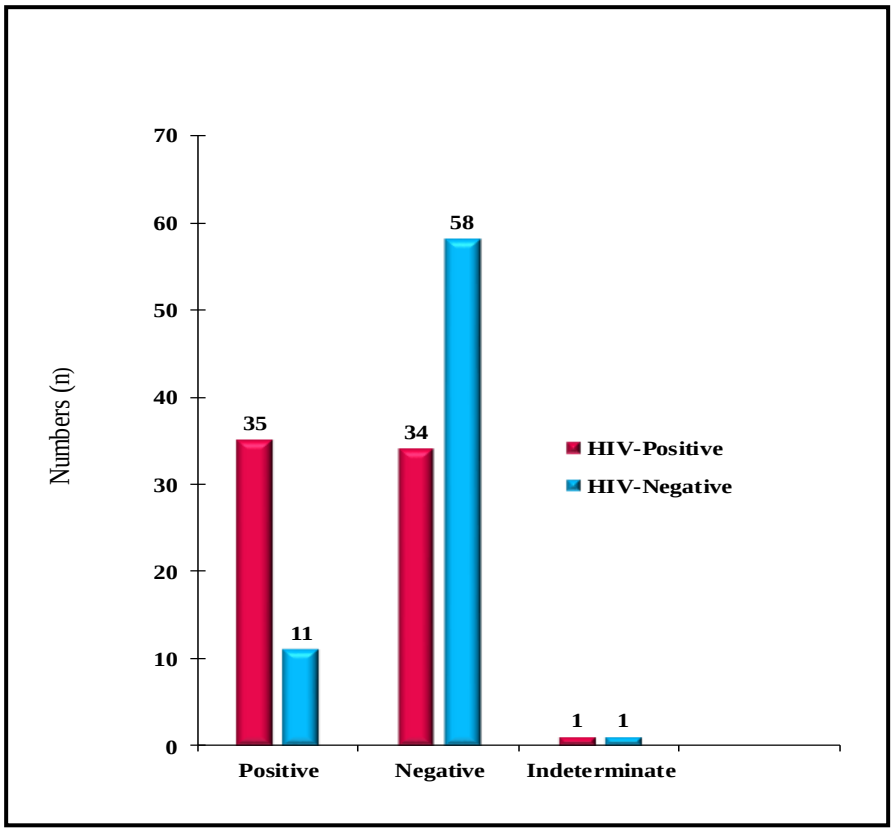

Figure 3.2: Number of participants reactive to the lytic K8.1 antigen and reported as positive, negative (not reactive) or indeterminate.

Latent ORF73 ELISA Assay

The results for the latent Orf73 assay are presented in Figure 3.3 for HIV-positive vs. HIV-negative participants.

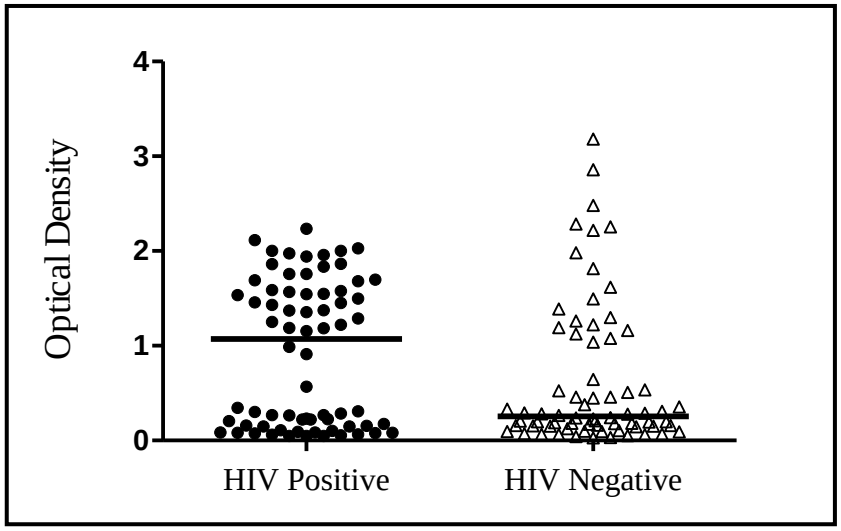

Figure 3.3: Latent Orf73 ELISA assay for HIV-positive vs. HIV-negative participants showing optical density values per participant.

A comparison of the latent assay between the two groups in Figure 3.4 showed that 46% (32/70) of the HIV-positive group were seropositive for latent antibodies and 54% (38/70) were seronegative. The HIV-negative group showed 28% (20/70) were seropositive, 64% (45/70) were seronegative whilst 8% (6/70) remained indeterminate. A significantly higher percentage of HIV-positive participants were positive for the latent KSHV antibody compared to those that were HIV-negative (p-value=0.0251, 95%CI, 0.0392 to 0.544).

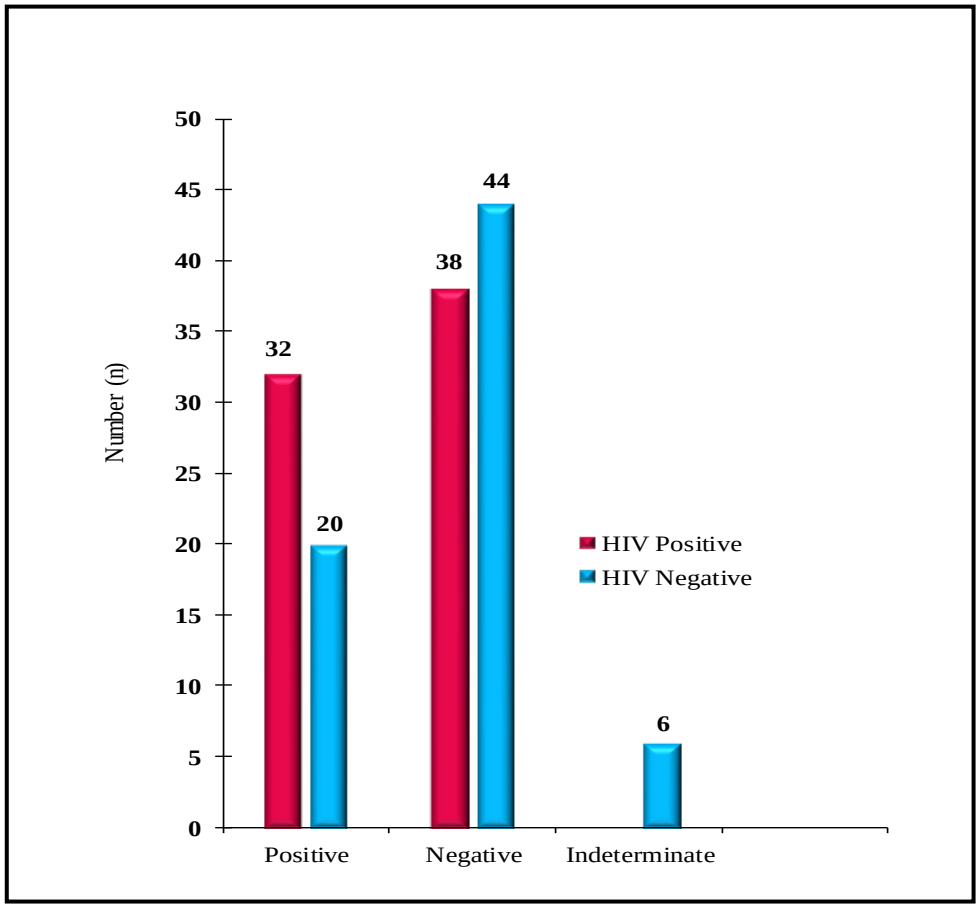

Figure 3.4: Number of participants reactive to the latent orf73 antigen and reported as positive, negative (not reactive) or indeterminate.

KSHV Seropositivity: Combined lytic and latent results

Results of the K8.1 and Orf73 assays were combined to give a final qualitative outcome for KSHV serology, extrapolated from Figure 3.2 and Figure 3.4 and presented in Figure3.5.

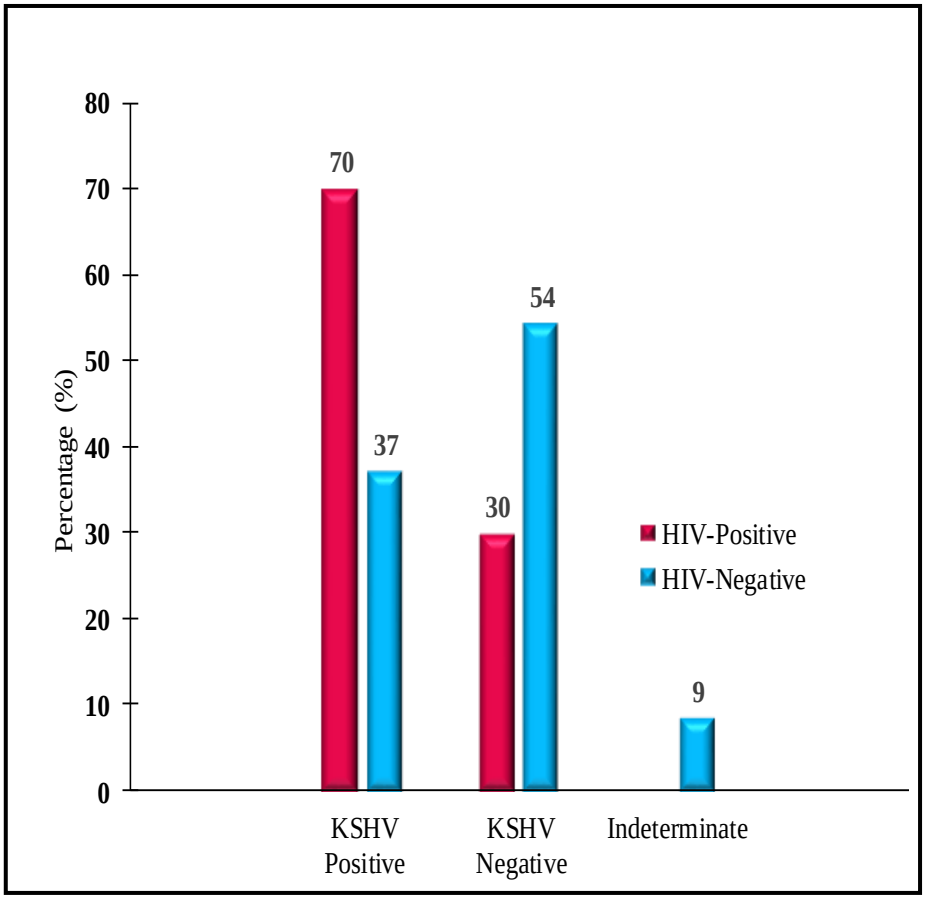

Figure 3.5: Combined lytic and latent ELISA results for HIV-positive vs. HIV-negative participants.

Total cohort Seroprevalence:

Seroprevalence for the entire cohort was calculated to be 54% (75/140) positive for KSHV, 42% (59/140) negative, and 4% (6/140) indeterminate.

The combined outcome of both the lytic and latent assays demonstrates that 70% (49/70) of HIV-positive participants were classified as KSHV-positive and 30% (21/70) as KSHV-negative. Whilst 37% (26/70) of the HIV-negative participants were KSHV-positive, 54% (38/70) were KSHV-negative and 9% (6/70) were indeterminate. Overall, HIV-positive participants had a significantly higher percentage KSHV positivity compared to those who were HIV-negative (OR=3.41, p-value=0.0009, $\alpha < 0.05$).

Of the 70 HIV-positive participants, 20 males and 29 female were KSHV positive compared the 10 males and 16 females of those HIV-negative (Figure 3.6). The overall KSHV seropositivity for males was 40% (30/75) compared to 60% (45/75) for females amongst the KSHV seropositive individuals. The proportion of males and females KSHV seropositive within the total cohort was 21.4% (30/140) vs 32.1% (45/140), respectively. Although there was no significant difference between KSHV seropositivity in males and females with regards to the participant numbers of the HIV-positive and HIV-negative groups, there were differences observed in lytic and latent antibody expression, as presented in Figure 3.7 and summarized in Table 3.2

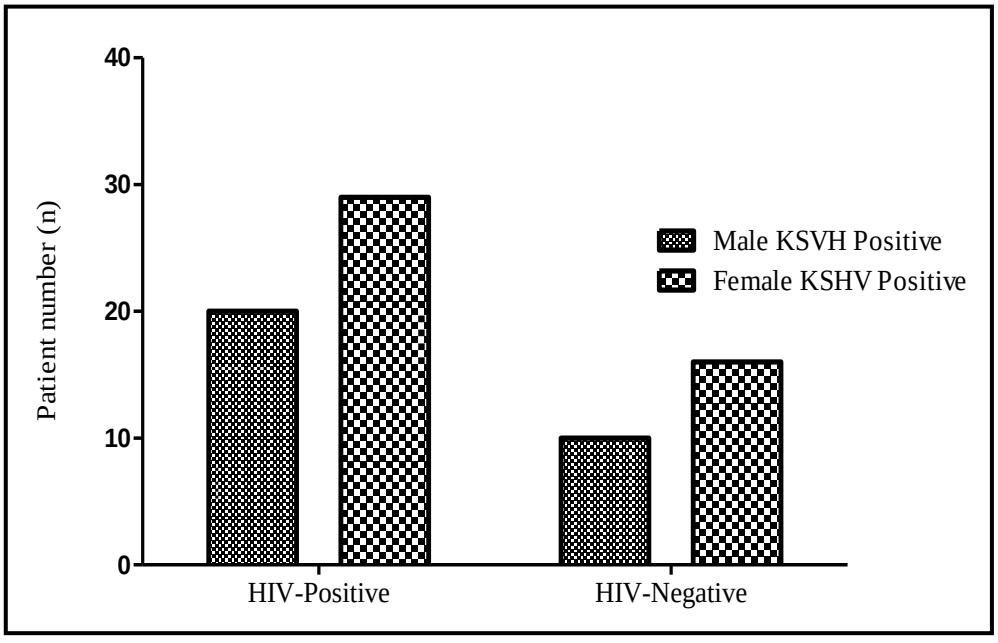

Figure 3.6: KSHV seropositivity of males vs. females in the HIV-positive and HIV-negative group.

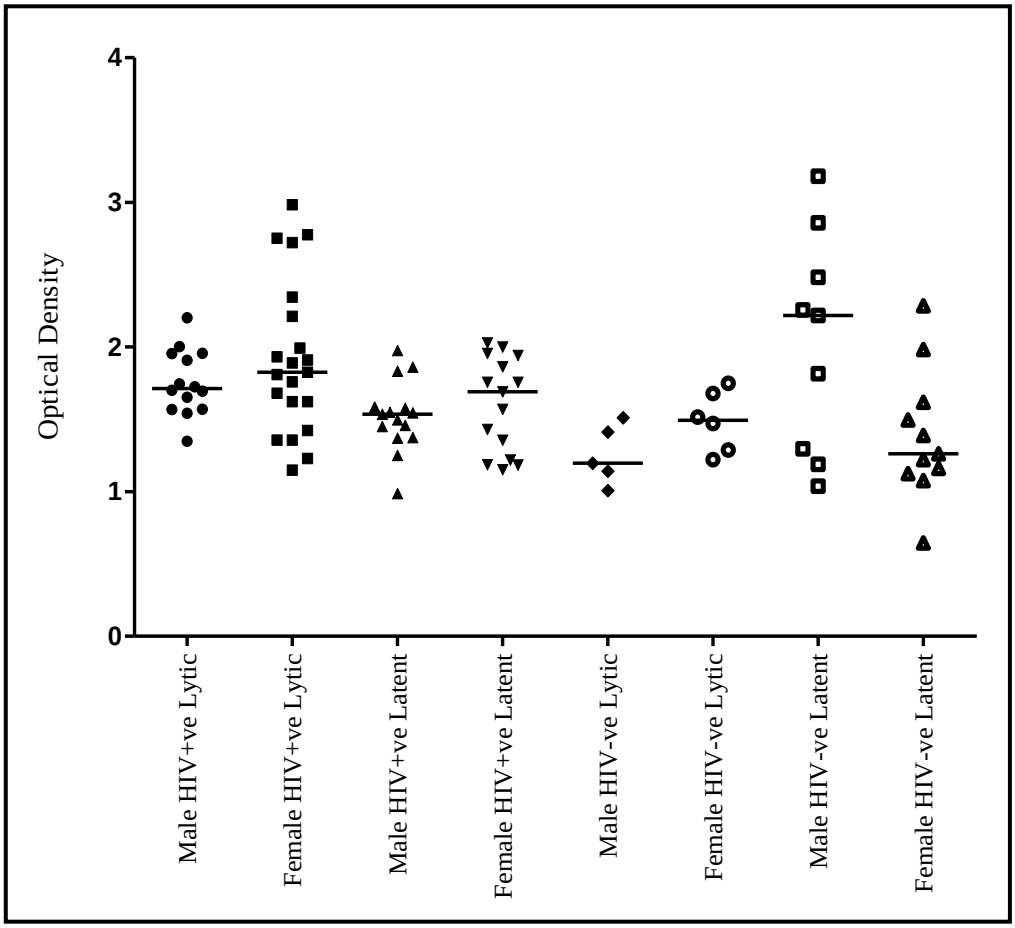

Figure 3.7: Comparison of lytic and latent ODs for HIV-positive and HIV-negative male vs. female.

The HIV-positive/lytic-positive males accounted for 20% (14/70) compared to the 30% (21/70) in females, while the latent-positive males were 21.4% (15/70) and the females 21.4% (15/70). The HIV-negative/lytic-positive males were 7% (5/70) compared to the females' 8.6% (6/70), while the latent-positive males accounted for 12.9% (9/70) compared to the 15.7% (11/70) in the females.

Table 3.2: Comparison of KSHV positive Male and Females in the HIV-positive and HIV-negative groups

Grouping Comparison	Lytic/Latent antibodies	Significance	P value
HIV-positive & HIV-negative males vs. females	Lytic vs Latent antibody	Yes	0.0008
	Lytic antibody	Yes	0.0067
	Latent antibody	Yes	0.0138
HIV-positive males vs. females	Lytic antibody	No	0.2808
	Latent antibody	No	0.4381
HIV-negative males vs. females	Lytic antibody	No	0.0964
	Latent antibody	Yes	0.0282
Male HIV-positive vs. male HIV-negative	Lytic antibody	Yes	0.0004
	Latent antibody	Yes	0.0225
Female HIV-positive vs. female HIV-negative	Lytic antibody	No	0.0656
	Latent antibody	No	0.1619

The statistical comparison using 1 way ANOVA of lytic versus latent antibody expression between males and females of the HIV-positive vs HIV-negative groups showed a significant difference between the means ($p=0.0008$), as well as a significant difference between the variances in the groups ($p<0.0001$, Bartlett's test for equal variance). A combination of male and female lytic antibody expression was marginally higher in mean and variance in the HIV-positive than the HIV-negative group ($p=0.0067$). A combination of male and female latent antibody expression was marginally higher ($p=0.0138$) in the HIV-negative group, including a higher variance ($p=0.0016$). Within the HIV-positive group, there was no significant difference between the males and females with respect to the lytic and latent antibody expression. However, there was a noticeable difference in the latent antibody expression between the males and females in the HIV-negative group. Male latent antibody expression was higher in the HIV-negative group ($p=0.0282$). The HIV-negative males latent antibody expression was also higher than the HIV-positive males ($p=0.0225$), including a significant variance ($p=0.0003$). The lytic antibody expression in HIV-positive males was significantly higher than HIV-negative males ($p=0.0004$). The females did not show any difference in lytic or latent expression with regards to HIV status, although a slightly higher variance ($p=0.0459$) was detected in HIV-positive female lytic antibodies.

3.4 Objective 3: To evaluate KSHV salivary shedding through cell associated KSHV viral loads in those who were KSHV-seropositive

Salivary samples of the 49 HIV-positive and 26 HIV-negative participants who were seropositive for KSHV antibodies were used to detect KSHV DNA, with an additional 4 samples that were randomly selected from the indeterminate KSHV category. This data is presented in Figures 3.8 - 3.11 and further summarized in Table 3.3 and Table 3.4.

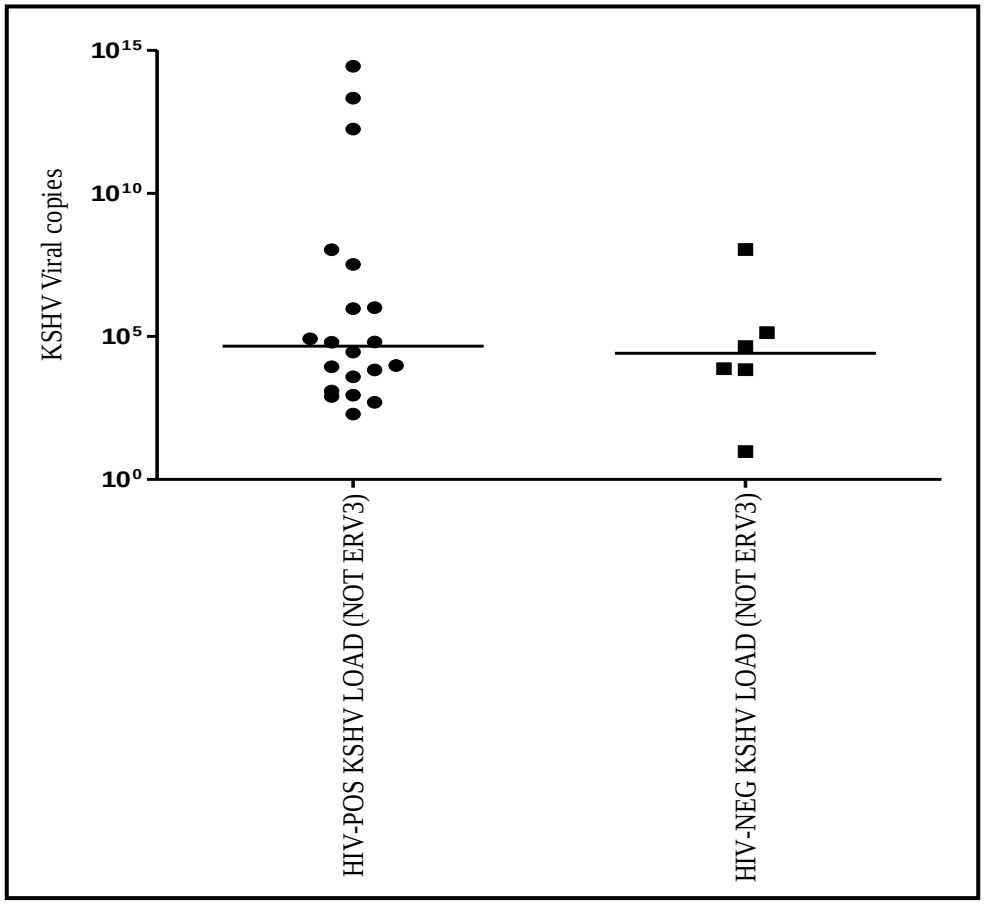

Figure 3.8: KSHV viral load (VL) quantification of HIV-positive and HIV-negative participants, not corrected for ERV3 housekeeping gene.

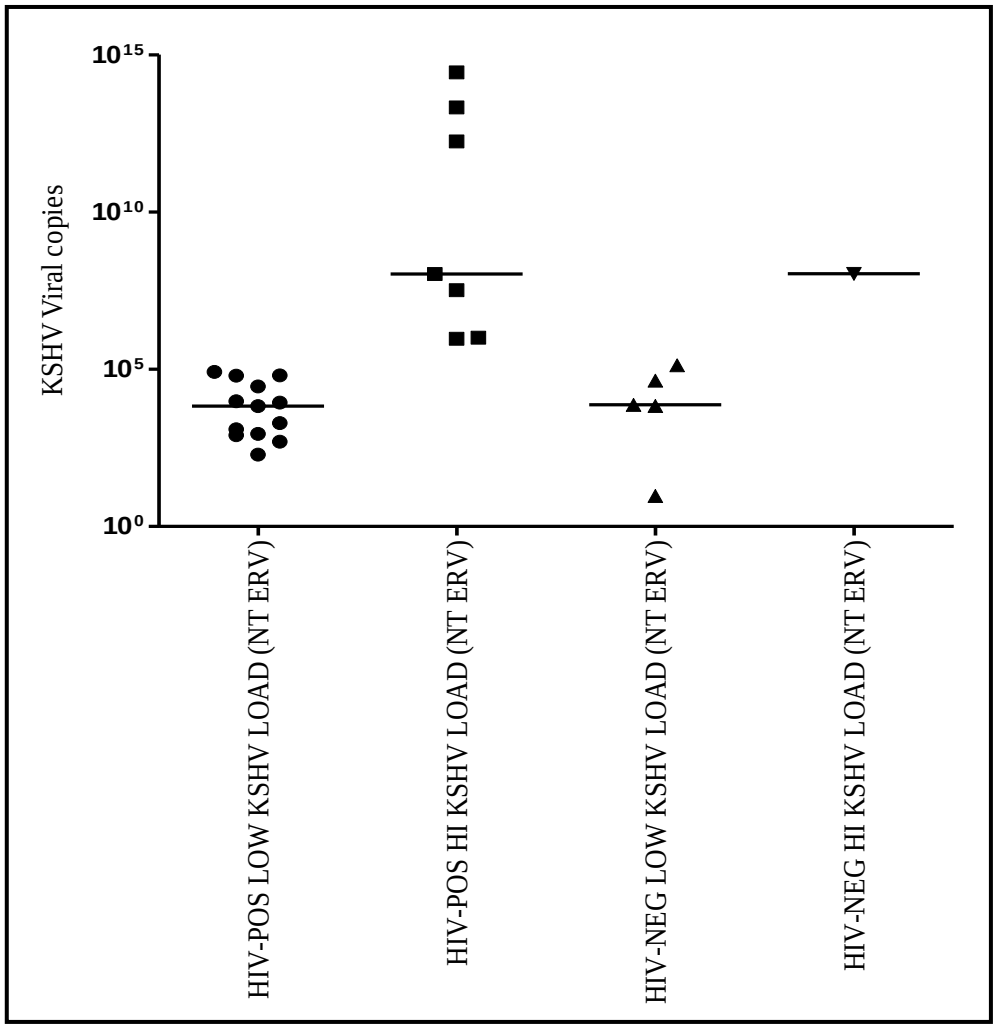

Figure 3.9: KSHV VL quantification of HIV-positive and HIV-negative participants, not corrected for ERV3 housekeeping gene and further categorized into low and high VL.

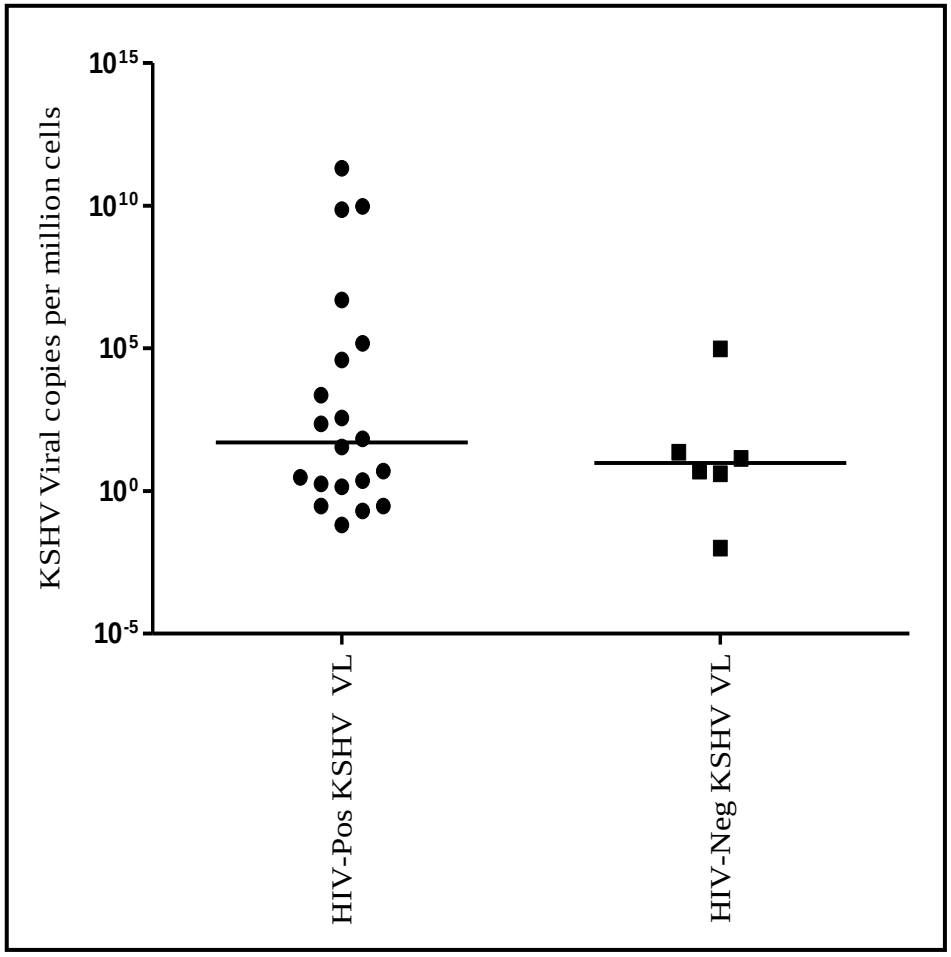

Figure 3.10: KSHV viral load (VL) quantification of HIV-positive and HIV-negative participants, corrected for ERV3 housekeeping gene.

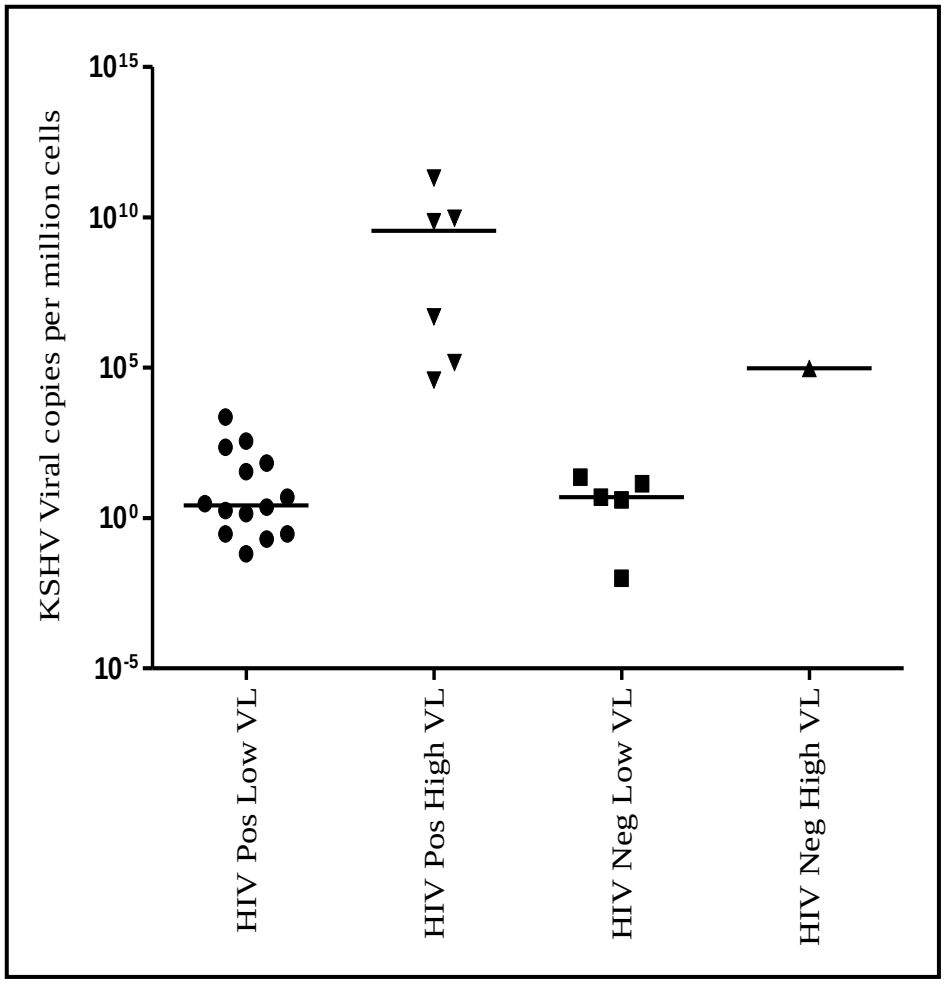

Figure 3.11: KSHV VL quantification of HIV-positive and HIV-negative participants corrected for ERV3 housekeeping and further categorized into low and high VL.

Table 3.3: Characteristics of co-infected (HIV/KSHV) participants analyzed for KSHV DNA

Variable (Median)	KSHV Shedding Category					
	Not Detectable– "Non Shedders"		Low Positive- "Low Shedders" (<3000copies/10^6 cells)		High Positive– "High Shedders" (>3000copies/10^6 cells)	
Gender (n)	Male (12)	Female (17)	Male (5)	Female (9)	Male (3)	Female (3)
Age (35) (Range: 36-53)	36.5	36	32	37	30	29
KSHV VL (51copies/10^6cells) (Range: 1 - 2.1x10^{11} copies/10^6cells)	*	*	1.4x10^0	3x10^0	7.20x10^9	4.91x10^6
K8.1 OD (1.623) (Range: 0.094- 2.984)	0.985	1.356	1.695	1.932	1.956	1.623
Orf73 OD (1.452) (Range:0.048- 2.236)	1.538	0.570	1.459	1.758	1.536	1.186

* *denotes undetectable KSHV VL*

Table 3.4: Characteristics of HIV-negative/KSHV-positive participants analyzed

Variable (Median)	KSHV Shedding Category				
	Not Detectable– "Non Shedders"		Low Positive- "Low Shedders" (<3000copies/10^6 cells)		High Positive– "High Shedders" (>3000copies/10^6 cells)
Gender (n)	Male (7)	Female (14)	Male (2)	Female (3)	Male (1)
Age (39) (Range: 22-77)	32	42	45	41	38
KSHV VL (9.5copies/10^6cells) (Range: 2 – 9.7x10^4 copies/10^6cells)	*	*	2.7x10^0	1.4x10^1	9.7x10^4
K8.1 OD (0.536) (Range: 0.072- 1.750)	0.617	0.456	1.279	1.288	1.512
Orf73 OD (1.176) (Range: 0.041- 2.860)	1.815	1.143	1.648	0.535	2.860

* *denotes undetectable KSHV VL*

Of the 75 individuals, regardless of HIV status, who were KSHV seropositive, 50 (66.7%) had an undetectable viral load while KSHV DNA was detected in 26 (34.7%). Of those, 19 had a low positive (<3000 KSHV copies/10^6 cells) and seven had a high positive viral load (>3000 KSHV copies/10^6 cells).

Of the 30 HIV-negative participants (Table 3.4), KSHV DNA was detected in six samples. The KSHV viral load ranged from 2 to 97 000 KSHV copies per 10^6 cells, with the median viral load of 9.5 KSHV copies/10^6 cells and an average viral load of 1.6×10^3 KSHV copies per 10^6 cells. There were seven males and 14 females in the "not detectable – non shedder" category with median ages of 32 years and 42 years, respectively. The median OD for lytic K8.1 and latent Orf73 for males within this category was 0.617 and 1.815, respectively. The median OD for lytic K8.1 and latent Orf73 for females within this category was 0.456 and 1.143, respectively. There were two male and three females in the "low positive – low shedder" category with median ages of 45 years and 41 years, respectively. The median OD for lytic K8.1 and latent Orf73 for males within this category was 1.279 and 1.648, respectively. The median OD for lytic K8.1 and latent Orf73 for females within this category was 1.288 and 0.535, respectively. One male in the "high positive – high shedder" category, aged 38 years had an OD for lytic K8.1 and latent Orf73 of 1.512 and 2.860, respectively. There was no significant difference in means or variance between the "not detectable", "low positive", and "high positive" OD values of HIV-negative participants.

Of the 49 HIV-positive participants (Table 3.3), KSHV DNA was detected in 20 samples. The KSHV viral load ranged from 1 to 2.0×10^{11} KSHV copies per 10^6 cells, with the median viral load of 51 KSHV copies/10^6 cells and an average viral load of 1.13×10^{10} KSHV copies per 10^6 cell. There were 12 males and 17 females in the "not detectable – non shedder" category, with median ages of 36.5 years and 36 years, respectively. The median OD for lytic K8.1 and latent Orf73 for males within this category was 0.985 and 1.538, respectively. The median OD for lytic K8.1 and latent Orf73 for females within this category was 1.356 and 0.570, respectively. There were five male and nine females in the "low positive – low shedder" category with median ages of 32 years and 37 years, respectively. The median OD for lytic K8.1 and latent Orf73 for males within this

category was 1.695 and 1.459, respectively. The median OD for lytic K8.1 and latent Orf73 for females within this category was 1.932 and 1.758, respectively. There were three males and three females in the "high positive – high shedder" category with median ages of 30 years and 29 years, respectively. The median OD for lytic K8.1 and latent Orf73 for males within this category was 1.956 and 1.536, respectively. The median OD for lytic K8.1 and latent Orf73 for females within this category was 1.623 and 1.186, respectively. The "low positive" and "high positive" OD values were significantly higher ($p=0.0209$) than the "not detectable" of the HIV-positive participants.

The viral load variance between the HIV-positive and HIV-negative groups was significant (p-value < 0.0001), with a higher KSHV viral load in the HIV-positive participants, making them 3.6 times more likely to shed KSHV. Nine of the 29 individuals from the "not detectable" KSHV shedding category (Table 3.3) were on ART (5 males and 4 females), as well as an additional female from the "low positive". This subgroup of 10 participants was further analyzed in Table 3.5 against other HIV-positive/ KSHV-negative participants on ART.

In the HIV-positive group, 17 participants were on ART, with 10 of these KSHV seropositive. Of these 10 participants, nine had an undetectable KSHV viral load, while only one female had a low KSHV viral load of 0.2 KSHV copies/10^6 cells (881 KSHV copies, not ERV3 corrected). Two KSHV/HIV-positive males had CD4 counts of 218 cell/mm^3 and 500 cell/mm^3.

Table 3.5: Participants on ART

Variable	KSHV-positive/HIV-positive		KSHV-negative/HIV-positive	
Gender (n)	Male (5)	Female (5)	Male (1)	Female (6)
Age (Range)	39 (35-53)	37 (33-43)	37	39.5 (29-52)
K8.1 OD Median (Range)	0.518 (0.144-1.567)	2.753 (1.149-2.984)	0.285	0.353 (0.116-0.418)
ORF73 OD Median (Range)	1.290 (0.912-1.863)	0.102 (0.074-0.264	0.218	0.181 (0.047-0.268)
CD4 Count (cell/mm^3)	218 and 500	*	*	340
HIV Viral load	*	*	*	Undetected
ARV Treatment	Regimen1	3TC, TDF, EFV	*	Regimen1, 3TC, TDF, EFV
Other Illness	STI	TB	STI	Heartburn

denotes unavailable information

Seven participants were in the KSHV-negative/HIV-positive category (1 male and 6 females), with one female's CD4 count of 340 cell/mm^3 and an undetected HIV viral load. The ART received in this group was also regimen 1 or 3TC, TDF, and EFV. All participants were first visits to the HCT clinic thereby limiting data for HIV-positive individuals on ART, which was obtained from participants' data sheets

3.5 Summary

The finding of this cross sectional study of adults in the eThekwini district of Durban, Kwazulu-Natal was that (54%) of the 140 participants tested positive for KSHV, with 33% being reactive to lytic K8.1, 37% to latent Orf73 and 21% to both. Of the HIV-positive group, 50% were seropositive for K8.1, and 46% for Orf73. In the HIV-negative group, 16% were seropositive for K8.1 and 29% for Orf73. The HIV-positive group demonstrated a significantly higher percentage KSHV seropositivity (70% vs 37%, p=0.0001).

Participants who were reactive to either lytic or latent antigens were analyzed for KSHV DNA. Of those who were KSHV seropositive, 70% were HIV-positive and 37% were HIV-negative. The KSHV DNA was detected in 41% HIV/KSHV-positive specimens with a viral load range from 1 to 2.0 x 10^{11} KSHV copies per 10^6 cells, two-thirds of whom were classified as low "viral shedders" and a third as high "viral shedders". Of the HIV-negative/KSHV-positive subjects, viraemia was detected in 23% specimens, with a load range from 2 to 97 000 KSHV copies per 10^6 cells with only one subject classified as a high "viral shedder". Only 24% of HIV-positive participants were on ART, 14% of those were KSHV seropositive with one low "viral shedder". The remaining 10% were KSHV seronegative.

CHAPTER 4. DISCUSSION

4.1 Introduction

This chapter discusses the findings of this study in comparison to other local and global studies. This chapter starts by discussing the participants' demographic details, after which the findings of each objective are addressed. The discussion includes the results of similar studies conducted in other provinces of South Africa, as well as in other countries.

4.2 Objective 1: To establish participants HIV status and medical history-demographic data

The demographic details of the HIV-positive and HIV-negative groups were comparable with regards to the number and age. A study in SSA showed a peaking KSHV seroprevalence at the age range 33-39 years.[7] Similarly, our study showed HIV co-infected males at the age range of 30-37 and females at 26-49 years. The number of females in the cohort was almost double that of the male cohort. This ratio was also seen in the study conducted in the Gauteng and North West Provinces of South Africa.[70] The majority of the HIV-positive and HIV-negative participants in our study were Black South Africans, and this was representative of the South African population as well as the HIV-positive population.[2, 71]

Importantly noted was the significantly higher percentage of participants who were unemployed in the HIV-positive group (40%), as compared to the 11% in the HIV-negative group. This is in keeping with the past research in South Africa, where at least one in five HIV-positive individuals were unemployed.[72, 73]

KZN is the epicenter of the HIV epidemic in South Africa[2, 74], hence choosing an urban clinic in central Durban, was appropriate to conduct this cross-sectional study.

4.3 KSHV seroprevalence determined by KSHV ELISA

This study explored for reactivity to both lytic and latent antigens of KSHV to increase the sensitivity and specificity for identifying KSHV-positive participants'. The total cohort seroprevalence was marginally higher (6-20%) compared to Gauteng and North West Provinces, and Hlabisa District in KZN.[66, 70]

When analyzing seropositivity to KSHV as being positive for either lytic or latent antibodies, two-thirds of HIV-positive individuals were infected with KSHV, similar to West African Ghanaians.[60] The HIV-positive/KSHV seropositive was higher than the Johannesburg cohort and antenatal clinics in Gauteng, 70% vs 48%, 36.1%.[9, 69] KSHV seropositivity (37%) of HIV-negative individuals was almost 10 times higher than in the USA, Southeast Asia and Caribbean, but correlated with Zambia, Uganda and Ghana (37.5%, 38.8% and 41.9% respectively).[53] Our HIV-negative group was greater in KSHV seropositivity than reported in a Ghanaian study (37% vs 23.7%).[60] Regarding lytic antibody differences between HIV-positive and HIV-negative individuals, 50% of those who were HIV-positive expressed the lytic antibody, which was higher than the Johannesburg cohort.[9]

Based on these findings, the HIV-positive individuals had a greater lytic antibody expression than those HIV-negative but with no difference between males and females. This was in contrast to the Johannesburg cohort, which showed 66% male and 34% female lytic antibody levels amongst their HIV-positive participants. However, the Johannesburg cohort consisted only of HIV-positive subjects, making it difficult to draw a comparison with an HIV-negative population.

The latent antibody expression in the HIV-positive individuals was 30% higher than the Johannesburg cohort [9], with no significant difference between the latent antibody expression between the males and females, although the HIV-negative males showed a higher latent antigen affinity than the females. While this study has shown raised expressions of lytic and latent antibodies in HIV-positive individuals, there was no conclusive differences in reactivity to either antigens between the males and females within the HIV-positive and HIV-negative groups.

The study confirms the findings that overall, HIV-positive individuals are more likely to be KSHV positive than HIV-negative individuals [9, 20, 60, 66, 69], hence placing them at greater risk of developing Kaposi's Sarcoma.[38, 75]

4.4 Objective 3: To evaluate KSHV salivary shedding through cell associated KSHV viral loads in those who were KSHV-seropositive

KSHV DNA was detected in 35% of the cohort from salivary specimens, which suggests that KSHV seropositivity does not necessarily imply viral shedding, as seen in another local study.[9] KSHV shedding categories were formulated in order to interpret and compare the large variation between the KSHV viral load results. The positive KSHV "shedders" were aged between 29-44 years, which appears to be a common age range for HIV/KSHV co-infections.[37, 70] The KSHV viral load on average was higher in HIV-positive group, however a statistical analysis could not be drawn between the two groups as the HIV-negative had fewer detectable viral loads. The KSHV viral load variance between the two groups was significant (p-value < 0.0001). Similar to this cohort, other studies have documented higher KSHV viral loads in HIV-positive individuals, with the likely implication for KS pathogenesis, [11, 34, 35] with saliva being a possible route of transmission.[35, 42, 47]

There were 24% HIV-positive participants on ART and 14% expressed KSHV antibodies, with a low positive KSHV DNA detected in only one participant. There exists a possibility that the ART may have suppressive factors affecting KSHV expression and replication, ultimately decreasing KSHV viremia, as shown in other studies.[76-80] Four additional individuals were analyzed from the HIV-negative group to detect KSHV DNA, these participants having fallen into the indeterminate category by the KSHV ELISA assay. A low positive KSHV DNA was detected in only one HIV-negative, indeterminate KSHV participant.

The KSHV viral load quantification in HIV-negative individuals, although lower than in the HIV-positive individuals, may warrant further KS screening and monitoring. The significantly higher KSHV viral load in HIV-positive participants not on ART, may indicate a greater risk of progressing to KS.[11, 34, 76, 81]

4.5 Summary

The study demographics correlated with many similar studies in this field with males and females in their mid-twenties to late forties, co-infected with HIV and KSHV. However, contradictory to some studies, the female population was higher and the younger individuals were most affected by the co-infection. The HIV-positive group showed an almost four times higher unemployment rate than the HIV-negative group as shown in previous studies as well.

Based on this urban South African cohort, just over half the population is positive for KSHV. A third of HIV-negative individuals and two-thirds of HIV-positive individuals are KSHV positive based on being positive for either lytic or latent antibodies. It is evident that HIV-positive individuals present more frequently with KSHV positivity ($p < 0.0009$).

KSHV DNA was detected in just over a third of those KSHV/HIV-positive with a large variance and extremely higher viral burden. KSHV DNA detection was much lower in KSHV positive/HIV-negative individuals with a lower variance and lower viral titres. The HIV-positive individuals showed a significantly higher viral burden and variance (p-value < 0.0001) compared to the KSHV positive/HIV-negative. Historical studies have documented higher KSHV viral loads with progression to clinical KS.

Although participants on ART had lower KSHV viral loads compared to those not on ART, using ART as a therapeutic approach to control KSHV infectivity needs further investigation.

The HIV-positive and high viral shedding participants are at high risk of clinical Kaposi's sarcoma and we recommend that they be screened for this malignancy by healthcare workers. In addition testing of HIV-positive adults for KSHV is a recommendation so that those at risk can be followed up more closely for development of KSHV related disease.

CHAPTER 5. CONCLUSION

South Africa is faced with many challenges in addressing the HIV/AIDs epidemic, including opportunistic infections, HIV associated malignancies and diseases that hinder the quality of patients' lives. Early identification of those at risk of any of these associated conditions is crucial to decreasing this burden. Kaposi's sarcoma is the commonest malignancy associated with HIV in Sub-Saharan Africa. Despite access to treatment the outcome of this condition remains poor. KwaZulu-Natal is the epicenter of the HIV epidemic in South Africa and therefore evaluation of the KSHV (the causative agent in KS) seroprevalence in this population is vital to decreasing the burden of this malignancy.

A high KSHV seroprevalence was demonstrated amongst adults attending an urban HCT clinic in KwaZulu-Natal, South Africa. Those co-infected with HIV presented with a higher KSHV viral load and are at a higher risk of developing clinical Kaposi's sarcoma. We strongly recommend that all HIV infected patients be screened to prevent the spread of this oncogenic virus and those infected be monitored and treated aggressively to decrease the burden of KS.

Limitations to the study

This cross-sectional study only enabled the analysis of samples from one time point. A larger, longitudinal study over a longer time span, with a more extensive specimen and data collection would contribute to our understanding of the associations and risks with KSHV infection. Incomplete data on antiretroviral treatment, immune response and employment status limited the evaluation of these as risk factors for KSHV viral shedding and seroprevalence.

Future recommendations

The following recommendations are made as a result of the study findings:

- Prevention is the key to curbing the spread of KSHV infection and the subsequent development of KS. This can be achieved by educating the general population and especially the HIV population on KSHV infection and possible modes of infection and transmission. Campaigns to promote education on KSHV amongst health care workers especially at a primary care level are essential. This can be done in forms of information sharing workshops and roadshows. Dissemination of pamphlets at clinics, hospitals, schools and tertiary institutes (guest lectures).
- HIV-positive participants and those with a high KSHV shedding are at high risk of clinical Kaposi's sarcoma, and we recommend that they be screened for this malignancy by healthcare workers.

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APPENDIX I. PATIENT INFORMATION SHEET (English)

INFORMATION DOCUMENT

(Please refer to the UKZN Biomedical Ethics Terms of Reference at http://research.ukzn.ac.za/ResearchEthics11415.aspx)

Study title: SEROPREVALENCE AND VIRAL QUANTIFICATION OF KAPOSI'S SARCOMA ASSOCIATED HERPESVIRUS (KSHV) IN A HUMAN IMMUNODEFICIENCY VIRUS (HIV) POSITIVE ADULT SOUTH AFRICAN COHORT.

Good-day to you and thank you for affording us your time.

INTRODUCTION:

In order to take part of this research study, you need to understand the risks and benefits so that you can make an informed decision. This is known as informed consent. Once you understand the study, if you agree to participate, you will be asked to sign this form. Your decision to take part in the study is voluntary. This means that you are free to choose if you will take part in the study and this will not affect any other treatment you receive.

The study is being conducted by Dr. Anisa Mosam and Mrs. Shoohana Singh from the Department of Dermatology, UKZN. The study is sponsored by CAPRISA.

PURPOSE AND BACKGROUND:

This study is will collect information on a virus called Human Herpes Virus 8 (HHV8) in people with and without HIV. In some individuals that have the virus and HIV, it may cause a cancer called Kaposi's Sarcoma. It is hoped that this information will enable us to develop appropriate research studies in HHV8 and Kaposi's Sarcoma.

HOW MANY PEOPLE WILL TAKE PART IN THE STUDY?

70 HIV positive and 70 HIV negative individuals

WHAT WILL HAPPEN IN THE STUDY?

Once you agree to take part in this study, the following will happen:
Personal information such as age, sex, and ethnicity, medical history, and laboratory information including your HIV test results, will be collected on a data sheet.
You will be required to donate one blood and saliva sample only.
These samples will be tested to see if you have the HHV8

WHAT ARE THE STUDY RISKS?

You may experience a little discomfort during the blood draw, but the saliva collection is totally non-invasive.

WHAT ARE THE BENEFITS OF THE STUDY?

There is no direct medical benefit to you if you agree to take part in this study. We hope that what is learned from this study will help other patients with HIV and HHV8 in the future.

WHAT OTHER ALTERNATIVES ARE THERE?

You are free not to take part in the study

PARTICIPATION IS ENTIRELY VOLUNTARY

PARTICIPATION IN RESEARCH IS VOLUNTARY.

You are not forced to take part in this study and you are free to withdraw at any stage of the study. Refusing to participate will not affect any care that you continue to receive.

WHAT ARE THE COSTS?

There will be no costs to you as a result of taking part in this study. You will be reimbursed an amount of R 50.00 (Fifty Rands) for transportation costs.

WHAT ABOUT CONFIDENTIALITY?

Every effort will be made to keep personal information confidential. Absolute confidentiality cannot be guaranteed. Personal information may be disclosed if required by law.
The Organization that may inspect and/or copy your research records for quality assurance and data analysis will be the Biomedical Research Ethics Committee.
A record of your results for this study will be kept in a confidential form at the CAPRISA Clinic located at the Durban Chest Clinic. The confidentiality of computer records is safely guarded. No information by which you can be identified will be released or published.

WHOM DO I CALL IF I HAVE QUESTIONS?

For more information regarding the research or research related risks or injuries, please feel free to ask the study doctor, Dr. A Mosam 031 360 3550, 031 260 4565 or Ms. Shoohana Singh on 031 260 7769 or 084 247 5783.

For questions about your rights as a research participant, contact the Institutional Review Board, **BIOMEDICAL RESEARCH AND ETHICS ADMINISTRATION** (which is a group of people who review the research to protect your rights) at 031 2604769, Private Bag X 54001 Durban 4000 or email: BREC@ukzn.ac.za

APPENDIX II. PATIENT INFORMATION SHEET (isiZulu)

INCWADI YOLWAZI (INFORMATION DOCUMENT)

(Please refer to the UKZN Biomedical Ethics Terms of Reference at http://research.ukzn.ac.za/ResearchEthics11415.aspx)

Isihloko Socwaningo: UKWANDA SEKUKONKE KUBANTU ABATHILE ESIKHATHINI ESITHILE NJENGOBA KUSUKE KUKALWE NGOKUHLOLWA KWEGAZI NOKUBALWA KWESIBALO SAMAGCIWANE KWESIFO ESIBIZWA NGE-KAPOSI' SARCOMA ESIHAMBISANA NEGCIWANE LE-HERPES (KSHV) KUBANTU BASENINGIZIMU AFRIKA ABELASHWA NDAWONYE NJENGEQEMBU ABANE-HIV NABANGENAYO.

Sawubona futhi sibonga nesikhathi sakho osipha sona.

ISINGENISO:

Ukuze ukwazi ukubamba iqhaza kulolu cwaningo, kufanele uqonde izingozi nezinzuzo ukuze uthathe isinqumo esakhelwe olwazini. Lokhu kwaziwa ngokuthi yimvume eyakhelwe olwazini. Uma usuluqonda ucwaningo, uma uvuma ukubamba iqhaza, uzocelwa ukuthi usayine lefomu. Isinqumo sakho sokubamba iqhaza ocwaningweni ngokokuzithandela ngokwakho. Lokhu kusho ukuthi ukhululekile ukukhetha ukuthi uzolibamba yini iqhaza ocwaningweni kanti lokhu ngeke kube nomthelela kunoma yimiphi imithi yokwelashwa oyitholayo.

Ucwaningo lwenziwa uDkt Anisa Mosam noNkk Shoohana Singh abavela emnyangweni womkhakha obhekene nokwelashwa kwesikhumba obizwa nge-Department of Dermatology e-UKZN. Ucwaningo luxhaswe yi-CAPRISA.

INHLOSO NESENDLALELA:

Lolu cwaningo luzoqoqa ulwazi ngegciwane elibizwa nge-Human Herpes Virus 8 (HHV8) kubantu abane-HIV nabangenayo. Kwabanye abantu abanegciwane ne-HIV,

lingadala isifo somdlavuza esibizwa nge-Kaposi's Sarcoma. Kwethenjwa ukuthi lolu lwazi luzosivumela ukuthi sisungule ucwaningo nge-HHV8 ne-Kaposi's Sarcoma.

BANGAKI ABANTU ABAZOBAMBA IQHAZA OCWANINGWENI?

Abantu abangama-70 abane-HIV nabangama-70 abangenayo.

KUZOKWENZEKANI OCWANINGWENI?

Uma uvuma ukubamba iqhaza kulolu cwaningo, okulandelayo kuzokwenzeka:
Ulwazi oluphathelene nawe njengeminyaka yobudala, ubulili, ubuzwe, umlando ophathelene nokwelashwa, nolwazi oluphathelene nelabhorethri kubandakanya imiphumela yokuhlolelwa i-HIV, luzofakwa ephepheni lemininingwane.
Uzocelwa ukuthi unikele ngesampula elilodwa kuphela legazi namathe.
La masampula azohlolwa ukubheka ukuthi ngabe unayo yini i-HHV8.

YIZIPHI IZINGOZI ZOCWANINGO?

Ungase uzwe ukungaphatheki kahle okuncane ngesikhathi kudonswa igazi, kodwa ukuthathwa kwamathe akunayo nencane inkinga.

YIZIPHI IZINZUZO ZOCWANINGO?

Akukho nzuzo yokwelashwa eqondile kuwe uma uvuma ukubamba iqhaza kulolu cwaningo. Sethemba ukuthi okufundwa kulolu cwaningo kuzosiza iziguli ezine-HIV ne-HHV8 ngomuso.

YIZIPHI EZINYE IZINDLELA EZIKHONA?

Ukhululekile ukungabambi iqhaza ocwaningweni

UKUHLANGANYELA KUNGOKOKUZITHANDELA NGOKUPHELELE

UKUHLANGANYELA OCWANINGWENI KUNGOKOKUZITHANDELA.
Awuphoqiwe ukubamba iqhaza kulolu cwaningo futhi ukhululekile ukuhoxa kunoma yiliphi ibanga locwaningo. Ukwenqaba kwakho ukubamba iqhaza ngeke kube nomthelela kunoma yikuphi ukunakekelwa oqhubeka nokukuthola.

ZIYINI IZINDLEKO?

Ngeke kube khona zindleko eziza kuwena ngenxa yokubamba iqhaza kulolu cwaningo. Uzonxeshezelwa ngenani lika-R50.00 (Amashumi amahlanu amarandi) yezindleko zokuhamba.

KWENZEKANI NGEMFIHLO?

Kuzokwenziwa yonke imizamo ukugcina ulwazi luyimfihlo. Angeke sikwazi ukuqinisekisa imfihlo ephelele. Ulwazi oluphathelene nawe lungadalulwa uma kudingwa umthetho.

INhlangano engahlola futhi/noma engakopisha amarekhodi akho ocwaningo ukwenzela ukuqinisekisa izinga nokuhlaziywa kwemininingwane kuyoba yi-Biomedical Research Ethics Committee.

Irekhodi lemiphumela yakho yalolu cwaningo liyogcinwa efomini eyimfihlo emtholampilo wase-CAPRISA osemtholampilo wesifo sesifuba waseThekwini i-Durban Chest Clinic. Ubumfihlo bamarekhodi ekhompyutha bubhekwe ngokuphephile. Akukho lwazi ongaziwa ngalo oluyodedelwa noma lushicilelwe.

NGITHINTA BANI UMA NGINEMIBUZO?

Olunye ulwazi mayelana nocwaningo noma izingozi noma ukulimala okuphathelene nocwaningo, ukhululekile ukuthi ubuze udokotela wocwaningo, Dkt A Mosam ku-031 360 3550, 031 260 2565 noma uNk Shoohana Singh ku-031 260 7769 noma ku-084 247 5783.

Ngemibuzo mayelana namalungelo njengombambiqhaza wocwaningo, thintana nebhodi lokubuyekeza lesikhungu okuyi-Institutional Review Board, ne-BIOMEDICAL RESEARCH AND ETHICS ADMNISTRATION (okuyiqembu labantu ababuyekeza ucwaningo ukuvikela amalungelo akho) ku-031 2604769, Private Bag X 54001 Durban 4000 noma kwi-email: BREC@ukzn.ac.za

APPENDIX III. CONSENT FORM (English)

CONSENT DOCUMENT

Consent to Participate in Research

SEROPREVALENCE AND VIRAL QUANTIFICATION OF KAPOSI'S SARCOMA ASSOCIATED HERPESVIRUS (KSHV) IN A HUMAN IMMUNODEFICIENCY VIRUS (HIV) POSITIVE AND NEGATIVE ADULT SOUTH AFRICAN COHORT.

I, _____agree to participate in this research study.
I agree that the study has been explained to me in a language that I can understand and I understand the study information sheet. I have discussed the advantages and disadvantages of participating in the study. I agree to divulging my HIV status, as well as personal and medical information for the purposes of this study only. I also agree to the collection of one blood and saliva samples.
I know that I can leave the research study at any time without prejudice. I will still be able to attend and make full use of all the facilities at the clinic as usual.

You may contact Dr. A Mosam 031 360 3550, 031 260 4565 or Shoohana Singh on 031 260 7769 or 084 247 5783 at any time if you have questions about the research or if you are injured as a result of the research.

You may contact the Biomedical Research Ethics Office on 031-260 4769 or 260 1074 or Email BREC@ukzn.ac.za if you have questions about your rights as a research participant.

Your participation in this research is voluntary, and you will not be penalized or lose benefits if you refuse to participate or decide to stop at any time.

If you agree to participate, you will be given a signed copy of this document and the participant information sheet which is a written summary of the research.

The research study, including the above information, has been described to me orally. I understand what my involvement in the study means and I voluntarily agree to participate. I have been given an opportunity to ask any questions that I might have about participation in the study.

_____ _____

Signature of Participant **Date**

_____ _____

Signature of Witness **Date**
(Where applicable)

_____ _____

Signature of Translator **Date**
(Where applicable)

APPENDIX IV. CONSENT FORM (isiZulu)

INCWADI YEMVUME (CONSENT DOCUMENT)

Imvume yokubamba iqhaza ocwaningweni

UKWANDA SEKUKONKE KUBANTU ABATHILE ESIKHATHINI ESITHILE NJENGOBA KUSUKE KUKALWE NGOKUHLOLWA KWEGAZI NOKUBALWA KWESIBALO SAMAGCIWANE KWESIFO ESIBIZWA NGE-KAPOSI' SARCOMA ESIHAMBISANA NEGCIWANE LE-HERPES (KSHV) KUBANTU BASENINGIZIMU AFRIKA ABELASHWA NDAWONYE NJENGEQEMBU ABANE-HIV NABANGENAYO.

Mina, _____ngiyavuma ukubamba iqhaza kulolu cwaningo.

Ngiyavuma ukuthi ngiye ngachazelwa ucwaningo ngolimi engiluqondayo futhi ngiyaliqonda iphepha lolwazi locwaningo. Ngixoxile ngokuzohlomulisa nokungeke okumayelana nokubamba iqhaza ocwaningweni. Ngiyavuma ukudalula isimo sami se-HIV, kanjalo nolwazi oluphathelene nami nalolo oluphathelene nokwelashwa ukwenzela lolu cwaningo kuphela. Ngiyavuma futhi ukuthi kuthathwe isampula elilodwa legazi nelamathe.

Ngiyazi ukuthi ngingashiya noma nini ocwaningweni ngaphandle kokubandlululwa. Ngisazokwazi ukuza futhi ngisebenzise ngokugcwele konke okwasemtholampilo njengokujwayelekile.

Ungathintana noDkt A Mosam ku-031 360 3550, 031 260 4565 noma no-Shoohana Singh ku-031 260 7769 noma ku-084 247 5783 noma nini uma unemibuzo ngocwaningo noma ulimala ngenxa yocwaningo.

Ungathintana ne-Biomedical Research Ethics Office ku-031 260 4769 noma ku-260 **1074** noma nge-email ethi BREC@ukzn.ac.za uma unemibuzo mayelana namalungelo akho njengombambiqhaza wocwaningo.

Ukubamba kwakho iqhaza kulolu cwaningo kungukuzithandela ngokwakho, futhi ngeke uhlawuliswe noma ungabe usasizakala uma wenqaba ukubamba iqhaza noma uthatha isinqumo sokuyeka noma nini.
Uma uvuma ukuhlanganyela, uzonikezwa ikhophi esayiniwe yale ncwadi kanye nephepha lolwazi oluphathelene nombambiqhaza/nomhlanganyeli okuyisifinqo esibhaliwe socwaningo.

Isifundo socwaningo, kubandakanya ulwazi olungenhla, ngiye ngachazelwa lona ngomlomo. Ngiyakuqonda ukubandakanyeka kwami ocwaningweni ukuthi kusho ukuthini futhi ngiyazivumela ngokuthanda kwami ukubamba iqhaza. Ngiye nganikezwa ithuba lokubuza noma yimiphi imibuzo engingaba nayo ngokubamba iqhaza ocwaningweni.

_____ _____

Isignesha yombambiqhaza **Usuku**

_____ _____

Isignesha kafakazi **Usuku**
(Lapho kufanele khona)

_____ _____

Isignesha yomhumushi **Usuku**
(Lapho kufanele khona)

APPENDIX V. DATA SHEET

HHV 8 STUDY DATA SHEET

- **DEMOGRAPHICS**

Date:	**PID/Study No: 2806-12-**			
Address:				
Phone no:	**Employment status:**			
Race:	**Age or D.O.B:**	**Gender**	**M**	**F**

- **IS THE PATIENT CURRENTLY ON ANTI RETROVIRAL TREATMENT?**

Y	**N**

- **HAS THE PATIENT EVER BEEN ON ANTI-RETROVIRAL TREATMENT?**

Y	**N**

IF YES, WHEN WAS IT STARTED AND ENDED AND WHAT WAS THE NAMES OF THE MEDICATION?

Regimen:	
Start Date:	**End Date:**

- **INVESTIGATIONS**

PLEASE PROVIDE CD4 AND HIV VIRAL LOAD RESULTS IF THE PATIENT HAD THESE TESTS DONE.

DATE		
CD4 Count		
HIV Viral Load		

DOES THE PATIENT COMPLAIN OF ANY OTHER

Y	**N**

ILLNESS?

IF YES, PLEASE RECORD ON TABLE WHAT THE ILLNESS IS HOW LONG (DURATION) THEY EXPERIENCED IT AND IF THEY RECEIVED TREATMENT FOR THE ILLNESS

ILLNESS/ CONDITION DURATION TREATMENT

- **IS THERE ANY OTHER IMPORTANT INFORMATION THAT THE PATIENT WISHES TO SHARE?**

NOTES:

_____

_____

_____

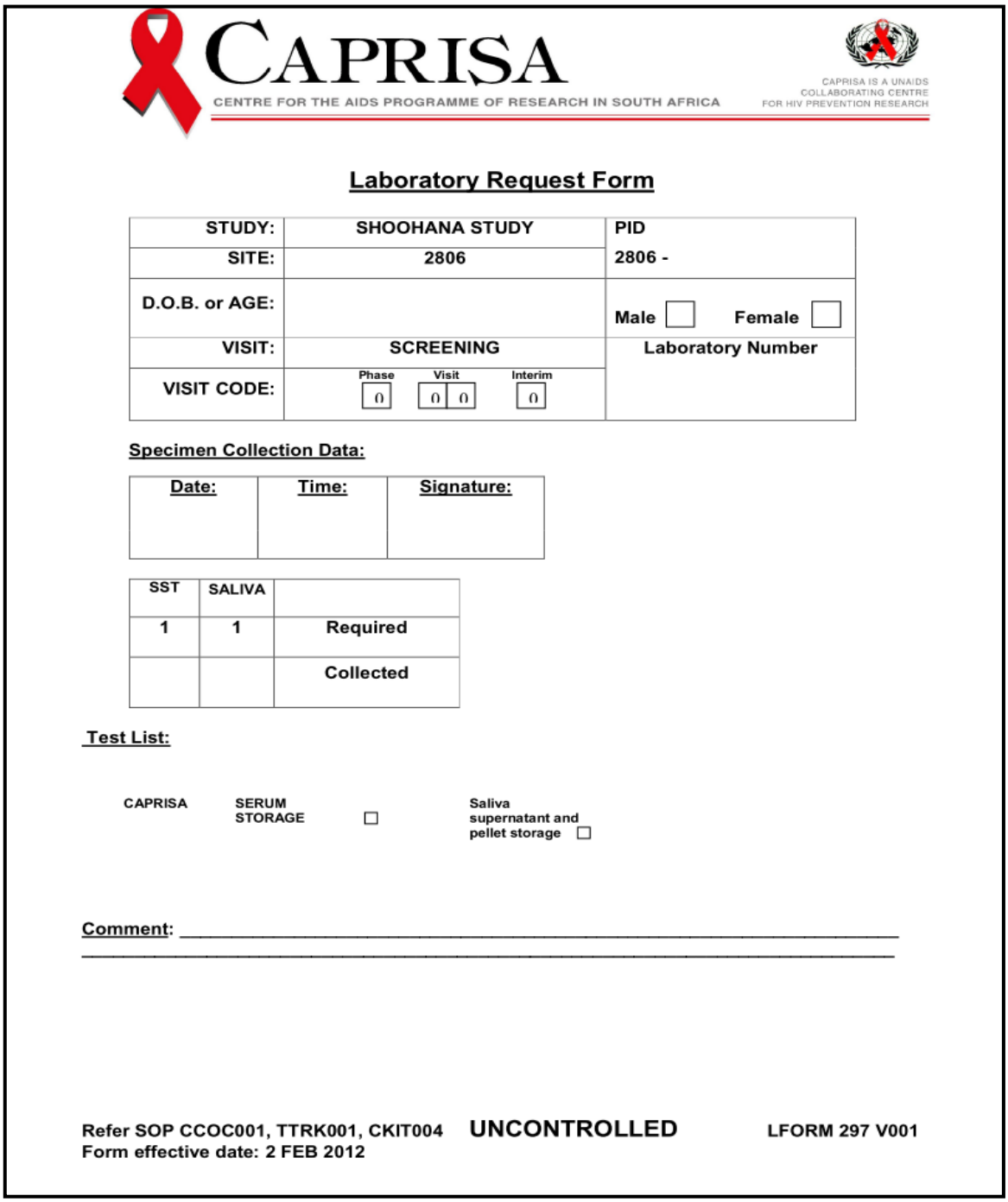
_____

_____

STAFF COMPLETION: _____ DATE:_____

Version 1.0 (25 Feb 2013)

APPENDIX VI. LABORATORY SPECIMEN TRACKING FORM



CAPRISA

CENTRE FOR THE AIDS PROGRAMME OF RESEARCH IN SOUTH AFRICA

CAPRISA IS A UNAIDS COLLABORATING CENTRE FOR HIV PREVENTION RESEARCH

Laboratory Request Form

STUDY:	SHOOHANA STUDY	PID
SITE:	2806	2806 -
D.O.B. or AGE:		Male ☐ Female ☐
VISIT:	SCREENING	Laboratory Number
VISIT CODE:	Phase 0 Visit 0 0 Interim 0	

Specimen Collection Data:

Date:	Time:	Signature:

SST	SALIVA	
1	1	Required
		Collected

Test List:

CAPRISA SERUM STORAGE ☐ Saliva supernatant and pellet storage ☐

Comment: _____

_____

Refer SOP CCOC001, TTRK001, CKIT004 UNCONTROLLED LFORM 297 V001
Form effective date: 2 FEB 2012

SUPPLEMENTARY FIGURES

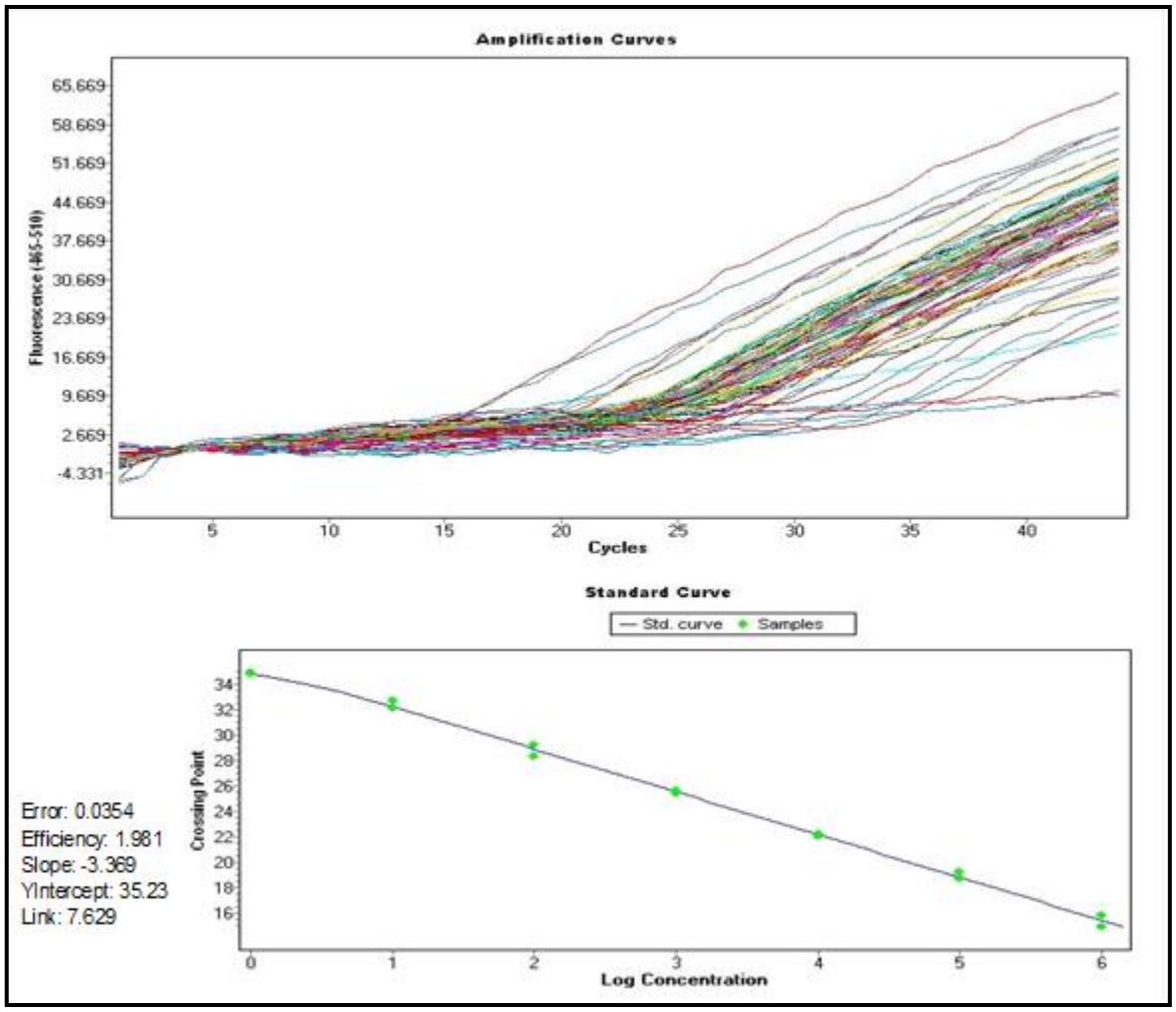


Figure S1: *Standard curve and Amplification curve for ERV3-housekeeping gene*

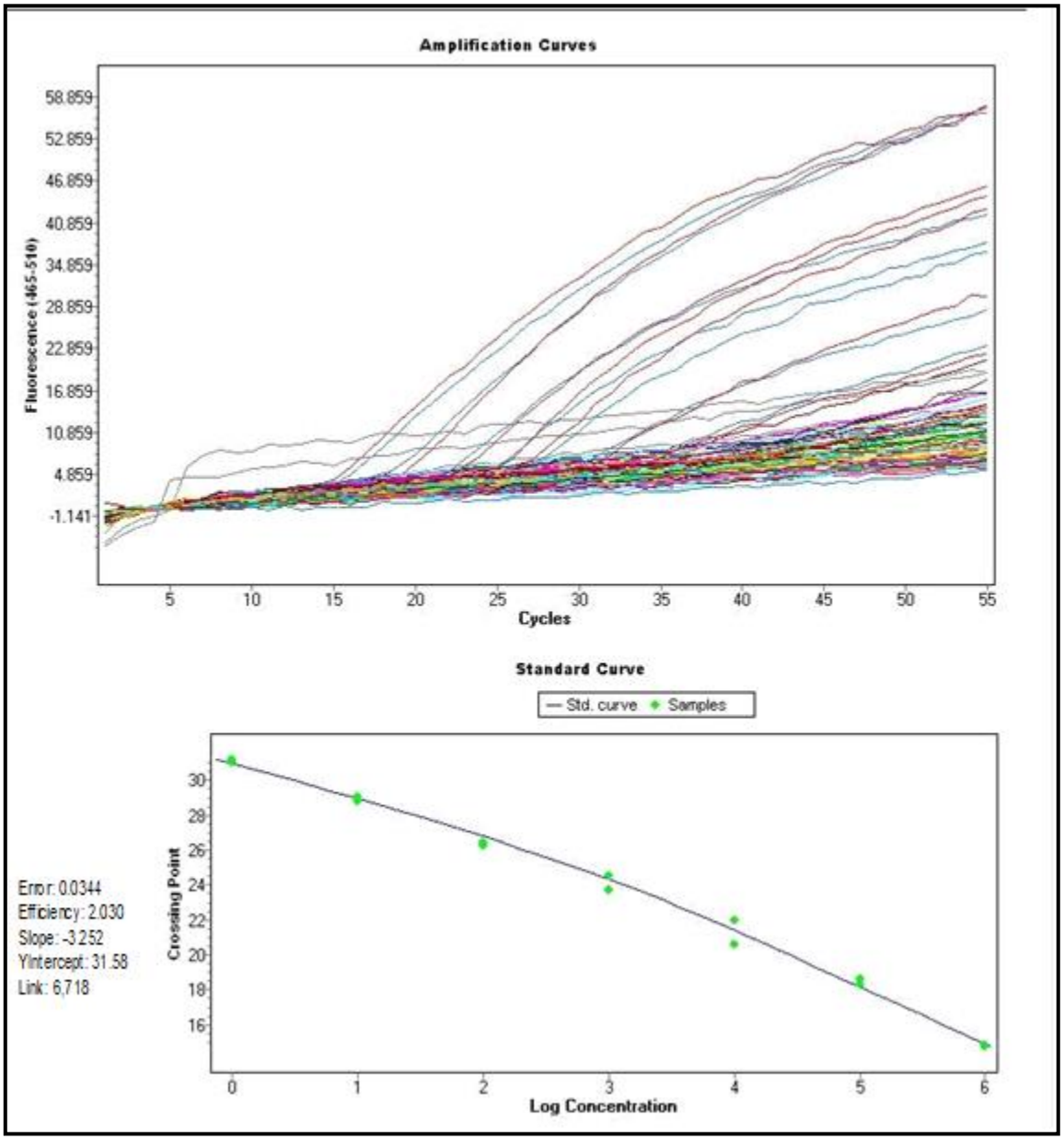


Figure S2: *Standard curve and Amplification curve for K6 – viral gene*