
# DETECTION AND CHARACTERIZATION OF HUMAN HERPES VIRUS-8 IN AN HIV-INFECTED COHORT IN CAMEROON

By

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A dissertation submitted in fulfilment of the requirements for the award of Master of Science degree in Microbiology to the Department of Microbiology, School of Mathematical and Natural Sciences, University of Venda, South Africa.

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## DECLARATION

I, Alayande Doyinmola Paul, declare that this dissertation is my own work in design and execution and that all references contained therein have been duly acknowledged. It is hereby submitted for the award of a Master of Science degree in Microbiology at the University of Venda, South Africa. It has not been submitted before for any degree or examination at this or any other University.

Signature_____ Date_____

Alayande Doyinmola Paul

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## DEDICATION

*This dissertation is dedicated to God Almighty,*

*my late father (Pastor S. F. Alayande), grandma (Yee Mekolani)*

*and my loving family.*

*I thank you all.*

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## *OH HOW WE "8" YOU*

Oh Herpesvirus 8,

Thou, we hate,

We know your story,

You grew up with some guys who don't even know your name (MSM)

Then in 1994, Chang and Moore christened you (discovery)

Since then, you became an icon to be reckoned with

Are you so promiscuous to the point of having sexual intercourse with every being (can infect all human), regardless of their immune and disease status?

Initially, we heard you were in love with Kaposi, a simple derma (associated with KS)

But now, you give deep kisses to MCD, PEL, MM, Sarcoidosis and even pulmo Tuberculosis (saliva exchange, disease associations)

Till date, we don't understand why and how you do it (means of transmission)

You made almighty HIV fall in love with you, (AIDS-KS)

Gave your blood to save aging Mediterranean, (classic and Iatrogenic KS in Mediterranean)

Chose Afrika as your residence yet you are found everywhere even in US (African/Endemic KS, Epidemic KS)

Well, we won't stop you from being promiscuous, but we will surely stop you from throwing your discus into every cause (gene expression; expressing yourself)

Here we are declaring war against you saying NO to your advances (risk factors)

We would neither blush nor hush but we would confront you with your weaknesses.

ALAYANDE DOYINMOLA PAUL

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## Abstract

**Background**: Human Herpes Virus-8 (HHV-8) and Human Immunodeficiency Virus (HIV) are endemic in sub-Saharan Africa. However, the prevalence of HHV-8 in HIV-infected individuals in sub-Saharan Africa has not been fully described and characterized.

**Objectives**: The objective of this study was to determine the seroprevalence and genetic subtypes of HHV-8 in an HIV-infected population in Cameroon.

**Methodology**: KSHV/HHV-8 Enzyme-linked Immunosorbent Assay (ELISA) kit (Advanced Biotechnologies Inc., USA) was used to detect IgG antibodies in the plasma of 406 HIV-infected outpatients of the Mutengene Baptist Health Centre, Cameroon. To detect the viral presence, a 233 bp fragment of the ORF 26 gene of HHV-8 was targeted by polymerase chain reaction (PCR) in total DNA purified from patients' whole blood. A 453 bp of the K1 gene was amplified by nested PCR, sequenced and phylogenetically analysed to infer subtypes. The online tool, Synonymous Non-synonymous Analysis Program (SNAP), was used to determine the rate of synonymous and nonsynonymous mutations in the K1 gene. The genetic variability among the derived K1 nucleotide sequences was determined by mean genetic distance analysis.

**Results**: Of the 406 participants, an HHV-8 seroprevalence of 79.1% was obtained. There was a statistically significant association of seroprevalence with age ($p$= 0.00), CD4+ cell count ($p$= 0.02), marital status ($p$= 0.02) and ownership of a transistor radio set ($p$= 0.00). Seventy samples (23.3%) were successfully amplified for ORF 26 gene confirming the presence of replicating virus. K1 sequences were obtained for 14 of the 20 (70%) K1 amplified DNAs. The mean genetic diversity of K1 sequences ranges from 0.0%-22.3%. Phylogenetic analysis revealed two infecting viral subtypes in the study cohort: subtype A5 (57.1%), and subtype B (35.7%). Greater positive selection and genetic diversity were observed in A5 subtype compared to B subtype of K1. Interestingly, one sample (BM 547) clustered with an unclassifiable sequence from South Africa.

**Conclusions and recommendation**: This study revealed the endemicity of HHV-8 infection in the studied population, with subtypes A5 and B as the most important epidemiological genetic variants. In addition, targeting the ORF 26 region by PCR could be an approach to detect replicating virus in individuals. Further studies should investigate the association between HHV-8 infection and KS development in the study area which is endemic for HIV. This study contributes data to the HIV/HHV-8 co-infection landscape in the study area and in Africa at large.

**Keywords:** Human Herpes Virus-8; Human Immunodeficiency Virus; Seroprevalence; Subtype; South-West; Littoral; Cameroon.

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## Abbreviations

| | |
|---|---|
| $^0$C | Degree Celsius |
| % | Percentage |
| µl | Microlitre |
| aa | Amino acid |
| AIDS | Acquired Immune Deficiency Syndrome |
| AIDS-KS | Acquired Immune Deficiency Syndrome associated-Kaposi's sarcoma |
| BLAST | Basic Local Alignment Search Tool |
| bp | base pairs |
| BCR | B-cell receptor |
| BHM | Baptist Health Mutengene |
| CCL | Chemokine ligand |
| cds | Coding Sequences |
| CMV | Cytomegalovirus |
| DNA | Deoxyribonucleic acid |
| EBV | Epstein-Barr virus |
| ELISA | Enzyme-linked Immunosorbent Assay |
| FLIP | FLICE Inhibitory Protein |
| G-C | Guanine-Cytosine |
| GPCR | G-protein-coupled receptor |
| GSK | Glycogen synthase kinase |
| HAART | Highly active antiretroviral therapy |
| HHV | Human Herpes Virus |
| HIV | Human immunodeficiency virus |
| HSV | Herpes simplex virus |
| HVS | Herpesvirus saimiri |
| IAP | inhibitor of apoptosis protein |
| ICTV | International Committee on Taxonomy of Viruses |
| IFA | Immunofluorescence Assay |
| IFN | Interferon |
| IgG | Immunoglobulin G |
| IL | Interleukin |
| IRF | Interferon regulator factor |
| ITAM | Immunoreceptor tyrosine-based activation Motif |
| KS | Kaposi's sarcoma |
| KSHV | Kaposi's sarcoma-associated Herpes Virus |

| | |
|---|---|
| LAMP | Latent antigen membrane protein |
| LANA | Latency-associated nuclear antigen |
| LUR | Long Unique Region |
| mA | Milliampere |
| MEGA | Molecular Evolutionary Genetics Analysis |
| mg | Milligram |
| MIP | Macrophage inflammatory protein |
| MIR | Modulator of immune recognition |
| ml | Millilitre |
| NB | Notice board |
| NCBI | National Center for Biotechnology Information |
| ng | nanogram |
| NJ | Neighbour Joining |
| nt | Nucleotide |
| MCD | Multicentric Castleman's Disease |
| mTOR | Mammalian target of rapamycin |
| ORFs | Open reading frames |
| PCR | Polymerase chain reaction |
| PEL | Primary Effusion Lymphoma |
| PI3K | Phosphatidylinositol 3-kinase |
| PKB/Akt | Protein Kinase-B |
| PTEN | Phosphatase and tensin homolog |
| SNAP | Synonymous Non-synonymous Analysis Program |
| SPSS | Statistical package for the social sciences |
| TAT | transcriptional transactivator |
| TNF | Tumour necrosis factor |
| TR | Terminal Repeats |
| UV | Ultra-violet |
| V | Volt |
| VEGF | Vascular endothelial growth factor |
| VZV | Varicella Zoster virus |

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# CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

## 1.1 INTRODUCTION

Infection with Human Herpes virus-8 (HHV-8) occurs in humans, regardless of the population being immunocompetent or immunosuppressed. Among the immunocompetent population, a long-term latent infection exists, although with asymptomatic shedding in saliva, with the possibility of transmission through different modes in different populations (Stolka *et al.,* 2014; Phipps *et al*., 2014; Minhas and Wood, 2014). However, in an immunosuppressed population such as HIV-infected, transplant recipients of organs; bone marrow and stem cells, lytic replication and persistent shedding of HHV-8 can result in Kaposi's sarcoma (KS) or other lymphoproliferative disorders (Isaacs *et al.*, 2016; Razonable, 2013; Luppi *et al.*, 2000a; Luppi *et al.*, 2000b; Chang *et al.*, 1994).

In an HIV-infected population, HHV-8 has been implicated as the etiological agent of AIDS-associated Kaposi's sarcoma (AIDS-KS) (Chang *et al.*, 1994) and two other lymphomas, that is, Primary Effusion Lymphoma (PEL) and Multicentric Castleman's Disease (MCD) in AIDS patients (Cesarman *et al.,* 1995; Soulier *et al.,* 1995). Based on serological assays, the prevalence of HHV-8 varies by geographical location. The seroprevalence ranges from 30-48% in HIV/AIDS population in North America to more than 60% in sub-Saharan Africa (Dedicoat and Newton, 2003; Ablashi *et al.*, 1999; Martin *et al.*, 1998; Gao *et al.*, 1996). Similarly, the prevalence of Kaposi's sarcoma varies widely among HHV-8 infected populations and reason(s) for this remain unknown (Dedicoat and Newton, 2003).

Although HHV-8 is characterized by high genetic variability across the whole genome, however, most phylogenetic studies employ either the conserved Open Reading Frame (ORF) 26 region or the hypervariable regions- ORF K1 and/or ORF K15 for identification and characterization (Tornesello *et al.*, 2010; Hayward and Zong, 2007; Lacoste *et al.*, 2000). Based on ORF K1 sequence analysis, molecular subtypes of HHV-8 (A, B, C, D, E, F and Z) have been found to vary geographically (Isaacs *et al.*, 2016; Kanno *et al.*, 2010; Hayward and Zong, 2007; Kajumbula *et al.*, 2006; Biggar *et al.*, 2000; Lacoste *et al.*, 2000).

## 1.2 LITERATURE REVIEW

### 1.2.1 CLASSIFICATION OF HERPES VIRUSES

Herpes virus is one of the largest human viruses ranging in size from 100-300nm. The double-stranded deoxyribonucleic acid (ds DNA) enveloped virus contains a linear genome encoding 100-200 genes. The name herpes was coined from a Greek word “Herpein” which means “to creep”, i.e. to develop gradually and imperceptibly (latently) in host’s cell (www.dictionary.com, May 3, 2015). Herpesviruses infect both vertebrates and invertebrates. The International Committee on Taxonomy of Viruses (ICTV) Herpesvirus Study Group categorised the order *Herpesvirales* into family *Herpesviridae* and subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* (Figure 1.1) based on their biological properties, genomic structure, sequence homology, presence of glycoprotein B gene or using the DNA sequence analysis of the DNA polymerase gene (Knipe *et al.*, 2001). However, many species are not assigned to any genera.

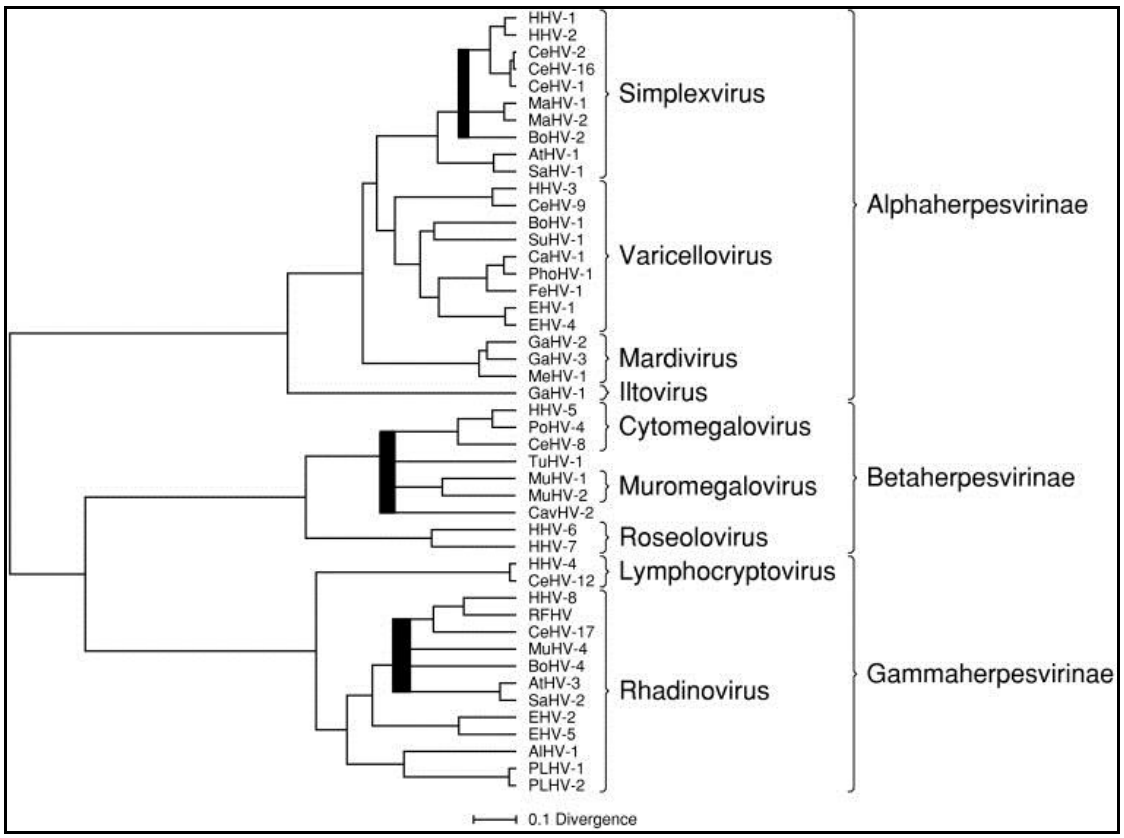



Figure 1.1 Composite phylogenetic tree for herpesviruses. The tree is based on amino acid sequence alignments of eight sets of homologous genes, constructed from maximum-likelihood trees for subsets of these genes, with molecular clock imposed (Adapted from McGeoch *et al.,* 2000).

### 1.2.2 CLASSIFICATION OF HUMAN HERPES VIRUSES

Of the more than 170 known herpesviruses, humans are infected routinely by 9, these are Herpes Simplex Virus types 1 and 2 (HHV-1, HHV-2), Varicella-Zoster Virus (HHV-3/VZV), Epstein-Barr Virus (HHV-4/EBV), Cytomegalovirus (HHV-5/CMV), Human Herpes Virus-6 (HHV-6A and HHV-6B), Human Herpes Virus 7 (HHV-7), Kaposi's sarcoma-associated Herpes Virus (HHV-8/KSHV) (Davison *et al.,* 2009; Davison *et al.*, 2005; Ackermann, 2004). Many a times, one herpesvirus species is found in association with different diseases (Table 1.1) (IARC, Working Group, 1997).

**Table 1.1 Classification of Human Herpesviruses into sub-family and associated diseases**

| Abbreviated ICTV Name | Name | Sub-Family | Genus | Associated diseases |
|---|---|---|---|---|
| HHV-1 | Herpes simplex virus 1 (HSV-1) | *α herpesvirinae* | Simplexvirus | Oral herpes, Genital herpes (predominantly orofacial) |
| HHV-2 | Herpes simplex virus 2 (HSV-2) | | | Oral herpes, Genital herpes (predominantly genital) |
| HHV-3 | Varicella Zoster virus (VZV) | | Varicellovirus | Chickenpox (in children), Shingles (in adults) |
| HHV-5 | Cytomegalovirus (CMV) | *β herpesvirinae* | Cytomegallovirus | Mononucleosis like syndrome, Retinitis |
| HHV-6A | Human lymphotropic virus | | Roseolavirus | Roseola infantum, Exanthema subitum |
| HHV-6B | | | | |
| HHV-7 | Human herpesvirus 7 (HHV-7) | | | Pityriasis rosea Iosidu excite |
| HHV-4 | Epstein-Barr virus (EBV) | *γ herpesvirinae* | Lymphocryptovirus | Mononucleosis, Burkitt's lymphoma, post-transplant lymphoproliferative syndrome, nasopharyngeal carcinoma |
| HHV-8 | Kaposi's sarcoma-associated virus (KSHV) | | Rhadinovirus | Kaposi's sarcoma, primary effusion lymphoma, Castleman's disease |

(Adapted from Blaškovičová and Labuda, 2014).

### 1.2.3 DISCOVERY OF HUMAN HERPES VIRUS-8 (HHV-8)

More than a century (1872) before HHV-8 was discovered, Kaposi's sarcoma (KS) was described by the Hungarian dermatologist, Dr. Moritz Kaposi as "idiopathic multiple pigmented sarcoma of the skin" (Kaposi, 1872). KS was described also in organ transplant recipients undertaking immunosuppressive therapy (Myers *et al.,* 1974), but not until a decade later, during the AIDS epidemic that KS became well known. Before 1994, there were suspicions that KS is caused by an infectious agent as this was backed by the presence of herpes-type virus particles in tumour specimen (Walter *et al.*, 1984; Giraldo *et al.*, 1980; 1972). The quest for the virus associated with KS led to the discovery of two small fragments of DNA present in almost all AIDS-KS specimens. The two DNA fragments- KS330Bam and KS631Bam possess DNA sequences unique to tissues of AIDS-KS patients (93%) and non-KS AIDS patients (15%) which suggested a new virus similar to, but different from known herpes virus sequences. Analysis of the DNA sequences revealed homology to capsid and tegument proteins of Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS) (Figure 1.2). The virus was named after the tumour it caused- Kaposi's sarcoma-associated Herpes Virus (KSHV) and for taxonomical purposes, it is called Human Herpes Virus-8 (HHV-8) (Chang *et al.*, 1994).

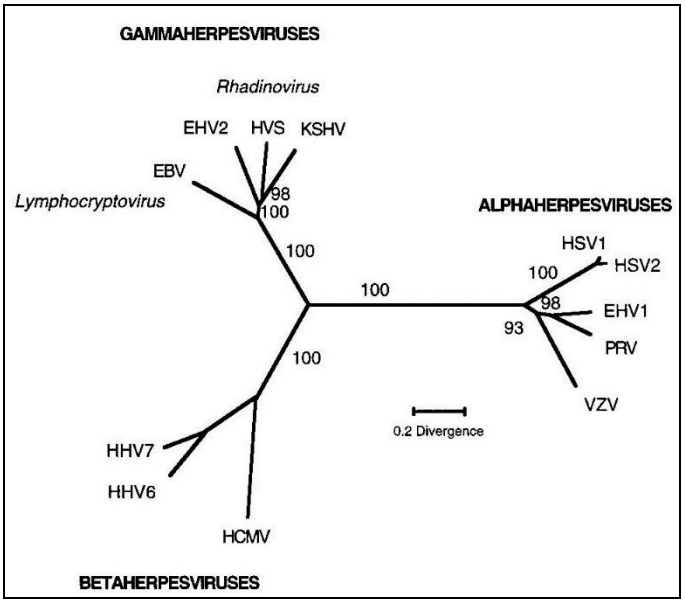



Figure 1.2 Phylogenetic tree of 12 representatives of herpesviruses. The radial, unrooted tree obtained by Neighbour Joining (NJ) method presents aligned amino acid sequences of the major capsid proteins with bootstrapping of 100 replicates. The figure clearly shows that HHV-8/KSHV is a gamma-herpesvirus and member of the genus *Rhadinovirus* (Moore *et al.,* 1996a).

### 1.2.4 STRUCTURE AND ORGANIZATION OF HHV-8 GENOME

HHV-8 is one of the largest human viruses known to date and it consists of four major components: a viral core; capsid; tegument and envelope. The central core contains a linear double stranded DNA (Deoxyribonucleic acid) embedded in a proteinaceous spindle (Sarid *et al.,* 1998). The viral DNA is enclosed by an icosahedral capsid of 100nm in diameter with 162 capsomeres. The capsomeres consist of 12 pentons and 150 hexons, containing five and six copies of the major capsid proteins respectively. The capsid is surrounded by an amorphous protein coat called tegument (Figure 1.3), which contains 30 or more viral proteins. The exterior of the tegument is surrounded by the lipid bilayer envelope, a glycoprotein-bearing membrane (Owen *et al.,* 2015; Sarid *et al.,* 2001).

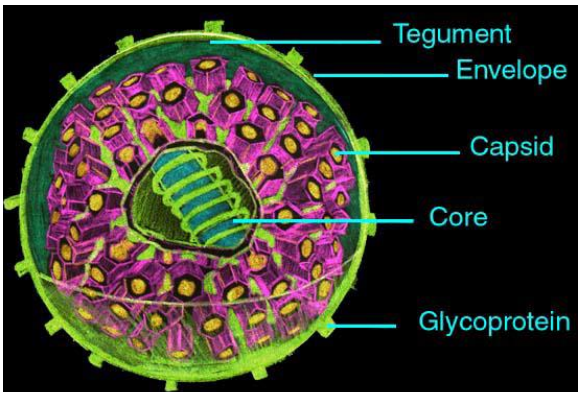



Figure 1.3 A model of the herpesvirus particle. All herpesvirus family members show the same general structure (Juli Solomon, www.brown.edu, accessed on July 2, 2015).

The genome of HHV-8 consists of 165-170,000 nucleic acid bases in length, with over 145 kilobases in the central long unique region (LUR) which contains all the Open Reading Frames (ORFs) and is flanked by approximately 20-30 kilobases of terminal repeat sequences (TR) at both ends of the genome (Moore and Chang, 2001; Russo *et al.,* 1996). The variation in the genome sizes of isolates is as a result of the number of TR the genome has, this ranges from 16 to 75 (Duprez *et al.,* 2007). The overall Guanine-Cytosine (G-C) content in the LUR and TR is 53.5% and 84.5% respectively. HHV-8 is similar to a closely related genus member, Herpesvirus saimiri (HVS) in structure and gene content (Ouyang *et al.*, 2014; Renwick *et al.*, 1999). Of the approximately 95 Open Reading Frames (ORFs) HHV-8 possesses, over 60 ORFs are homologous to other rhadinoviruses, while 25 encode novel proteins not observed in other human herpesviruses, 15 designated as K1 to K15 (Figure 1.4) are unique to HHV-8. Over 15 are pirated from cellular genes (Chang *et al*., 1996; Moore *et al.*, 1996b; Russo *et al.*, 1996). Transcription experiments with alternative splicing reveal new genes of HHV-8 with similar or modified functions (Pfeffer *et al.,* 2004; Gottwein, 2012).

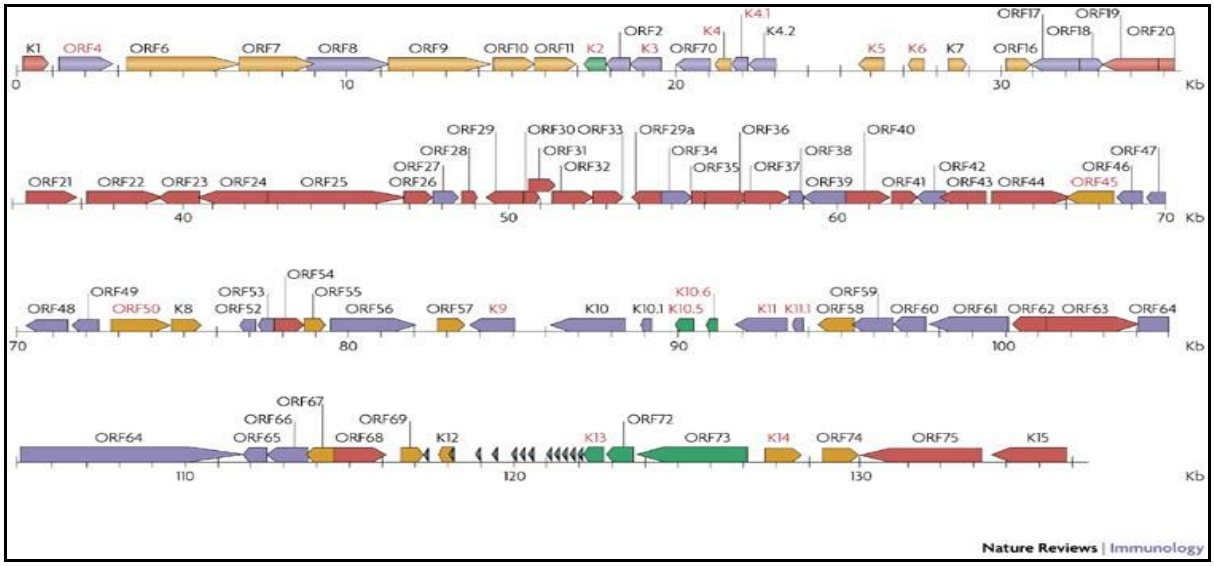



Figure 1.4 Structure of Human Herpes Virus-8 (HHV-8) genome (Coscoy, 2007).

### 1.2.5 GENES OF HHV-8 AND THEIR FUNCTIONS

HHV-8 possesses approximately 95 genes, some of which are shared and conserved with other herpesviruses, while some are pirated from the host genome (Fields *et al.,* 2013). Furthermore, HHV-8 possesses unique genes designated as open reading frames, ORF K1 to K15 with diverse functions (Table 1.2).

**Table 1.2 Unique genes of HHV-8, protein encoded and functions**

| Gene | Protein encoded | Functions |
|---|---|---|
| K1 | ITAM-containing signal transducing membrane glycoprotein | Transformation; inhibition of apoptosis; B cell activation; downregulation of surface B-cell receptor (BCR); immunomodulation; activation of PI3K/Akt/mTOR kinases; signalling |
| K2 | Interleukin-6 (vIL-6) | Anti-apoptosis, angioproliferation/angiogenesis, B cell proliferation, immunomodulation and autocrine/paracrine signalling |
| K3 | Modulator of immune recognition 1 (MIR1) | Immunomodulation and signalling |
| K4 | Interleukin 8-like CC chemokine; vCCL-3; vMIP-Ibeta/vMIP-III | Anti-apoptosis, angioproliferation/angiogenesis, immunomodulation and signalling |
| K4.1 | Interleukin 8-like CC chemokine; macrophage inflammatory protein (vMIP-II) | Anti-apoptosis, angioproliferation/angiogenesis, immunomodulation and signalling |
| K5 | E3 ubiquitin ligase; membrane protein; MIR2 | Immunomodulation and signalling |
| K6 | Interleukin 8-like CC chemokine; vCCL-1; vMIP-I | Anti-apoptosis, angioproliferation/angiogenesis, immunomodulation and signalling |
| K7 | Survivin: inhibitor of apoptosis protein (vIAP) | Anti-apoptosis; inhibition of vGPCR (G-protein-coupled receptor) expression and function |
| K8.1 | Glycoprotein | Switch from latent to lytic infection |
| K9 | Interferon regulator factor (vIRF-1) | Anti-apoptosis, transformation angioproliferation/angiogenesis, proliferation, immunomodulation, transactivation and signalling |
| K10.5 | LANA-2 | Anti-apoptosis and signalling |
| K11.5 | vIRF-2 | Anti-apoptosis, immunomodulation and signalling |
| K12 | Kaposin A, B and C | Transformation and signalling |
| K13 | FLICE Inhibitory Protein (vFLIP) | Anti-apoptosis, immunomodulation and signalling |
| K14 | OX-2 homolog (vOX2) | Signalling |
| K15 | Latent antigen membrane protein (LAMP) | Immunomodulation |

Refer to abbreviation: PI3K/Akt/mTOR (Adapted from Wen and Damania, 2010; Edelman, 2005).

### 1.2.6 MOLECULAR EPIDEMIOLOGY OF HHV-8 SUBTYPES AND VARIANTS

It is a fact that the genome of HHV-8 displays an elevated level of genetic diversity and the clustering of subtypes at certain loci as this is observed in genomes of several human herpesviruses. However, the diversity observed in HHV-8 is higher than what is found in other human herpesviruses. This has created the need to understand this phenomenon with the belief that it might give insights into the evolution of the genome over a short timescale, the biology, and pathogenesis of diseases associated with HHV-8 (Hayward and Zong, 2007).

The highest level of genetic variation is observed at both ends of the genome, (5' and 3' termini). That is, ORF K1 at the extreme left hand side (LHS) and ORF K15 at the extreme right hand side (RHS). ORF K15 encodes a 500-amino acid latency associated membrane protein (LAMP) but lacks the immunoreceptor tyrosine-based activation motif (ITAM) present in ORF K1 protein. ORF K15 is characterized into three alleles- P (Prototype), M (Minor) and N (Novel). The P and M alleles were described based on 70% amino acid divergence observed between HHV-8 isolates but with low level of variability (Kakoola *et al*., 2001; Poole *et al*., 1999; Hayward, 1999). The predominant subtype is the P allele, the M allele found in samples is approximately 20% while the N allele is very rare (Olp *et al.,* 2015; Alagiozoglou *et al.,* 2000).

ORF K1 is the first translatable gene of the genome. K1 gene has a long N-terminal signal sequence extracellular domain, a single transmembrane domain, a short (37 amino acid containing) conserved C terminal domain which contains an ITAM necessary for signal transduction (Lee *et al.,* 1998). Apart from signaling, other functions of K1 is summarised in Figure 1.5.

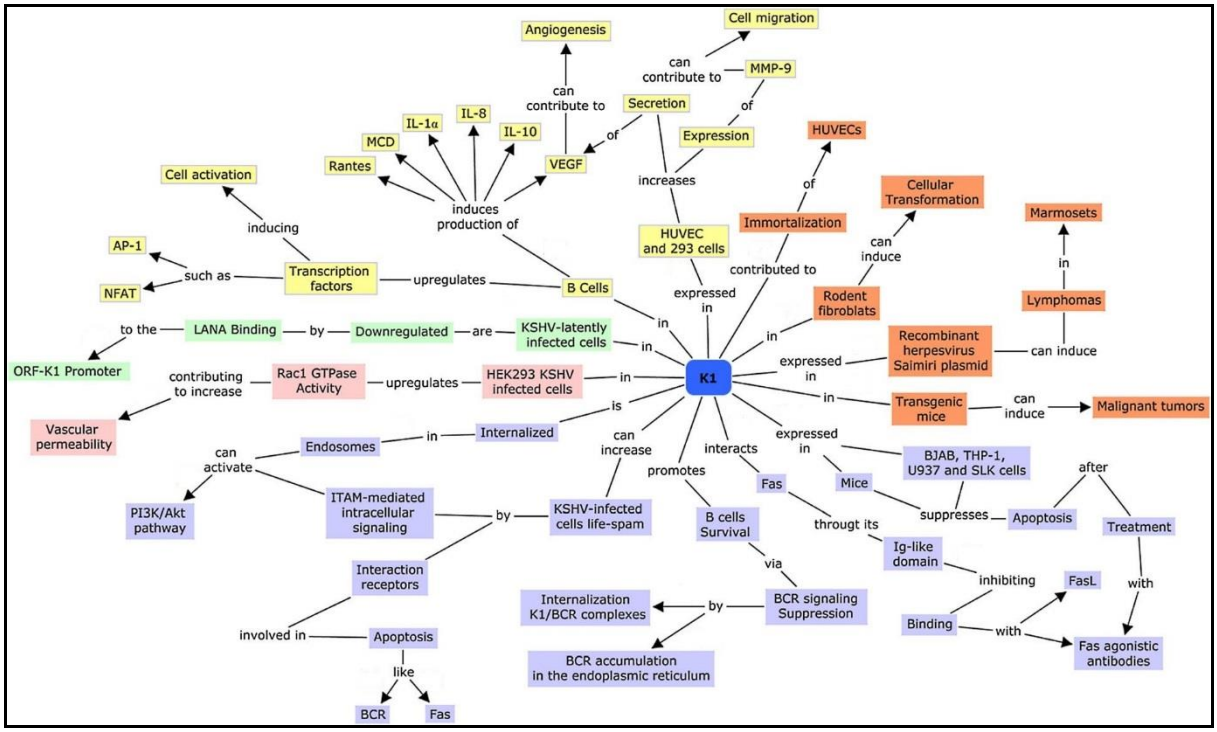



Figure 1.5 A concept map (CMap) summarizing functions of K1, produced with CMap Tools Software, Institute for Human and Machine Cognition, Ocala, FL, USA. (Adapted from Sousa-Squiavinato *et al.,* 2015).

K1 is also referred to as variable ITAM-containing protein (VIP). It encodes an early lytic transmembrane glycoprotein with an immunoglobulin receptor (ITAM) and 289 amino acids (Russo *et al.*, 1996; Zong *et al.*, 1999). ORF K1 gene is referred to as a hypervariable gene exhibiting 0.4-44% amino acid variability resulting from 85% nucleotide substitution between different HHV-8 isolates probably due to the strong selective pressure that acts on the gene (Olp *et al.,* 2015; Cook *et al.,* 1999). Although variability is observed throughout the whole gene, these amino acid (aa) variations and nucleotide substitutions are concentrated in two hypervariable regions of its extracellular matrix- Variable Region 1 (aa 54-92) and Variable Region 2 (aa 199-227). Within VR1 (Figure 1.6), VR * loop (aa 52-76) was defined (Ouyang *et al*., 2014; Hayward and Zong, 2007; Zong *et al.*, 1999). The two hypervariable regions are separated by conserved regions {C1 (aa 22-54) and C2 (aa 94-121)}. Furthermore, the Ig domain and the transmembrane domain within the cytoplasmic tail was defined.

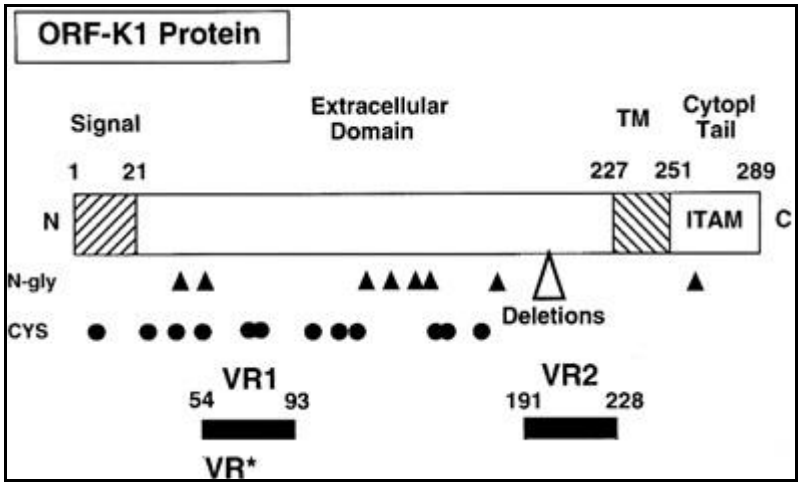



Figure 1.6 Predicted domain structure and key features of the hypervariable 289-amino-acid ORF K1 protein encoded between genomic nucleotide coordinates 105 and 974. Hatched bars denote signal peptide and transmembrane (TM) domains with amino acid boundaries indicated. Predicted N-glycosylation sites (NXS/NXT) (solid triangles) and the 12 conserved Cys residues (solid circles) are indicated. Cytopl, cytoplasmic. Locations of highly variable VR1 and VR2 domains (Lower panel) and the proposed hypervariable VR * domain (Adapted from Hayward and Zong, 2007).

Moreover, a much lower rate of polymorphisms (1-5%) was observed in nine discrete loci (approximately 5.6%) within the central region of the genome. Recently, four genes (K4.2, K8.1, K11/vIRF, K12/Kaposin) within the central conserved region of the genome were identified to display high levels of nonsynonymous mutations (Olp *et al.,* 2015). ORF 26 gene is one of the nine centrally conserved regions of the genome that shows between 1-2% polymorphisms between HHV-8 isolates (Zong *et al.,* 1997). ORF 26, the minor capsid protein gene was the first to be amplified (Chang *et al.,* 1994). Sequence analysis of the gene has revealed 9 subtypes (A/C, B1, B2, D/E, J, K/M, N, Q, R) which cluster with geographic origin of samples and shows a strong linkage with K1 subtype, for instance B2 of ORF 26 is linked to B3 and A5 of K1 (Tornesello *et al.,* 2010).

Several studies on molecular genetics of HHV-8 based on ORF K1 sequence analysis have found various molecular subtypes of HHV-8 (A, B, C, D, E, F and Z) in close association with geography and ethnicity probably due to human migrations (White *et al.*, 2008; Hayward and Zong, 2007; Whitby *et al.*, 2004; Meng *et al.*, 2001; Biggar *et al.*, 2000; Zong *et al.*, 1999; Kasolo *et al.*, 1998). Also, there is evidence of subtypes and variants association with particular diseases and their progressions (Mancuso *et al.*, 2008; Metaxa-Mariatou *et al.*, 2005; Gazouli *et al.*, 2004). In sub-Saharan Africa, the most prevalent subtypes are B and A5 (Figure 1.7), which have been found almost exclusively in individuals residing in Africa, while subtypes A and C are frequently seen in AIDS-KS cases in United States, Europe and northern Asia. Subtype D has been found in individuals of Pacific Island origin; subtype E in Brazilian Amerindians; subtype F in Uganda and subtype Z in Zambia (Kanno *et al.*, 2010; Hayward and Zong, 2007; Kajumbula *et al.*, 2006; Meng *et al.*, 2001; Biggar *et al.*, 2000;

Cook *et al.*, 1999; Kasolo *et al.*, 1998). Although subtype B is predominantly found in Africa, subtype B has been reported in Cuba, Caucasians and in South America countries which include Brazil, French Guiana and Venezuela (Kouri *et al.,* 2012; Ishak *et al.,* 2007; Hernadez *et al.,* 2003; Lacoste *et al.,* 2000).

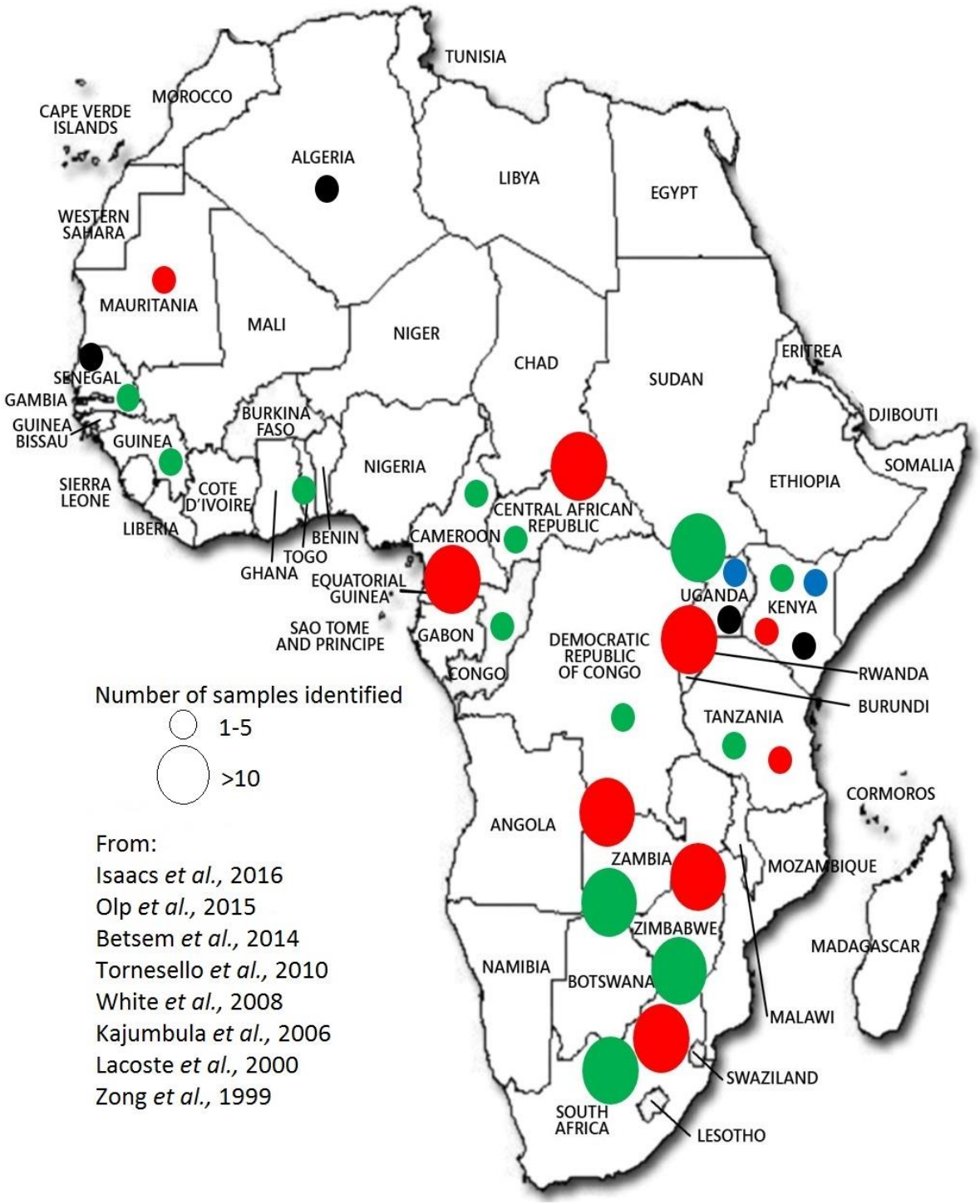



Figure 1.7 Map of Africa with the geographic distribution of the different ORF K1-subtypes of HHV-8. NB: red circle represents subtype A5, green circle for subtype B, black for subtype C and blue for subtype F. NB: Subtypes were identified in individuals with/without KS or any other HHV-8 associated diseases.

### 1.2.7 DETECTION TECHNIQUES OF HHV-8

Since there is no "gold standard" method of detection of HHV-8 infection, HHV-8 infection is detected using either a molecular or serological method. The molecular detection of HHV-8 is achieved by Polymerase Chain Reaction (PCR), the primary method used in the detection of HHV-8 DNA from KS biopsies (Chang *et al.,* 1994). Studies based on detection of HHV-8 by amplification revealed geographic variation in HHV-8 seroprevalence, however, lacks sensitivity to determine the rates of variation (Renwick *et al.,* 1999). Other molecular methods such as Southern blot or dot-blot hybridization are less sensitive and not suitable for specimens with low concentrations of the virus (Biggar *et al.,* 2000; Moore *et al.,* 1996b). Furthermore, detection of HHV-8 in the population at high risk of KS requires a more sensitive and less expensive method. Various serological assays are in use but with varying successes. These include Immunofluorescence assay (IFA), Enzyme-linked Immunosorbent Assay (ELISA), Western blot and Immunohistochemistry (IHC) (Mbondji-wonje *et al.,* 2013; Pereira *et al,* 2013; Araujo *et al.,* 2003). ELISA, IFA, IHC and western blot function on different chemistries and use different cell lines and antigenic sources to detect anti-HHV-8 antibodies in different specimens. However, each technique has their disadvantages which make them unsuitable as a gold standard for detection of HHV-8.

### 1.2.8 SEROEPIDEMIOLOGY OF HHV-8

Globally, epidemiology pattern of HHV-8 is uneven but follows that of KS and countries at high risk of KS report high prevalence of HHV-8. In adult healthy population, the prevalence of HHV-8 varies widely as oppose to prevalence observed in groups at increased risk of developing KS. In the American, Asians and European populations, HHV-8 appears to be rare, as it ranges from 3-6.5% in Hungary, Switzerland, United Kingdom, France, Spain and Germany. However, in HIV-infected individuals, it ranges from 16% in women in Germany to 31% in UK's homosexuals. In blood donors of Italy and Greece, prevalence was 35% (Gambus *et al.*, 2001; Juhasz *et al.*, 2001; Preiser *et al.*, 2001).

In the United States, the prevalence of HHV-8 ranges from 22-35% in the HIV-infected homosexual population (Gao *et al.*, 1996; Kedes *et al.*, 1996; Lennette *et al.*, 1996). KS is not common in children, heterosexual men and women of US, Asia and Europe but it is prevalent among the homosexual and bisexual populations groups. In contrast, moderate prevalence of approximately 15-25% is observed in Mediterranean regions where classic KS is prevalent (Chironna *et al.,* 2006; Larocca *et al.,* 2005; Cattani *et al.,* 2003) while high prevalence has been reported in sub-Saharan African's adults and children of all age groups using serological and molecular laboratory techniques (Mbondji Wonje *et al.,* 2013; Malope *et al.*, 2007; Dedicoat and Newton, 2003).

HHV-8 infection is ubiquitous in African regions with increased incidences of endemic Kaposi's sarcoma in the general population and AIDS-associated Kaposi's sarcoma in HIV/AIDS population (Mbondji Wonje *et al.,* 2013; Sitas *et al.*, 1999; *Gao et al.,* 1996). In contrast to observations in US (Blauvelt *et al.,* 1997), studies have showed that HHV-8 seroprevalence increases from childhood (27.5% at 4 years of age) to adulthood (48% above 15 years of age) in African regions (Andreoni *et al.,* 1999; Gessain *et al.,* 1999; Wilkinson *et al.,* 1999). In Central Africa, Cameroon in particular, where HIV and HHV-8 infections are endemic, the seroprevalence of HHV-8 in the general population ranges from 28-62% and in HIV-infected population, the prevalence is above 60% (Betsem *et al.,* 2014; Mbondji Wonje *et al.,* 2013; Bestetti *et al.,* 1998).

Although most continents have explored the seroprevalence of HHV-8 infection in both the general population and populations at different levels of risk of developing any of its associated diseases, continuous surveillance is necessary as there are increasing number of infected population (especially in endemic regions) and diseases associated with HHV-8 (Betsem *et al*., 2014; Ouyang *et al.*, 2014; Minhas and Wood, 2014; Mbondji-wonje *et al.*, 2013). Nonetheless, care must be taken in comparing the seroprevalence of HHV-8 as seropositivity can be limited by any of the following; antigen against antibodies tested for (lytic or latent antigen); the sensitivity and specificity of the assay used which could either be Enzyme-linked Immunosorbent Assay (ELISA) or Immunofluorescence Assay (IFA); the geographic/ethnic origin, the population studied, and the health or disease condition of the studied population (Burbelo *et al.,* 2010; Edelman, 2005).

### 1.2.9 RISK FACTORS AND TRANSMISSION OF HHV-8 INFECTIONS

There is still no conclusive report on risk factors for HHV-8 infections in the healthy population, however, associated risk factors could be of genetic and/or environmental source (in relation to behavioural, cultural, or social conditions). These include KS/HHV-8 endemic regions such as Africa and Mediterranean regions, KS partner, male gender, close contacts (deep kissing) with HHV-8 infected person, a major locus on chromosome 3p22, children crowding and family history of cancer (Bhutani *et al.,* 2015; Shebl *et al.,* 2013; Pedergnana *et al.,* 2012). Although HHV-8 infection is a pre-requisite but not sufficient to cause development of KS, people infected with HHV-8 infection are at higher risk of KS, these groups include immunodeficient and/or immunosuppressed individuals due to HIV/AIDS and organ transplant, infected mothers, caregivers, children and household members, male of sub-Saharan Africa region, men sleeping with men (MSM), people with high risk sexual

behaviours, drug users and patients with haemophilia (Wang *et al.,* 2016; Begré *et al.*, 2016; Bhutani *et al.,* 2015; Shebl *et al.,* 2013).

Previous studies have shown that the risk of KS is higher when HIV seroconversion precedes HHV-8 seroconversion or when the HIV/HHV-8 co-infected individual becomes more immunosuppressed (Jacobson *et al.,* 2000; Gao *et al.,* 1996; Whitby *et al.,* 1995). Risks of KS are influenced by high HHV-8 viral loads, high HHV-8 titres, high HIV RNA load and other host factors not well yet understood (Quinlivan *et al.,* 2002; Gao *et al.,* 1996). In an HHV-8 infected individual, there are some co-factors in development of KS, these include age, ethnic group, biting flies e.g. malaria parasite, volcanic soil, parasitic infections (chronic schistosomiasis), phorbol ester plants, household behaviours, high socio-economic status and environmental factors (Wakeham *et al.,* 2011; Whitby *et al.,* 2007; Mbulaiteye *et al.,* 2005). There is a possibility of associations between the virulence of a strain and the disease outcome, its progression and its expression.

There are three major modes of transmission of HHV-8; sexual, non-sexual and parenteral transmission. Despite the report of sexual transmission of HHV-8 in homosexual population from several cross-sectional and prospective cohort studies (Zhang *et al.,* 2013; Nawar *et al.*, 2005; Casper and Hutchinson*,* 2006; Smith *et al.,* 1999; Martin *et al.;* 1998), it's still hard to delineate the exact mode of HHV-8 transmission. Although HHV-8 seroprevalence is associated with sexual activity, high number of sexual partners and sexually transmitted diseases (STIs) such as Herpes Simplex Virus-2 (HSV-2), Hepatitis A, Hepatitis B, Hepatitis C and Syphilis, (Xie *et al.,* 2011; Zhang *et al.,* 2011; Zavitsanou *et al.,* 2007; Hladik *et al.,* 2006) however, evidence for sexual transmission among heterosexuals has not been consistent (Kakavand-ghalehnoei *et al.,* 2016; Shebl *et al.,* 2011; Eltom *et al.*, 2002; Casper *et al,* 2002; Kedes *et al.,* 1996). The precise route of transmission in the homosexual population is still under investigation though the mode of transmission is suspected to either be a combination or exclusively of oro- oral or anogenital insertive or receptive, insertive or receptive oro-genital, oro-anal sex. However, saliva has a role to play in the transmission of HHV-8 (Phipps *et al.,* 2014). A study in South Africa reported an increase in HHV-8 DNA shedding in saliva among humans carrying these Human Leukocyte Antigens (HLA) alleles; HLA-A*6801, HLA-A*4301, and HLA-DRB1*04 (Alkharsah *et al.,* 2007).

In sub-Saharan Africa regions, evidence of non-sexual transmission of HHV-8 is prevalent especially in childhood transmission, mother to child transmission and contact-to-contact transmission between children (Andreoni *et al.,* 1999; Gessain *et al.,* 1999; Wilkinson *et al.,* 1998). Infections in children less than 10 years are probably due to mother-to-child

transmission, but the precise mode of transmission either pre, peri-, post-partum is still under investigation (Melser *et al.,* 2015; Bourboulia *et al.,* 1998). However, this does not rule out the existence of sexual transmission of HHV-8 in high risk population of Africans (Shebl *et al.,* 2011). Previous studies have shown that horizontal transmission via HHV-8 contaminated blood during blood transfusion (Hladik *et al.,* 2012; Hladik *et al.,* 2006; Zavitsanou *et al.,* 2006) occurs. Also, there are evidence of HHV-8 antibodies in organ recipients after transplantation, which is attributed to either HHV-8 infected organs/donors or recipient's infection prior to transplantation (Andreoni *et al.,* 2001; Regamey *et al.,* 1998; Parravicini *et al.,* 1997).

### 1.2.10 LIFECYCLE OF HHV-8

Herpesviruses display both lytic and latent phases in their life cycles (Figure 1.8). Furthermore, they establish a lifetime latent infection within specific cells in the host, with periodic lytic replication or shedding. For example, latency is established in sensory nerve ganglia by α-herpesviruses, while β-herpesviruses establish latent infection in secretory glands and γ-herpesviruses establish latency in lymphocytes (Pellet and Roizman, 2013). The viral pathogenesis of HHV-8 is established by the interaction between the viruses, the host cells and immune system of the host. HHV-8 attachment to the host cell surface is achieved by interactions between the viral glycoproteins (gB and K8.1, gH/gL, gM/gN, ORF 4) and the attachment receptor (heparan sulfate, HS) on the host cell surface (Birkmann *et al*., 2001; Wang *et al*., 2001).

HHV-8 utilizes several receptors in the extracellular matrix to gain entry into different human cell types called antigen presenting cells, for example; endothelial cells, B-cells, monocytes, monocyte-derived dendritic cells(MDDC) to adapt to different immunological environments that it encounters in the host. Dendritic- cell-specific ICAM-3- grabbing non-integrin (DC-SIGN) and Integrin α3β1 has been revealed as entry receptors for HHV-8 infections of monocytes, and monocyte-derived macrophages, MDDC, B cells, foreskin fibroblasts and vascular endothelial cells (Kerur *et al*., 2010; Rappocciolo *et al*., 2008; Akula *et al*., 2002). Another receptor, ephrin receptor tyrosine kinase A2 which interacts with viral glycoprotein L and H dimer on HS-negative was discovered (Hahn *et al*., 2012). The reasons for the broad range of cellular targets of HHV-8 might be due to the ubiquitous nature of heparan sulfate (HS) expression on host cells (Campbell *et al*., 2014; Akula *et al.*, 2001).

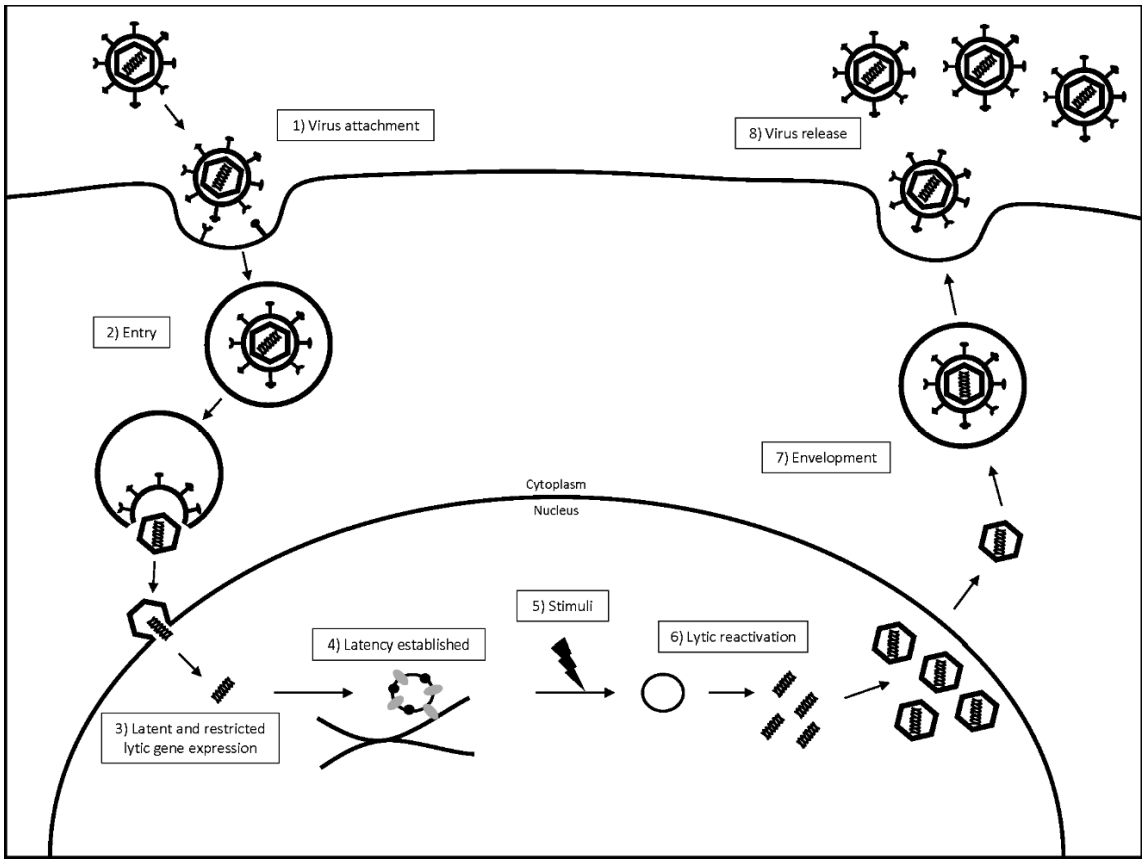



Figure 1.8 Life cycle of HHV-8 (Olp *et al.*, 2015).

The default program for HHV-8 infection is the latent cycle. During latency, latent genes and limited lytic genes are expressed to ensure the survival of the host cell and maintain segregation of the viral genome during host cell division (Damania and Cesarman, 2013). During this period, HHV-8 genome exists as an episome in the nucleus of the infected cells and not as a provirus in the host (Cesarman *et al.*, 1995). The episome is maintained by the latent gene, latency-associated nuclear antigen (LANA-1) and cellular histones. Latency-associated nuclear antigen (LANA-1) suppresses the expression of lytic genes needed for viral production and assembly. In addition, LANA-1 interacts with various cellular proteins such as p53 and Rb to inhibit their cellular functions, thereby protecting infected cells from apoptosis. In the advent of certain conditions such as immunodeficiency and immunosuppression, the infected cells may readily undergo uncontrolled proliferation (Chen *et al.,* 2010). Physiological stimuli involved in reactivation or switching of HHV-8 from latent to lytic phase (*in vivo*) are not yet fully understood, however, hypoxia, apoptosis, cellular stresses, inflammation, co-infections and some HHV-8 viral genes (ORF 50/Rta, ORF 57, K9) are reported to play some vital roles (Majerciak and Zheng, 2016; Uppal *et al.,* 2014; Sun *et al.,* 1999; Gao *et al.,* 1997). Nonetheless, the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) and histone deacetylase (HDAC) inhibitors, TLR 7/8

ligands and sodium butyrate are routinely used to reactivate virus replication *in vitro* (Purushothaman *et al.,* 2015; Cohen *et al.,* 2006; Renne *et al.,* 1996). The entire HHV-8 genome is expressed in a similar fashion to other herpesviruses during the lytic cycle (Jenner *et al.,* 2001). Reports from recent studies indicate a low level of K1 expression during latency, which contrasts the Knowledge of K1 gene been expressed only during the lytic phase (Arias *et al.,* 2014; Chandriani and Ganem 2010; Bowser *et al.,* 2002).

### 1.2.11 HHV-8 ASSOCIATED DISEASES

Having been categorized as a class I carcinogen, due to its causal link to KS and other two B-cell lymphoproliferative disorders, Primary Effusion Lymphoma (PEL) and Multicentric Castleman's Disease (MCD), HHV-8 disease association increases (Bouvard *et al.,* 2009; Cesarman *et al.,* 1995; Soulier *et al.,* 1995).

Although Kaposi's sarcoma (KS) was described in 1872, there was no association between the disease and HHV-8 until 1994 (Chang *et al.,* 1994). KS is a rare tumour which occurs in different forms and shapes of endothelial and spindle cells in red, brown or purple pigment, primarily on the skin and also in the mucosal membranes and visceral organs (Ablashi *et a*l., 2002). Based on pathological and epidemiological context, KS has been classified into four forms, namely; classic, iatrogenic endemic and epidemic KS. The association between KS and HHV-8 has been confirmed in nearly all forms of KS. Classic KS (cKS) was described by Moritz Kaposi, (Kaposi, 1872) as rare "idiopathic multiple pigmented sarcoma of the skin" present on lower extremities of older (above 65 years old) Jewish or Mediterranean men (Ganem, 2010). Iatrogenic/transplant-associated KS (IKS) is observed in solid-organ transplant recipients with immune suppression due to immunosuppressant regimens used (reviewed in Schulz and Cesarman, 2015). Although IKS is rare, an epidemiological study in the United States observed a 54-fold higher risk of developing KS in transplant recipients compared to the general population (Mbulaiteye and Engels, 2006). African/Endemic KS (EKS) was described in 1962, before the AIDS epidemic as a form prevalent in sub-Saharan Africa region (Oettle, 1962). Countries across equatorial Africa with high prevalence and incidence of Endemic KS are termed "KS belt", these includes Uganda, Zaire, Cameroon, Tanzania, and the southern African countries: South Africa, Zimbabwe, Zambia and Malawi (Cook-Mozaffari *et al.,* 1998). EKS is seen in both children and adults of African origin, but in different clinical forms with high morbidity and mortality (reviewed in Schulz and Cesarman, 2015; Friedman-Kien *et al.,* 1990). HIV-1/Acquired Immunodeficiency Syndrome associated-KS (AIDS-KS) is the most aggressive form of KS and one of commonest cancer in HIV and HHV-8 endemic regions of Africa (Giffin and Damania, 2014). A study of HIV and HHV-8 co-infection in homosexual men revealed the probability that in 10 years' time, about 50% of the

population will develop KS and also, that the development of KS can precede severe immunodeficiency or AIDS (Martin *et al.,* 1998). Active replications of HHV-8 in blood and increase detection of its viral DNA are strongly correlated with increased risk of progression to KS (Ambroziak *et al.,* 1995; Whitby *et al.,* 1995).

Shortly after HHV-8 was discovered, HHV-8 DNA sequences were identified in Primary Effusion Lymphoma (PEL), also referred to as body-cavity based lymphoma (BCBL) because of its presence in the pleural, pericardial and peritoneal cavities (Cesarman *et al.,* 1995). Unlike KS, PEL is of B cell origin, aggressive and progress rapidly with approximately 2-6 months mean survival time for patients and can cause high mortality (Simonelli *et al.,* 2003). It is a rare form of Non-Hodgkin's Lymphoma (NHL) found in AIDS patients. PEL can contain single positive HHV-8 cells or double positive HHV-8/EBV cells. PEL is of importance in HHV-8 research in the development of HHV-8 harbouring cell lines (BCBL-1, BC-1, BC-3) with copy numbers ranging from 50-150 per infected cells (Staudt *et al.,* 2004; Renne *et al.,* 1996; Cesarman *et al.,* 1995).

Also in 1995, a rare variant of Castleman's disease, with multiple lymphadenopathies, later named Multicentric Castleman's Disease (MCD) was found in association with HHV-8 (Soulier *et al.,* 1995). Although, there are two clinical variants of MCD, however, the most common variant, the hyaline vascular type which occurs as a solitary mass is not associated with HHV-8. The plasmablastic MCD is a polyclonal disorder which can be aggressive and progress rapidly. Features of MCD include high HHV-8 viral loads in the blood which is followed by excessive viral Interleukin-6 (vIL-6), human Interleukin-6 (hIL-6) and Interleukin-10 (IL-10) (Polizzotto *et al.,* 2012; Oksenhendler *et al.,* 2000). Often times, KS and NHL develop as secondary malignancies in patients with MCD (Soulier *et al.,* 1995).

Although KS continues to lead the global cancer burden in HIV/AIDS population, however, more diseases have been identified with replication of HHV-8 these include Kaposi's sarcoma Immune Reconstitution Inflammatory Syndrome (KS-IRIS), KSHV Cytokine Inflammatory Syndrome (KCIS), Sarcoidosis, Prostate cancer (Pca), Multiple Myeloma (MM) and more with controversial reports on their association with HHV-8 (Cattelan *et al.,* 2016; Su *et al.* 2015; Ismail *et al.,*2011; Uldrick *et al.*, 2010; Bower *et al.*, 2005; Di Alberti *et al.* 1997).

### 1.2.12 HHV-8 AND HIV-1 CO-INFECTION IN AIDS-KS

It has been known since the earliest days of AIDS pandemic that HIV-infected individuals are at greater risk of developing Kaposi's sarcoma (KS) (Engels *et al.,* 2008). The risk of developing KS in an HIV-infected population is 20,000 times that of the general population and 70 times of other immunosuppressed populations (Dukers and Rezza, 2003; Beral *et al.*, 1990). Previous studies have revealed that HHV-8/HIV-1 co-infected individuals almost exclusively develop KS (Zeng *et al.,* 2007; Ariyoshi *et al.,* 1998). The ability of HIV-1 to induce tumour is associated with chronic inflammation in HHV-8 infected patients (Fiume *et al.,* 2015), for instance, increase in Interferon γ (IFN-γ), Interleukins, 1 and 6 (IL-1 and IL-6) and tumour necrosis factor a (TNF-a) have been reported in AIDS-KS. AIDS-KS is a form of KS resulting from the interaction of HIV-1 with HHV-8. The effect of HIV-1 Tat (transcriptional transactivator) on HHV-8 infection cannot be overemphasized. Some of its effects include enhancement of HHV-8 infectivity, promotion of angiogenesis by induction of several cytokines, interaction with HHV-8 proteins to modulate multiple signaling pathways (Aoki and Tosato, 2007). Reactivation of HHV-8 lytic cycle can be achieved by penetration of HIV-1 Tat into various target cells to induce cytokines and growth factors expressions (Debaisieux *et al.,* 2012; Zeng *et al.,* 2007; Gallo, 1998). HIV-1 Tat co-operate with vascular endothelial growth factor (VEGF) to induce the formation of KS lesions in mice and the protein increase the proliferation of endothelial cells in KS cell lines (Albini *et al.,* 1996).

PI3K/PTEN/GSK-3β signaling pathway is regulated to promote angiogenesis and tumorigenesis by the interaction between HHV-8 vIL6 and HIV-1 Tat (Zhou *et al.,* 2013). Furthermore, HHV-8 oncoproteins, vGPCR and Kaposin A cooperates with HIV-1 Tat to promote tumorigenesis (Guo *et a*l., 2004; Chen *et al.,* 2009). Much recently, promotion of angiogenesis was observed as a result of an interaction between HHV-8 lytic oncogene K1 and HIV-1 Tat, this was achieved by induction of cellular miR8911-5p by HIV-1 Tat and HHV-8 K1 to activate NF-κB signaling pathway (Yao *et al.,* 2015). Xue and colleagues (2014) reported HIV-1 Nef synergizes with HHV-8 vIL6 to induce AKT signaling in order to cause tumorigenesis and induce cell proliferation. The damages resulting from the undesirable interaction between HIV-1 and HHV-8 could be limited with a better understanding of the mechanisms of their interactions and the understanding could be of pharmacological importance especially in regions where both infections are endemic (Thakker and Verma, 2016).

## 1.3 STUDY RATIONALE

Infection with Human Herpes Virus 8 (HHV-8) occurs in immunocompetent and immunocompromised individuals. HHV-8 causes Kaposi's sarcoma (KS) and other lymphoproliferative disorders in humans irrespective of their HIV status. Early acquisition of HHV-8 from infected individuals, persistence in infected individuals and transmission of the virus via non-sexual routes are the major causes of the high prevalence of HHV-8 in the general population of sub-Saharan Africa especially Central and East Africa. However, high prevalent rates have also been observed in some parts of Asia and the Mediterranean regions but the prevalence is low in the general population of Western Europe and United States (Minhas and Wood, 2014).

In HIV/AIDS associated-Kaposi's sarcoma (AIDS-KS) population, HHV-8 antibodies have consistently been found to be prevalent especially in the homosexual population (Kedes *et al.*, 1996; Lennette *et al.*, 1996). Several studies have employed ORF K1, ORF K14.1/K15, ORF 25, ORF 26 and ORF 75 genes to classify HHV-8 isolates into subtypes and clades (Isaacs *et al.*, 2016; Olp *et al.*, 2015; Betsem *et al.*, 2014; Kajumbula *et al.*, 2006).

Due to the high genetic variability observed across the whole genome, subtyping of HHV-8 based on conserved region (ORF 26) has not revealed the whole picture in the evolutionary relatedness of HHV-8 subtypes. Also, the same PCR primers were used in targeting the relatively conserved regions in different studies (Tornesello *et al.,* 2010; Zong *et al.*, 1999). In addition, previous molecular epidemiological studies of HHV-8, have relied on Kaposi's sarcoma biopsy specimens and these do not provide information on demography and immunological state and socio-economic status of the participants. Such studies only examine the viral strains found in symptomatic cases of HHV-8 associated diseases. Findings from such studies do not give a true representation of the prevalence of HHV-8 in an HIV-infected population. Moreover, molecular studies on peripheral blood mononuclear cells (PBMCs) do not present the true picture of prevalence in asymptomatic non-viremia infected subjects.

Despite the high seroprevalence of HHV-8 (28-62%) in Cameroon, screening for HHV-8 in Central Africa is restricted to research purposes and is not done routinely (Dedicoat and Newton, 2003) to date. It is, therefore, acknowledged that HIV/AIDS associated-Kaposi's sarcoma (AIDS-KS) and/or other lymphoproliferative diseases can easily develop in patients who are dually infected with HIV/HHV-8 and either have limited access to highly active antiretroviral therapy (HAART), failing treatment or have developed drug resistance. Hence,

continuous surveillance of HHV-8 in HIV-infected population, identification of HHV-8 subtypes and mutations involved becomes a necessity.

Therefore, this study seeks to add data to the biology of HHV-8 in the HIV/AIDS population of Africa by investigating its seroprevalence, subtypes and genetic variants in an HIV-infected cohort of Cameroon, Central Africa.

## 1.4 OBJECTIVES OF THE STUDY

### 1.4.1 GENERAL OBJECTIVE

The main objective of the study was to describe the epidemiology and genetic diversity of HHV-8 in an HIV-infected population in Cameroon, Central Africa.

### 1.4.2 SPECIFIC OBJECTIVES

- To determine the seroprevalence of HHV-8 in HIV-infected individuals of Cameroon.
- To phylogenetically define the genetic subtypes of HHV-8 based on ORF K1 gene.

# CHAPTER TWO: MATERIALS AND METHODS

## 2.1 ETHICAL CONSIDERATIONS

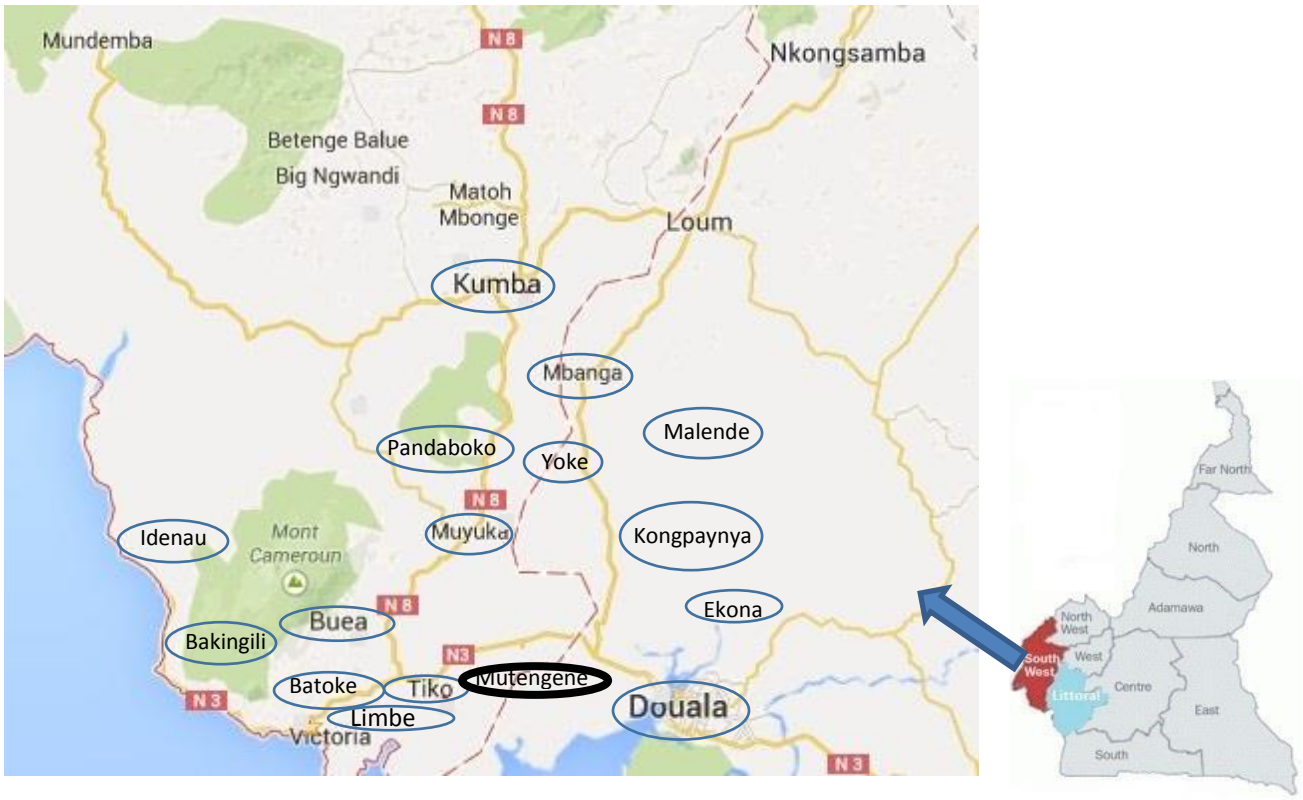
The ethical approval was obtained from the institutional review board of the University of Buea, Cameroon (IRB2012-01) and from Research Ethics Committee of the University of Venda, South Africa (SMNS/14/MBY/21/2110). Consent forms for participants above and below 18 years of age were signed by participants and legal guardians, respectively.

## 2.2 STUDY SITE AND POPULATION

The Baptist Health Centre in Mutengene (BHM), Cameroon, provides care to HIV-infected patients with provenance from several towns in the South-West and Littoral regions of Cameroon including Bafia, Banga, Batoke, Bomaka, Buea, Douala, Ekona, Kumba, Likoko, Likomba, Limbe, Malende, Missellele, Moliwe, Muea, Mutengene, Muyenge, Muyuka, Pendaboko, Souza, Tiko and Yoke (Figure 2.1). The study population is a cohort of HIV-infected outpatients from BHM. The cohort comprises individuals of both sexes and age 2 to 68 years.

The South-West region of Cameroon is located at the coordinates 4°10′N 9°14′E. The region is home to 1,534,232 people (cameroon.opendataforafrica.org), whose official language is English. It is considered the best tourist region in the country with beaches, Korup National Park, Mount Koupe, Wildlife Centres, Botanical Gardens, Golf Course and the University of Buea.

The Littoral region of Cameroon is located at the coordinates 4°0′N 10°0′E of the equator. The population of this region was estimated at 3,309,558 during the 2015 census (cameroon.opendataforafrica.org). Its capital, Douala, is the biggest city in Cameroon, where the major international airport and the University of Douala are located. The two regions (South-West and Littoral), serve as home and stop-over points for tourists, travellers, drivers and logging vehicle operators who target sex workers while entering or leaving Cameroon for other countries (https://en.wikipedia.org, accessed on May 23, 2015).




(a) (b)

Figure 2.1 Geographical location of the study population's provenance. (a) A figure representative of the enlarged view of South-West region of Cameroon showing the towns (circled blue) where the study participants originated and Mutengene (circled black) where Baptist Health centre (BHM) is located. (b) A map of Cameroon showing South-West (red) and Littoral (blue) regions where study participants originated. (Adapted from Google maps, 21 June 2015 21:18 pm).

## 2.3 STUDY DESIGN

This was a retrospective, laboratory based, cross-sectional study. Archived blood samples from study participants recruited between January and July, 2013 were used.

## 2.4 SAMPLE COLLECTION AND TRANSPORT

Five millilitres of venous blood from each consenting participant was collected in EDTA-containing vacutainer tubes at BHM, Cameroon. In addition, participants' demographic, socio-economic and immunologic data were collected. The blood samples were shipped, received on dry ice and stored at -20$^0$C in the HIV/AIDS global health (HAGH) laboratory of University of Venda, South Africa.

## 2.5 DETECTION OF ANTIBODIES TO HHV-8 ANTIGENS

The detection of HHV-8 in plasma samples from HIV-infected participants in this study was achieved by using KSHV/HHV-8 Immunoglobulin-G (IgG) antibody ELISA kit [Advanced Biotechnologies Inc. (ABI), Columbia, MD, USA] according to manufacturer's instructions. This is a quantitative HHV-8 whole virus extract/lysate which contained latent and lytic proteins. The ELISA plates were coated with antigens of KS-1 cell line (EBV/HIV negative)

from a patient with body cavity lymphoma (Said *et al.*, 1996). The ELISA is highly specific and sensitive, reproducible and does not cross-react with Epstein-Barr Virus (EBV) or any other human herpesviruses (Chatlynne *et al.*, 1998).

Briefly, ten microliters (10 µl) of plasma, positive and negative controls were added to 990 µl of 1X specimen diluent to make 1:100 dilutions. After the dilution, 100 µl of the diluted blank, plasma samples, positive and negative controls were transferred to wells of the antigen-coated plate for any specific antibodies present to bind to the antigen-coated plate. After the first incubation at $37^0$C for 30 minutes (immunological complexes formed), the wells were washed to remove unbound sample components. The second incubation (at $37^0$C for 30 minutes) involved the addition of 100 µl of horseradish peroxidase (HRP), to bind to any antibody-antigen complexes present in the wells. Washing of the wells was done to remove unbound conjugate (HRP). For the third incubation (at room temperature for 30 minutes), 100 µl of tetramethylbenzidine (TMB) was added to the wells. This enzyme-mediated cleavage resulted in a colour change. The reaction was stopped by addition of 100 µl of stop solution to each well. The wells with the colour change from blue to yellow predicted positive samples (i.e. contain antibodies to HHV-8) while wells which remain almost colourless predicted negative samples (i.e. do not contain antibodies to HHV-8).

The intensity of the colour is proportional to the level of HHV-8 IgG antibodies in a sample (Manual for ABI KSHV/HHV-8 IgG antibody ELISA kit, updated 2012). The absorbance of the IgG antibodies (known as the optical density value) was measured at a 450nm wavelength in a microplate reader (VersaMax; Molecular Devices, Silicon Valley, CA, USA).

### 2.6 EXTRACTION OF DNA FROM WHOLE BLOOD

The Quick-gDNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) was used to extract DNA from total cells of all HHV-8 seropositive samples according to the manufacturer's instructions. The extracted DNA (50 µl) was stored in sterile 1.5 ml microcentrifuge tube at -$20^0$C for conventional polymerase chain reaction.

### 2.7 AMPLIFICATION OF HHV-8 DNA

A 233 bp fragment of ORF 26 gene was amplified to detect the presence of HHV-8 DNA while fragments of 453 bp of K1 gene were amplified to detect the genetic variation and characterize the isolates.

A protocol by Chang and colleagues (1994) was optimised to amplify ORF 26 region of HHV-8 in a nested PCR with the reaction mixtures containing 1.25X PCR buffer, 0.1 µM

forward and reverse primers, 0.1 µM dNTP Mix (Invitrogen), 1.5 mM Magnesium Chloride, 0.5 U/µl Taq polymerase (Invitrogen) and 2µl of DNA template (Qiagen, Valencia, USA) between 50-200ng/µl in a 20µl final volume. The thermal cycling conditions for both rounds of PCR were as follow: 110$^0$C heat lid, an initial denaturation at 94$^0$C for 3 minutes, followed by 35 cycles of denaturation at 94$^0$C for 60 seconds, annealing at 57$^0$C for 60 seconds and extension at 72$^0$C for 60 seconds. This was followed by a final elongation at 72$^0$C for 10 minutes. The final volume of master mix (20 µl) was maintained for both rounds of PCR but with different sets of internal primers (Table 2.1). The reaction mixtures and thermal cycling conditions for amplification of K1 were similar to that of ORF 26 except for 0.2 µM of forward and reverse primers, 0.2 mM of dNTP Mix (Invitrogen) and an annealing temperature of 58$^0$C. The amplification was carried out in a Bio-Rad T-100 thermal cycler (Bio-Rad, California, USA).

**Table 2.1 HHV-8 primers for ORF K1 and ORF 26 amplifications**

| Gene | Size (bp) | Primers | Ext/Int Primers | Nucleotide Sequence (5'-3') | References |
|---|---|---|---|---|---|
| ORF K1 | 499 | PMC20 F | External Primers | GTCTTTCAGACCTTGTTGG | Cook *et al.,* 1999 |
| | | K1/428/2 R | | CCCGTTAGAACAAGTATA | |
| | 453 | PMC20A F | Internal Primers | GACCTTGTTGGACATCCTG | |
| | | K1/427/2 R | | GTATTTAGTTTGTGACACGG | |
| ORF 26 | 365 | KSA F | External Primers | CCTCTGACAACCTTCAGATA | Chang *et al.,* 1994 |
| | | KSB R | | GTACATGGACAGATCGTCAA | |
| | 233 | KS1 F | Internal Primers | AGCCGAAAGGATTCCACCAT | |
| | | KS2 R | | TCCGTGTTGTCTACGTCCAG | |

Key: ORF-Open Reading Frame, F-Forward, R-Reverse, bp-base pairs

All processes and procedures involved were carried out aseptically. Nuclease-free water and known HHV-8 positive samples were included in all reactions performed as negative and positive controls respectively. Also, workbenches were cleaned with DNA-away (Molecular BioProducts, CA), RNase-away (Molecular BioProducts, CA) and 70% ethanol before and after experiments. Decontamination was achieved using bleach, alcohol and UV-light when necessary.

## 2.8 VISUALIZATION OF AMPLIFIED PRODUCTS

To resolve all PCR products, gel electrophoresis was used. A 1.5% agarose gel was prepared in 1X TAE buffer (0.04M Tris-Acetate, 0.001M EDTA) and stained with 0.5µg/ml of ethidium bromide (Promega, Madison, USA). Two microliters of each amplicon were mixed with 0.1 µg/µl of DNA loading dye (Promega, Madison, USA) and loaded into each well. The gel electrophoresis was set up for migration of the amplicons from the negative to the positive anode. The amplicons were verified for expected size at 300 mA and 100 V for 35 minutes. A 100bp plus (marker, Benchtop) molecular base ladder (Invitrogen) was used to

confirm size of amplicons and the agarose gels were visualized at 322nm (at this wavelength, ethidium bromide intercalates DNA and fluoresces brightly) with the G-Box UV trans-illumination gel documentation system and the gel photo captured by the Genesnap program version 06-2.d.1 (Syngene, Germany).

## 2.9 PURIFICATION AND VISUALIZATION OF HHV-8 AMPLICONS

The nested amplicons of ORF 26 (233bp) and K1 gene (453bp) were purified using the Qiaquick PCR purification kit (Qiagen, Valencia, USA) following the manufacturer's instructions. The purified products were loaded on a 2% agarose gel, ran at 300mA and 100V for 35 minutes and visualized for the expected band sizes. The purified amplicons were stored at -20$^0$C until sequenced.

## 2.10 SANGER SEQUENCING OF HHV-8 AMPLICONS

The ABI Prism® BigDye™ Terminator version 3.1 ready reaction sequencing kit (Applied Biosystems, Warrington, UK) was used to sequence both strands of the viral DNA template from the nested purified K1 products. Sequences were analysed with an ABI 3500 XL Dx Sequencer, BigDye Terminator Cycle genetic analyser (Perkin Elmer, CA) (Applied Biosystem) using POP$^{TM}$-7 polymer and 24-capillary array. The sequencing was done using the internal primers for ORF 26 and K1 gene (Table 2.1). This direct population-based sequencing reaction is based on the dideoxy method (Sanger *et al*., 1977) and was carried out at Inqaba Biotec (Pretoria, South Africa).

## 2.11 DATA ANALYSIS

### 2.11.1 STATISTICAL ANALYSIS OF HHV-8 IgG ELISA RESULTS

Seroprevalence estimation was based on both lytic and latent positivity of the plasma samples screened for IgG antibodies to HHV-8 using KSHV/HHV-8 IgG ELISA kit (ABI, Columbia, MD, USA). The data obtained from the microplate reader were interpreted by relating each samples' Optical Density (OD) value to the Cut-off Value (CV) to obtain the OD ratio. Samples with OD ratio ≤0.75 were considered negative or non-reactive and those with OD ratio ≥1.00 were considered positive or reactive samples. Five samples with OD ratio between 0.76 and 0.99 were considered equivocal or borderline and were retested in duplicates.

The distribution of HHV-8 prevalence according to age, gender, region, marital status, HIV clinical stage, use of HAART, CD4+ cell count, house type, toilet type, employment status, own a transistor radio set, own a refrigerator, own a television (TV) and own a car was

analysed on a univariate level using the Statistical Package for the Social Sciences (SPSS) version 22 (IBM Corp., 2013) and the probability value for the relationship between HHV-8 seropositivity and available variables were calculated using Chi-square and considered statistically significant for *p* values less than 0.05 ($p < 0.05$).

### 2.11.2 SEQUENCE ASSEMBLY AND ANALYSIS OF K1 GENE

Before editing the 'raw' sequences, the sequences were blasted against the public dataset using the online NCBI BLAST tool (http://www.ncbi.nlm.nih.gov/blast) in order to confirm the sequencing and check for contamination. Sequences were deemed not contaminated if aligned with HHV-8 isolates. The resulting forward and reverse DNA sequences of K1 gene obtained from electropherograms were assembled, manually edited and translated into predicted amino acid with BioEdit software version 7.2.5 (Hall, 1999), SeqMan Pro II and Seqbuilder programs in the Lasergene software, version 8.0 (DNASTAR Inc., Maduson, Wisconsin, USA).

Previously described sequences from Cameroon and subtype representative reference sequences were obtained from GenBank. The study sequences, previously described HHV-8 sequences from Africa and western countries were aligned using CLUSTAL W incorporated in the MEGA program version 6.0 (Tamura *et al.*, 2013) as well as BIOEDIT software version 7.2.5 (Hall, 1999). Both MEGA and BIOEDIT programs were used for control check. An online tool, BioAfrica Oxford HHV8 Automated Subtyping Tool version 2.0 (http://bioafrica.mrc.ac.za/rega-genotype/html/indexhhv8.html, accessed on September 23, 2016) was used to determine the subtypes (Alcantara *et al.,* 2009; De Oliveira *et al.,* 2005). To confirm subtypes determined by the online tool, maximum likelihood phylogenetic trees were generated from the patients' sequences, along with representative reference sequences of all subtypes obtained from GenBank. The reliability of the trees was assessed by bootstrapping of 1000 replicates. The genetic subtypes of the sequences obtained in this study were confirmed from the phylogenetic trees drawn in MEGA program version 6.0 (Tamura *et al.*, 2013).

The online tool, Synonymous Non-synonymous Analysis Program (SNAP) version 2.1.1 (https://www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html, accessed on January 3, 2017) was used to determine the synonymous and nonsynonymous mutation ratio and rates (Korber, 2000), of obtained sequences and data obtained were analysed and drawn to scale on Microsoft Excel, 2016 (Microsoft Corporation, Washington, USA).

# CHAPTER THREE: RESULTS

## 3.1 DATA OF THE STUDY PARTICIPANTS

A total of 406 HIV-infected individuals participated in this study. Of the 406 participants, 301 (74.1%) were females, 103 (25.4%) were males while the gender status of 2 (0.5%) participants was not provided. The mean age of participants in the study was 34.1 years (range, 2-68 years). Demographic, immunologic and socio-economic variables of the study population are summarized in Tables 3.1 (a and b).

**Table 3.1 (a) Demographic and immunologic data of the study population**

| **Characteristics** | **No (%)** |
|---|---|
| | |
| **Region** | |
| South-West | 386 (95.1) |
| Littoral | 18 (4.4) |
| Unknown | 2 (0.5) |
| **Gender** | |
| Male | 103 (25.4) |
| Female | 301 (74.1) |
| Unknown | 2 (0.5) |
| **Age (years)** | |
| ≤14 | 37 (9.1) |
| 15-29 | 82 (20.2) |
| 30-44 | 209 (51.5) |
| 45-59 | 68 (16.7) |
| ≥60 | 8 (2.0) |
| Unknown | 2 (0.5) |
| **Marital Status** | |
| Single | 151 (37.2) |
| Married | 199 (49.0) |
| Divorced | 9 (2.2) |
| Widow/Widower | 45 (11.1) |
| Unknown | 2 (0.5) |
| **CD4+ Cell Count (cells/mm$^3$)** | |
| <500 | 303 (74.6) |
| ≥500 | 101 (24.9) |
| Unknown | 2 (0.5) |
| **HIV Stages** | |
| Stage 1 & 2 | 193 (47.5) |
| Stage 3 & 4 | 203 (50.0) |
| Unknown | 10 (2.5) |
| **On HAART** | |
| Yes | 323 (79.6) |
| No | 81 (19.9) |
| Unknown | 2 (0.5) |
| **Total** | **406** |

Key: CD-clusters of differentiation, Stage 1, 2, 3 and 4 based on World Health Organisation (WHO) classification of HIV stages. Note: Demographic and immunologic data were not available for 2 reactive samples: BM 11 and BM 207. Also, HIV clinical stage for 10 samples was not available.

**Table 3.1 (b) Socio-economic data of the study population**

| Characteristics | No (%) |
|---|---|
| | |
| **On HAART** | |
| Yes | 323 (79.6) |
| No | 81 (19.9) |
| Unknown | 2 (0.5) |
| **House type** | |
| Block | 255 (62.8) |
| Plank | 149 (36.7) |
| Unknown | 2 (0.5) |
| **Toilet type** | |
| Pit | 291 (71.7) |
| Flushing | 109 (26.8) |
| Other sources (W/S/B) | 4 (0.99) |
| Unknown | 2 (0.5) |
| **Employment Status** | |
| Employed | 80 (19.7) |
| Unemployed | 324 (79.8) |
| Unknown | 2 (0.5) |
| **Own a Refrigerator** | |
| Yes | 150 (36.9) |
| No | 254 (62.6) |
| Unknown | 2 (0.5) |
| **Own a Radio** | |
| Yes | 259 (63.8) |
| No | 145 (35.7) |
| Unknown | 2 (0.5) |
| **Own a TV** | |
| Yes | 353 (86.9) |
| No | 51 (12.6) |
| Unknown | 2 (0.5) |
| **Own a Car** | |
| Yes | 59 (14.5) |
| No | 345 (85.0) |
| Unknown | 2 (0.5) |
| **Total** | **406** |

Key: W/S/B- Water/Sea/Bush, TV-television. Note: Socio-economic data were not available for 2 reactive samples: BM 11 and BM 207.

### 3.2 SEROPREVALENCE OF HHV-8

Of the 406 samples screened for HHV-8 antigens using indirect ELISA technique, 321 (79.1%) were reactive while 85 (20.9%) were non-reactive. The seroprevalence of HHV-8 observed in the HIV-positive cohort of BHM, Cameroon in this study was 79.1%. Furthermore, distribution of HHV-8 based on its seropositivity, demographic, immunologic and socio-economic variables were assessed at the univariate level (Tables 3.2a and 3.2b). Age, marital status and CD4+ cell count and ownership of a transistor radio set were statistically associated with HHV-8 seroprevalence ($p < 0.05$). Two samples (0.6%, 2/321) which had anti-HHV-8 antibodies lacked epidemiological data.

Furthermore, a very high prevalence 79% and 77.8% was observed in South-West and Littoral region respectively (Table 3.2a), with no significant statistical difference ($p$ =0.99). Although more females (n=301) than males (n=103) were screened for anti-HHV-8 antibodies, the seroprevalence in males (79.6%) was slightly higher than that of the females (78.7%) but this difference was not statistically significant ($p$ =0.78). Sixteen participants in this study categorized as children (≤14 years old) had anti-HHV-8 antibodies (43.2%). There is a significant increase ($p$ =0.00) observed in HHV-8 seropositivity from adolescent (15 years old: 76.8%) to adulthood (≥60 years old: 100.0%).

The majority (82.9%, 165/199) of the married participants in this study had detectable antibodies against HHV-8 antigens (Table 3.2a). Also, a high prevalence (88.9%, 40/45) of HHV-8 was observed among participants who are widow/widower. A statistically significant association was observed between HHV-8 seropositivity and marital status ($p$ =0.02).

Although a moderately high seroprevalence of HHV-8 was observed in patients at HIV clinical stages 3 and 4 (79.3%) compared to stages 1 and 2 (77.7%), this association was not statistically significant ($p$ =0.70). There was, however, a significant association between higher HHV-8 prevalence and lower CD4 + Cell count ($p$ =0.02). Correlation between HHV-8 seroprevalence and use of HAART by participants was assessed, though higher prevalence was observed in ART-naïve participants (81.5% versus 78.3%) but was not statistically significant. Furthermore, the association between HHV-8 seropositivity and socio-economic status was accessed using variables such as employment status, house type, toilet type, ownership of a television (TV), transistor radio set, refrigerator and car, regrettably, none but one (ownership of a transistor radio set), was found associated with HHV-8 seropositivity. HHV-8 seroprevalence (Table 3.2b) was inversely associated with having a transistor radio set ($p$ =0.00).

**Table 3.2a Distribution of HHV-8 seropositivity and risk factors (univariate analysis)**

| Characteristics | Total (n) (%) | Reactive | Prevalence | Chi-Square ($X^2$) | *P*-Value |
|---|---|---|---|---|---|
| **Gender** | | | | | |
| Male | 103 (25.4) | 82 | 79.6% | 0.075 | 0.78 (NS) |
| Female | 301 (74.1) | 237 | 78.7% | | |
| **Age (years)** | | | | | |
| ≤14 | 37 (9.1) | 16 | 43.2% | 34.7291 | **0.00 (Sig)** |
| 15-29 | 82 (20.2) | 63 | 76.8% | | |
| 30-44 | 209 (51.5) | 174 | 83.3% | | |
| 45-59 | 68 (16.7) | 58 | 85.3% | | |
| ≥60 | 8 (2.0) | 8 | 100.0% | | |
| **Marital Status** | | | | | |
| Single | 151 (37.2) | 108 | 71.5% | 10.3893 | **0.02 (Sig)** |
| Married | 199 (49.0) | 165 | 82.9% | | |
| Divorced | 9 (2.2) | 6 | 66.7% | | |
| Widow/widower | 45 (11.1) | 40 | 88.9% | | |
| **Region** | | | | | |
| South-West | 386 (95.1) | 305 | 79.0% | 0.0002 | 0.99 (NS) |
| Littoral | 18 (4.4) | 14 | 77.8% | | |
| **CD4+ Count (cells/mm$^3$)** | | | | | |
| <500 | 303 (74.6) | 247 | 81.5% | 5.0585 | **0.02 (Sig)** |
| ≥500 | 101 (24.9) | 72 | 71.3% | | |
| **HIV Stages** | | | | | |
| Stage 1 & 2 | 193 (47.5) | 150 | 77.7% | 0.1472 | 0.70 (NS) |
| Stage 3 & 4 | 203 (50.0) | 161 | 79.3% | | |
| **On HAART** | | | | | |
| Yes | 323 (79.6) | 253 | 78.3% | 0.6716 | 0.41 (NS) |
| No | 81 (19.9) | 66 | 81.5% | | |
| | | | | | |
| **Total** | **406** | **321** | **79.1%** | | |

Key: CD-clusters of differentiation, NS- Not significant, Sig- Significant, *p* value significant at $p < 0.05$, stage 1, 2, 3 and 4 based on World Health Organisation (WHO) classification of HIV stages. Note: Demographic and immunologic data were not available for 2 reactive samples: BM 11 and BM 207. Also, HIV clinical stage for 10 samples was not available.

**Table 3.2b Distribution of HHV-8 seropositivity and risk factors (univariate analysis)**

| Characteristics | Total (n) (%) | Reactive | Prevalence | Chi-Square ($X^2$) | *P*-Value |
|---|---|---|---|---|---|
| **House type** | | | | | |
| Block | 255 (62.8) | 197 | 77.3% | 1.2106 | 0.27 (NS) |
| Plank | 149 (36.7) | 122 | 81.9% | | |
| **Toilet type** | | | | | |
| Pit | 291 (71.7) | 236 | 81.1% | 2.872 | 0.24 (NS) |
| Flushing | 109 (26.8) | 80 | 73.4% | | |
| Other sources (W/S/B) | 4 (0.99) | 3 | 75.0% | | |
| **Employment Status** | | | | | |
| Employed | 80 (19.7) | 67 | 83.8% | 1.3775 | 0.24 (NS) |
| Unemployed | 324 (79.8) | 252 | 77.8% | | |
| **Own a Refrigerator** | | | | | |
| Yes | 150 (36.9) | 113 | 75.3% | 1.8893 | 0.17 (NS) |
| No | 254 (62.6) | 206 | 81.1% | | |
| **Own a Radio** | | | | | |
| Yes | 259 (63.8) | 200 | 77.2% | 28.0725 | **0.00 (Sig)** |
| No | 145 (35.7) | 119 | 82.1% | | |
| **Own a TV** | | | | | |
| Yes | 353 (86.9) | 275 | 77.9% | 1.8795 | 0.17 (NS) |
| No | 51 (12.6) | 44 | 86.3% | | |
| **Own a Car** | | | | | |
| Yes | 59 (14.5) | 46 | 77.9% | 0.0411 | 0.84 (NS) |
| No | 345 (85.0) | 273 | 79.1% | | |
| | | | | | |
| **No data** | 2 (0.5) | 2 | | | |
| **Total** | **406** | **321** | **79.1%** | | |

Key: W/S/B- Water/Sea/Bush, TV-television, NS- Not significant, Sig- Significant, *p* value significant at $p < 0.05$, Note: socio-economic data were not available for 2 reactive samples: BM 11 and BM 207.

### 3.3 AMPLIFICATION OF HHV-8 DNA FROM TOTAL CELLS

Two lytic genes of HHV-8, that is, ORF 26, a gene that shows homology with other herpesviruses, was amplified for detection and confirmation of HHV-8 DNA in the study population (Figure 3.1), while K1, HHV-8 unique gene was amplified for characterization of the isolates (Figure 3.2). Of the 321 samples reactive to HHV-8 IgG ELISA, DNA was extracted from 300 total cells and subjected to amplification of a 233 bp of ORF 26 gene of HHV-8 by PCR. The remaining 21 samples (6.5%, 21/321) were lysed and could not be extracted or amplified. Seventy (70) samples were successfully amplified (23.3%, 70/300). The extracted DNA of the ORF 26 amplified samples (n=70) were further subjected to amplification of the ORF K1 gene.

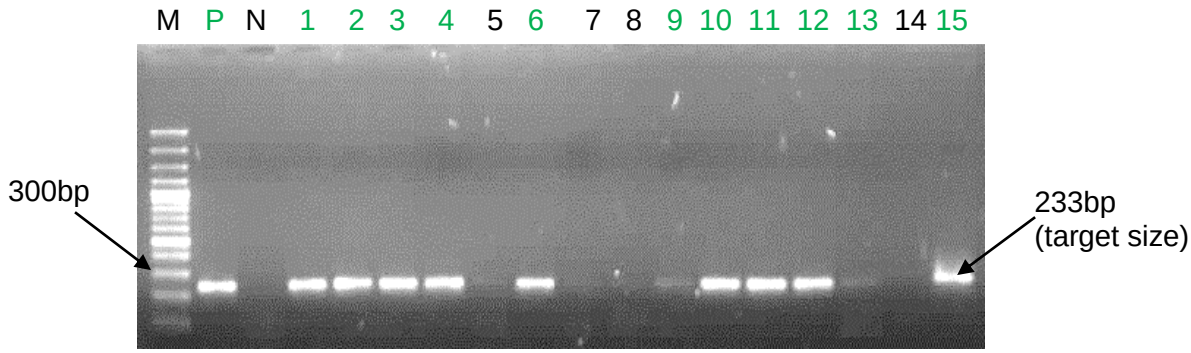



Figure 3.1 Representative gel electrophoresis photo of the amplified fragment of ORF 26 gene of HHV-8 on a 1.5% agarose gel and 100bp plus ladder (Invitrogen) molecular marker. Note: M-molecular marker, N-negative control (PCR Water), P-positive control (known HHV-8 DNA containing sample). Samples containing HHV-8 DNA are represented in lane 1-4, 6, 9-13 and 15 (green) and those not successfully amplified were represented in lane 5,7,8 and 14 (black).

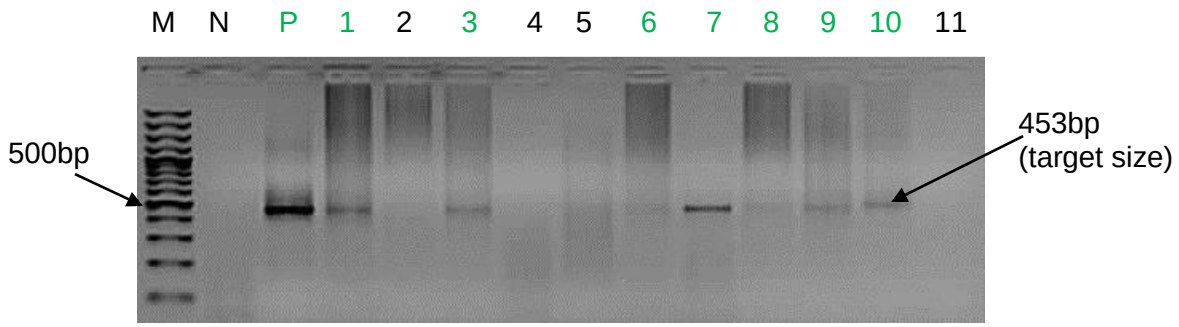



Figure 3.2 Representative gel electrophoresis photo of the amplified fragment of ORF K1 of HHV-8 on a 1.5% agarose gel and 100bp plus ladder (Invitrogen) molecular marker. Note: M-molecular marker, N-negative control (PCR Water), P-positive control (known HHV-8 DNA containing sample). HHV-8 DNA positive lanes are lane 1, 3, 6-9 and 10. Samples containing HHV-8 DNA are represented in lane 1,3, 6-10 (green) and those not successfully amplified were represented in lane 4,5 and 11 (black).

## 3.4 PURIFICATION AND SEQUENCING OF AMPLICONS

Two of the 70 ORF 26 amplicons were purified, sequenced and compared with published sequences to confirm amplicons as HHV-8 DNA. Twenty amplified products of K1 gene were purified and visualized (Figure 3.3) on a 2% agarose gel electrophoresis. Of these K1 purified amplicons, 14 were successfully sequenced in both forward and reverse directions using Sanger sequencing method and subtyped phylogenetically (Table 3.3).

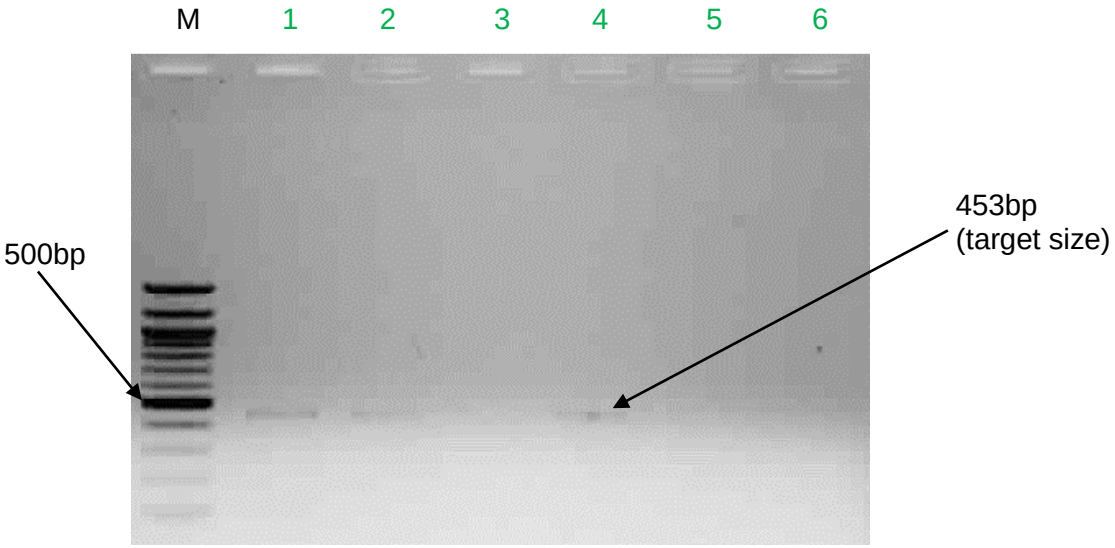



Figure 3.3 Representative gel electrophoresis photo of the purified fragment of ORF K1 of HHV-8 on a 2% agarose gel and 100bp plus ladder (Invitrogen) molecular marker. Note: M-molecular marker. Samples containing HHV-8 DNA are represented in lane 1-6 (green). Although, the bands were not visible enough but purified amplicons possess enough DNA for successful sequencing.

## 3.5 PHYLOGENETIC ANALYSIS OF HHV-8

Nucleotide sequences of each sample were uploaded in BioAfrica Oxford HHV8 Automated Subtyping Tool version 2.0 (http://bioafrica.mrc.ac.za/rega-genotype/html/indexhhv8.html) to determine the subtypes (Alcantara *et al.,* 2009; De Oliveira *et al.,* 2005). The subtypes assigned from the online tool were confirmed phylogenetically (Figure 3.4). The 14 sequences obtained in this study were aligned with sequences from previous studies (Isaacs *et al.,* 2016; Olp *et al.,* 2015; Kajumbula *et al.,* 2006; Lacoste *et al.,* 2000; Cook *et al.,* 1999; Zong *et al.,* 1999) and the phylogenetic relationship was determined. The phylogenetic tree shows the relationship between nucleotide sequences obtained in this study and sequences from Western and African countries such as New Zealand, Taiwan, Brazil, USA, Spain, France, Cameroon, Central African Republic (CAR), Uganda, South Africa, Zambia, Zaire (now Democratic Republic of Congo) and French Guiana (Figure 3.4).

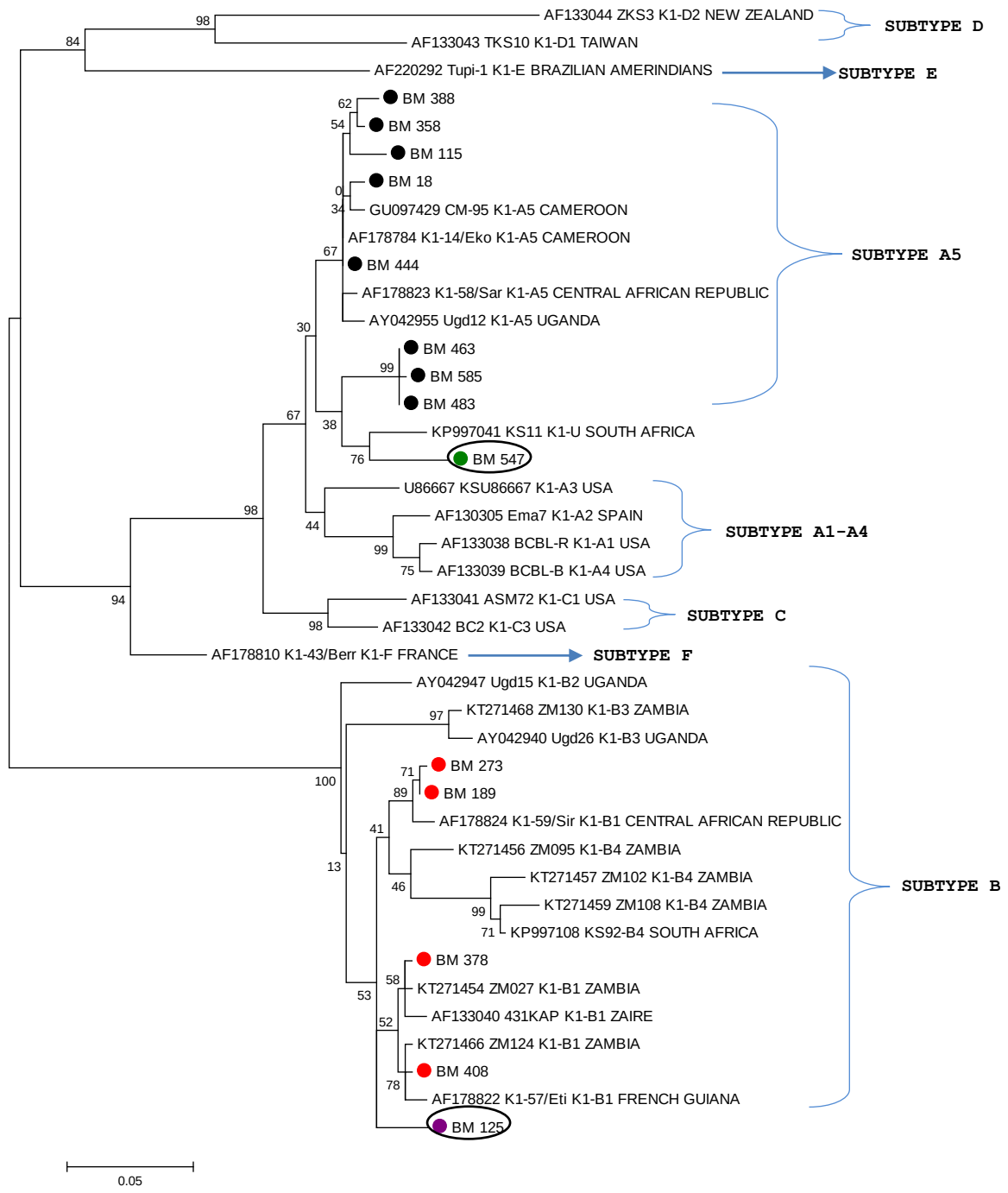



Figure 3.4 Phylogenetic relationships between 14 HHV-8 sequences in this study. The phylogenetic tree includes the 14 426 bp HHV-8 K1 consensus sequences and worldwide A to F sub-types prototypes from healthy persons and KS patients. Once the sequences were obtained, a multiple sequence alignment (1.6 Clustal) was performed with the SEQMAN Pro II (V 8) and BIOEDIT programs (v.7.2.5) on the basis of a previous **nucleotides** alignment created from the original sequences. The pairwise distance was estimated using Maximum Composite Likelihood (MCL) approach (Kimura 2-Parameter model + a discrete Gamma distribution with 5 rate categories). The phylogeny was derived by the **Maximum Likelihood** (ML) method, performed in the MEGA program (v 6) (Tamura *et al.*, 2013) and the reliability of the inferred tree was evaluated by bootstrap analysis on 1000 replicates. Eight isolates were aligned to A5 subtype (black circles), BM 547 (green circle) as unclassified, four isolates as B1 (red circles) and BM 125 as B1/B4 (purple circle). ZKS3 was used as an outlier. The tree was drawn to scale with 0.05 nucleotide replacements per site.

### 3.6 PREVALENCE OF HHV-8 K1 SUBTYPES

Of the 14 K1 sequences obtained in this study, thirteen (92.9%) sequences grouped with either subtype A or B references, supported by bootstrap values of 98% and 100%, respectively (Figure 3.4). Insertions, deletions or stop codons were not observed in K1 region amplified. The two major subtypes found in this population were subtype A5 (n=8; 57.1 %) and subtype B (n=5; 35.7 %). However, one sequence did not group with any of the known subtypes. One B subtype sequence, (BM 125) did not cluster with designated B subgroups in the tree constructed while another termed "unclassified" (BM 547) did not cluster with any designated subtype (Figure 3.4). None of the subtyped sequences is of patients from Littoral region (0/14, 0%) and only one male (1/14, 7.1%) sequence was successfully subtyped (Table 3.3). Furthermore, distribution of HHV-8 based on K1 subtypes identified, demographic, immunologic and socio-economic variables were assessed at the univariate level as shown in Table 3.4. None of the variables was statistically associated with HHV-8 K1 subtypes.

**Table 3.3 Demographic and immunologic characteristics of K1 amplicons and K1 Subtype**

| S/N | Sample Code | Age | Sex | Marital Status | CD4+ Count | HIV Stage | K1 Subtype |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| 1 | BM018 | 38 | Female | Divorced | 568 | 1 | A5 |
| 2 | BM115 | 48 | Female | Single | 207 | 2 | A5 |
| 3 | BM125 | 36 | Female | Married | 132 | 2 | B1/B4 |
| 4 | BM189 | 28 | Female | Single | 104 | 3 | B1 |
| 5 | BM273 | 48 | Female | Single | 349 | 3 | B1 |
| 6 | BM358 | 37 | Female | Single | 16 | 3 | A5 |
| 7 | BM378 | 43 | Male | Widower | 185 | 4 | B1 |
| 8 | BM388 | 38 | Female | Single | 68 | 4 | A5 |
| 9 | BM408 | 29 | Female | Single | 259 | 1 | B1 |
| 10 | BM444 | 49 | Female | Single | 689 | 1 | A5 |
| 11 | BM463 | 31 | Female | Married | 200 | 4 | A5 |
| 12 | BM483 | 21 | Female | Single | 162 | 4 | A5 |
| 13 | BM547 | 5 | Female | Single | 811 | N/A | U |
| 14 | BM585 | 30 | Female | Married | 173 | 3 | A5 |

Key: N/A-Not Available, U-unclassified, B1/B4-undefined subgroup.

**Table 3.4 Distribution of HHV-8 subtypes and risk factors (univariate analysis)**

| Characteristics | Total (n) (%) | Subtype A (%) | Subtype B (%) | Chi-Square ($X^2$) | *P*-Value |
|---|---|---|---|---|---|
| **Gender** | | | | | |
| Male | 1 (7.1) | 0 (0.0) | 1 (100.0) | 1.9385 | 0.16 (NS) |
| Female | 13 (92.9) | 9 (69.2) | 4 (30.8) | | |
| **Age** | | | | | |
| ≤14 | 1 (7.1) | 1(100.0) | 0 (0.0) | 1.9704 | 0.58 (NS) |
| 15-29 | 3 (21.4) | 1 (33.3) | 2 (66.7) | | |
| 30-44 | 7 (50.0) | 5 (71.4) | 2 (28.6) | | |
| ≥45-59 | 3 (21.4) | 2 (66.7) | 1 (33.3) | | |
| **Marital Status** | | | | | |
| Single | 9(64.3) | 6 (66.7) | 3 (33.3) | 2.3852 | 0.49 (NS) |
| Married | 1 (7.1) | 1 (100.0) | 0 (0.0) | | |
| Divorced | 3 (21.4) | 2 (66.7) | 1 (33.3) | | |
| Widow/widower | 1 (7.1) | 0 (0.0) | 1 (100.0) | | |
| **CD4+ Count** | | | | | |
| <500 | 11 (78.6) | 6 (54.5) | 5 (45.5) | 2.1212 | 0.15 (NS) |
| ≥500 | 3 (21.4) | 3 (100.0) | 0 (0.0) | | |
| **HIV Stages** | | | | | |
| Stage 1 & 2 | 5 (35.7) | 3 (60.0) | 2 (40.0) | 0.0081 | 0.93 (NS) |
| Stage 3 & 4 | 8 (57.1) | 5 (62.5) | 3 (37.5) | | |
| Unknown | 1 (7.1) | 1 (100.0) | 0 (0.0) | | |
| **On HAART** | | | | | |
| Yes | 5 (35.7) | 2 (40.0) | 3 (60.0) | 1.998 | 0.16 (NS) |
| No | 9 (64.3) | 7 (77.8) | 2 (22.2) | | |
| **House type** | | | | | |
| Block | 10 (71.4) | 5 (50.0) | 5 (50.0) | 3.1111 | 0.08 (NS) |
| Plank | 4 (28.6) | 4 (100.0) | 0 (0.0) | | |
| **Toilet type** | | | | | |
| Pit | 10 (71.4) | 5 (50.0) | 5 (50.0) | 3.1111 | 0.21 (NS) |
| Flushing | 3 (21.4) | 3 (100.0) | 0 (0.0) | | |
| Bush | 1 (7.1) | 1 (100.0) | 0 (0.0) | | |
| **Employment Status** | | | | | |
| Employed | 4 (28.6) | 3 (75.0) | 1 (25.0) | 0.28 | 0.08 (NS) |
| Unemployed | 10 (71.4) | 6 (60.0) | 4 (40.0) | | |
| **Own a Refrigerator** | | | | | |
| Yes | 6 (42.9) | 5 (83.3) | 1 (16.7) | 1.6593 | 0.20 (NS) |
| No | 8 (57.1) | 4 (50.0) | 4 (50.0) | | |
| **Own a TV** | | | | | |
| Yes | 13 (92.9) | 8 (61.5) | 5 (38.5) | 0.5983 | 0.44 (NS) |
| No | 1 (7.1) | 1 (100.0) | 0 (0.0) | | |
| **Own a Radio** | | | | | |
| Yes | 6 (42.9) | 4 (66.7) | 2 (33.3) | 0.0259 | 0.87 (NS) |
| No | 8 (57.1) | 5 (62.5) | 3 (37.5) | | |
| **TOTAL** | **14** | **9** | **5** | | |

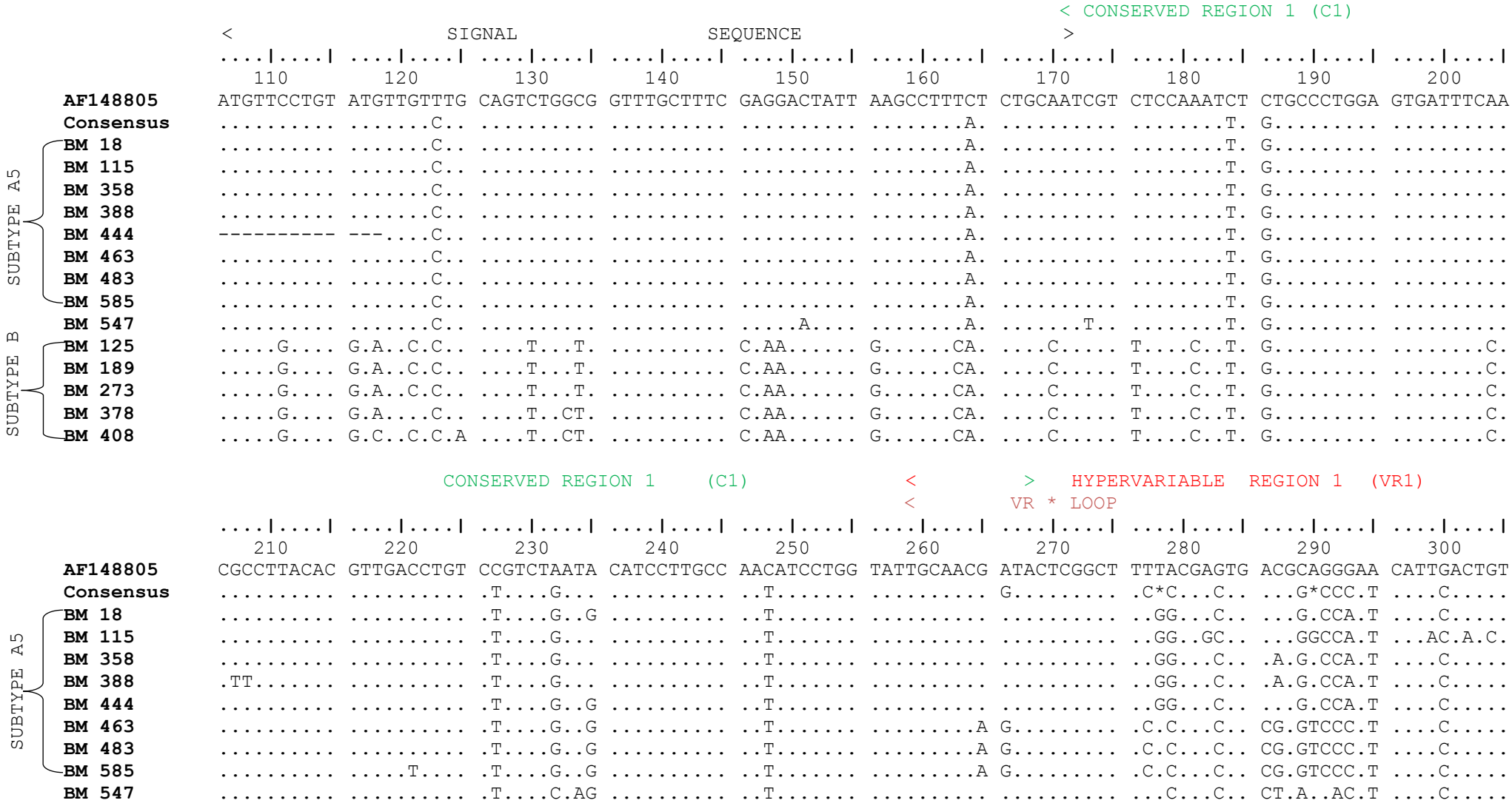
Key: CD-clusters of differentiation, TV-television, NS- Not significant, Sig- Significant, p value significant at $p < 0.05$, stage 1, 2, 3 and 4 based on World Health Organisation (WHO) classification of HIV stages.

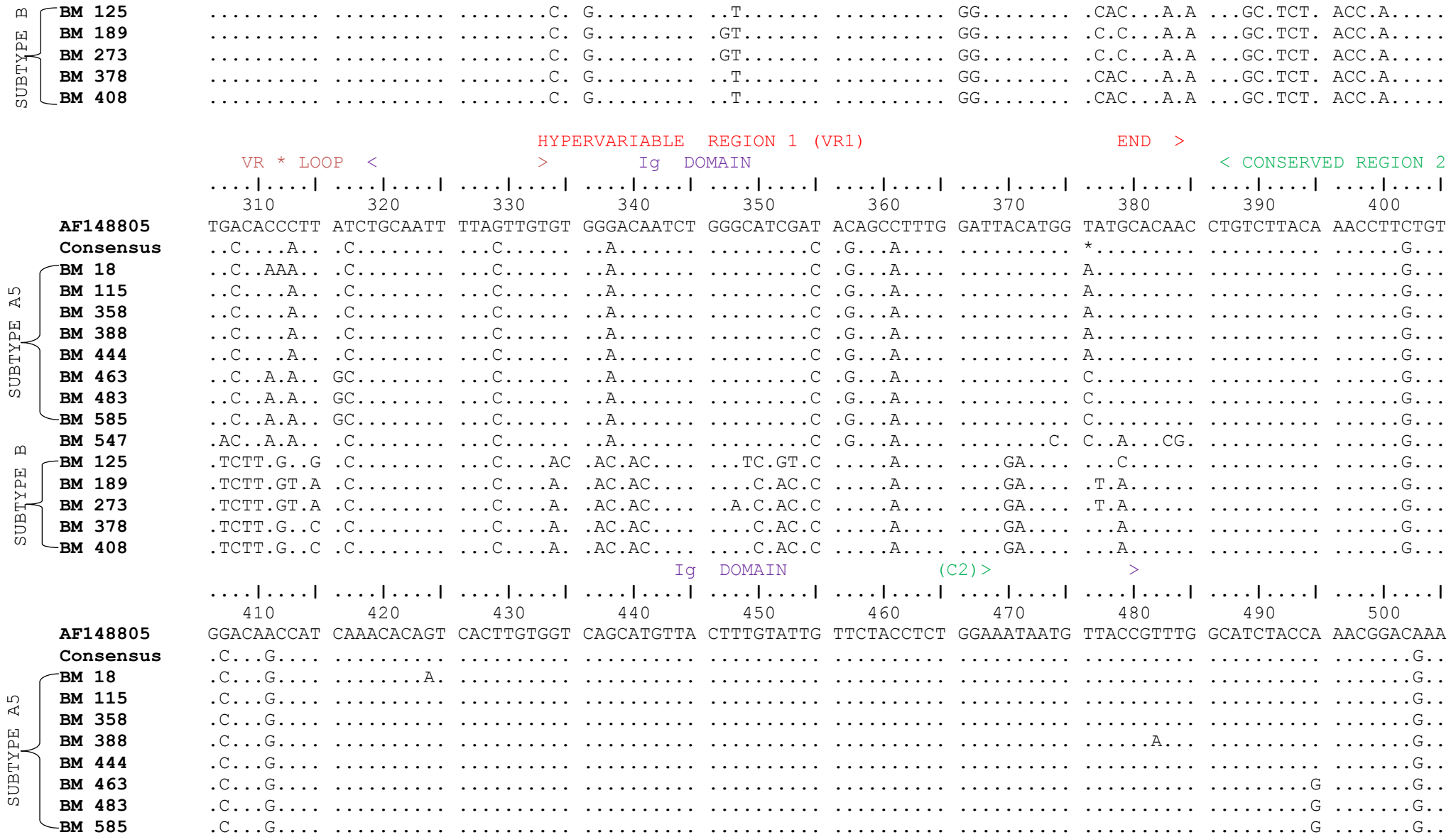
## 3.7 GENETIC VARIABILITY OF AMPLIFIED K1 REGION

Prior to amplification of K1 gene in samples, the known HHV-8 DNA of a sample was re-amplified, sequenced and used as a positive control. The new sequence generated was identical to the previous one, this indicates that there was no misincorporation of nucleotides during PCR and that the genetic variability of K1 observed in this study is real. All K1 sequences obtained were different from each other in terms of their nucleotide and amino acid sequences except for BM 463 and BM 483 which were 100% identical, albeit, from different patients (Figure 3.5). Another sample similar to BM 463 and BM 483 is BM 585 (Figure 3.6), but different from both at nucleotide position 221 (C vs. T) and at amino acid position 39 (T vs. I). The 14 K1 sequences display 0.0% (BM 463 vs. BM 483) to 22.3% (BM 547 vs. BM 273) nucleotide divergence which resulted into 0.0% (BM 463 vs. BM 483) and 44.7% (BM 547 vs. BM 408) amino acid divergence among pairwise compilations (Table 3.7). The mean genetic distance (intra-subtype variability) for subtype A sequences ranges from 0.000 to 0.064 and from 0.002 to 0.034 for subtype B sequences (Table 3.8 and 3.9).

The K1 nucleotide sequences (426 bp) with their consensus aligned with HHV-8 reference prototype (AF148805) revealed the signal sequence (105-171 nt), the conserved region 1 (171-267 nt), VR1 (259-384 nt), VR * loop (259- 333 nt), Ig domain (319-480 nt) and conserved region 2 (387-468 nt) present in the extracellular domain (Figure 3.5). The consensus generated from sequences in this study differs from the global HHV-8 prototype at 32 positions which include T123C, C164A and A503G (Figure 3.5). Similarly, some genetic variations were observed between A5 and B sequences in this study and reference subtype sequences. The Cys bridges of both A and B sequences from this current study were conserved, except for BM 408 (C7Y). Also, variation was observed at NXS/T motif of an A5 sequence (BM 115; T66N). Alignment of A5 prototype with A5 consensus revealed that asparagine, N (an N-glycosylation residue) is replaced in all A5 sequences with Aspartic acid, D (N43D) within the conserved region 1 (C1) and another of such replacement was observed in VR * loop, (L70I). Other mutations observed in A5 sequences from this study varies across the isolates (Figure 3.6). From the alignment of B1 prototype with B consensus generated, we observed mutations in the signal peptide (V6L and L10V) and in VR1 (Q56R and Q89E). Furthermore, BM 547, differs from A5 prototype at 15 amino acid positions including N43Q and N91H. BM 125 differs from B1 prototype at 10 amino acid positions including D118N and from B4 prototype at 11 positions (Figure 3.7).

## 3.8 CONSENSUS OF ALIGNED HHV-8 K1 NUCLEIC ACID SEQUENCES OF STUDY PARTICIPANTS WITH HHV-8 PROTOTYPE (AF148805)





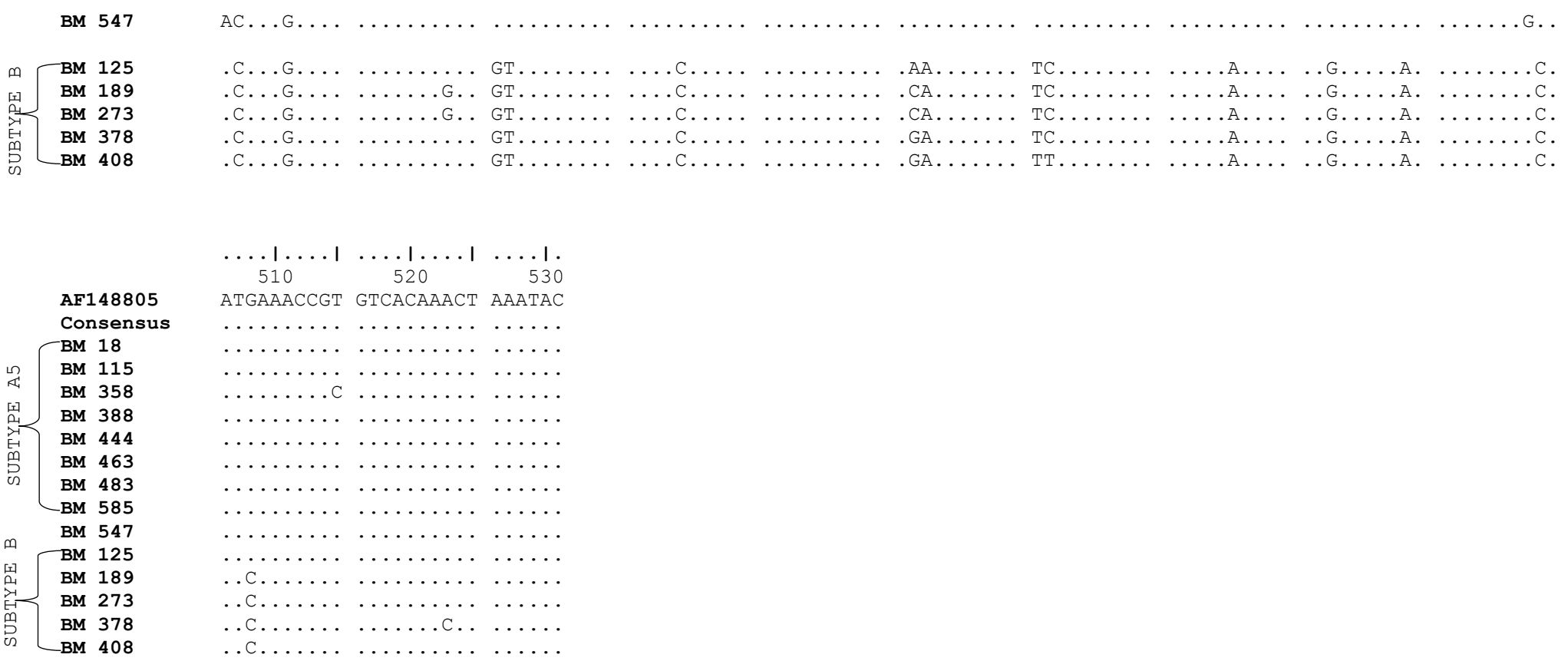



Figure 3.5 Consensus alignment of K1 nucleotide sequences. The sequences with the consensus generated from test sequences were aligned against HHV-8 prototype (AF148805). The signal sequence and the hypervariable region 1 of K1 were indicated. The dots (.) indicate identical nucleic acids while the asterisks (*) indicate non-consensus positions. The sequences were aligned using Clustal W multiple alignment with threshold frequency of 57% on BIOEDIT (V.7.2.5). The consensus differs from the global HHV-8 prototype at 32 positions which include T123C, C164A and A503G. NB: The sequence numbering (nt 105-531) was according to the nucleotide positions on the HHV-8 prototype (AF148805) and nt implies nucleotide.

### 3.9 CONSENSUS OF PREDICTED AMINO ACID SEQUENCES WITH A5 AND B1 PROTOTYPES

Sequences of K1 gene consisting of the signal peptide, VR1 and VR * loop present in the extracellular domain of gene was successfully amplified from 14 samples. These sequences with other previously published sequences obtained from GenBank were used for analysis. The amino acids (aa 1-142) obtained with the consensus generated were aligned with HHV-8 A5 and B1 prototypes (Figure 3.6). The alignments with A5 prototype (AAG01621) and B1 prototype (AAD30531) revealed the signal peptide (aa 1-22), the conserved region 1 (aa 22-54), VR1 (aa 52-93), VR * loop (aa 52-76), Ig domain (aa 72-125) and conserved region 2 (aa 94-121) present in the extracellular domain.

```
                                  <   CONSERVED REGION 1     (C1)    < >         HYPERVARIABLE REGION 1     (VR1)   > < (C2)
                 <   SIGNAL PEPTIDE     >                                <         VR * LOOP     <     >  Ig  DOMAIN
                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                          10         20         30         40         50         60         70         80         90        100
AAG01621-A5      MFLYVVCSLA VCFRGLLSLY LQSSPNLCPG VISTPYTLTC LSNASLPISW YCNDTRLWRL TDQSFTVATL TCNFTCVEQS GHRQSIWITW NAQPVLQTLC
Consensus        .......... .......... .......... .......... ..D....... .......... .........NI .......... .......... ..........
BM 18            .......... .......... .......... .......... ..D....... .......... .........KI .......... .......... ..........
BM 115           .......... .......... .......... .......... ..DT...... .......... .G..YNL..I .......... .......... ..........
BM 358           .......... .......... .......... .......... ..DT...... .......... K........I .......... .......... ..........
BM 388           .......... .......... .......... ....S..... ..DT...... .......... K........I .......... .......... ..........
BM 444           ----X..... .......... .......... .......... ..D....... .......... .........I .......... .......... ..........
BM 463           .......... .......... .......... .......... ..D....... ...S...L.. RVP.....NI A.......... .......... H.........
BM 483           .......... .......... .......... .......... ..D....... ...S...L.. RVP.....NI A.......... .......... H.........
BM 585           .......... .......... .......... ........I. ..D....... ...S...L.. RVP.....NI A.......... .......... H.........
BM 547           .......... .....I.... ..L....... .......... ..Q....... .......F.. LKT....TNI .......... .........S HTP.......

AAD30531-B1      MLLCIVCSLL VCFPKLLSLH LPSFPHLCPG VISTPYTLTC PSNRSLPISW YCNGTQLHRI TASNLTVSSL TCNFTCMTTS GPTHSIWIQW YTQPVLQTLC
Consensus        .....L...V .......... .......... .......... .......... .....R.... .......... .......... ........E. ..........
BM 125           .....L...V .......... .......... .......... .......... .....R.... .......... ......T... .SV.....E. .P........
BM 189           .....L...V .......... .......... .......... .......V.. .....R.L.. .......... .......... ........E. F.........
BM 273           .....L...V .......... .......... .......... .......V.. .....R.L.. .......... .......... ........E. F.........
BM 378           .......... .......... .......... .......... .......... .....R.... .......... .......... ........E. ..........
BM 408           ....LLY... .......... .......... .......... .......... .....R.... .......... .......... ........E. ..........

SUBTYPE A5
SUBTYPE B
```

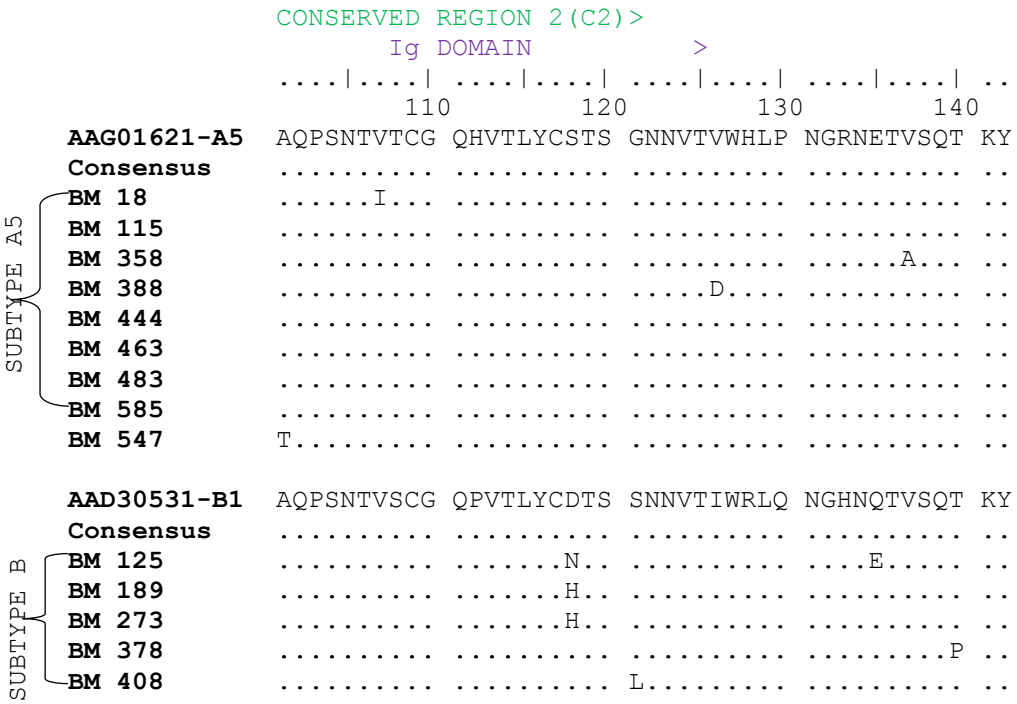



Figure 3.6 Alignment of predicted amino acid sequences of K1. The signal peptide, VR * loop (boxed) and the hypervariable region 1 (VR1) were indicated. The sequences were aligned against a consensus generated from the test sequence, HHV-8 subtype A5 (AAG01621) and subtype B1 (AAD30531) prototypes. The dots (.) indicate identical amino acids. The two conserved NXS/T motifs (at position 53/55 and 64/66) between the conserved Cys bridge residues of VR * loop (boxed) are indicated in bold letters. The sequences were aligned using Clustal W multiple alignment with threshold frequency of 57% on BIOEDIT (V.7.2.5). The consensuses differ from the global A5 and B1 prototype at 3 and 4 positions respectively which include N43D, T69N, L70I, V6L, L10V, Q56R and Q89E. NB: The sequence numbering (aa 1-142) was according to the amino acid positions on the A5 and B1 prototypes (AAG01621 and AAD30531) and aa implies amino acid.

### 3.10 SYNONYMOUS AND NONSYNONYMOUS MUTATIONS IN K1 GENE

From the alignments results, some genetic mutations were observed and reported in Table 3.5 and Figure 3.7. Synonymous and nonsynonymous mutations were detected in amplified K1 region (142 aa; 49% of the complete K1 gene). In the fragment of K1 amplified, A5 viruses displayed higher nonsynonymous mutation rates (1.33) than synonymous mutation rates (0.5) which contrast observation in B viruses, with higher synonymous mutation rate (1) than the nonsynonymous rate (0.8). Similarly, the VR1 (codons 52 to 93) of K1 displays synonymous and nonsynonymous mutations in both A5 and B viruses. Although within the VR1, A5 viruses have higher nonsynonymous mutation rates than the B viruses (1.33 verses 0.8), however, the B viruses have higher synonymous mutation rates than the A5 viruses (1 verses 0.28) (Figure 3.7). Both synonymous and nonsynonymous mutations were observed in K1 of subtype B, although uncommon in VR1 but occur at a lower rate (Figures 3.6 and 3.7a). In summary, nonsynonymous mutations were more common than synonymous mutations in both subtypes A5 and B (Tables 3.5 and 3.6). The subtype A5 sequences obtained have a lower dS/dN ratio of 1.0638 but higher median value of 0.5454 compared to dS/dN ratio of 1.2724 and median value of 0.2979 in subtype B sequences (Figures 3.7 and 3.8).

**Table 3.5 List of codons in K1 sequences that resulted in synonymous mutations**

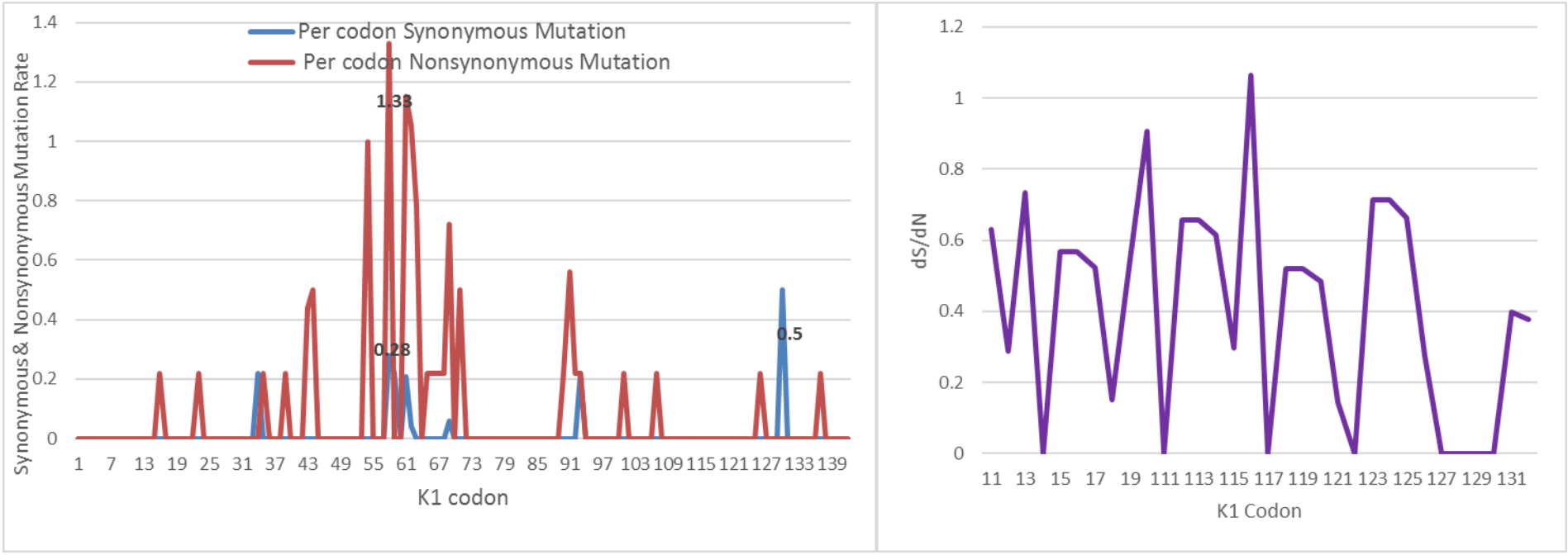
| Sample code | Nucleotide positions on reference prototype | Change in codons | Predicted amino acid |
|---|---|---|---|
| **Subtype A5 samples** | | | |
| BM 388 | nt 205-207 | acG→acT | Threonine (T) |
| BM 463 | nt 277-279 | ttA→ctC | Leucine (L) |
| BM 483 | nt 277-279 | ttA→ctC | Leucine (L) |
| BM 585 | nt 277-279 | ttA→ctC | Leucine (L) |
| BM 115 | nt 280-282 | cgA→cgG | Arginine (R) |
| BM 463 | nt 493-495 | ccA→ccG | Proline (P) |
| BM 483 | nt 493-495 | ccA→ccG | Proline (P) |
| BM 585 | nt 493-495 | ccA→ccG | Proline (P) |
| **Subtype B samples** | | | |
| All B subtypes | nt 232-234 | aaT→aaC | Asparagine (N) |
| All B subtypes | nt 298-300 | ttG→ctA | Leucine (L) |
| BM 125 | nt 313-315 | ctT→ctG | Leucine (L) |
| BM 189 | nt 313-315 | ctT→ttA | Leucine (L) |
| BM 273 | nt 313-315 | ctT→ttA | Leucine (L) |
| BM 273 | nt 346-348 | ggG→ggA | Glycine (G) |
| BM 189 | nt 421-423 | acA→acG | Threonine (T) |
| BM 273 | nt 421-423 | acA→acG | Threonine (T) |
| All B subtypes | nt 424-426 | gtC→gtG | Valine (V) |

Key: nt- nucleotide, A-Adenine, C-Cytosine, T-Thymine, G-Guanine, nucleotide changes in block letters.

**Table 3.6 List of some nonsynonymous mutations in K1 region amplified**

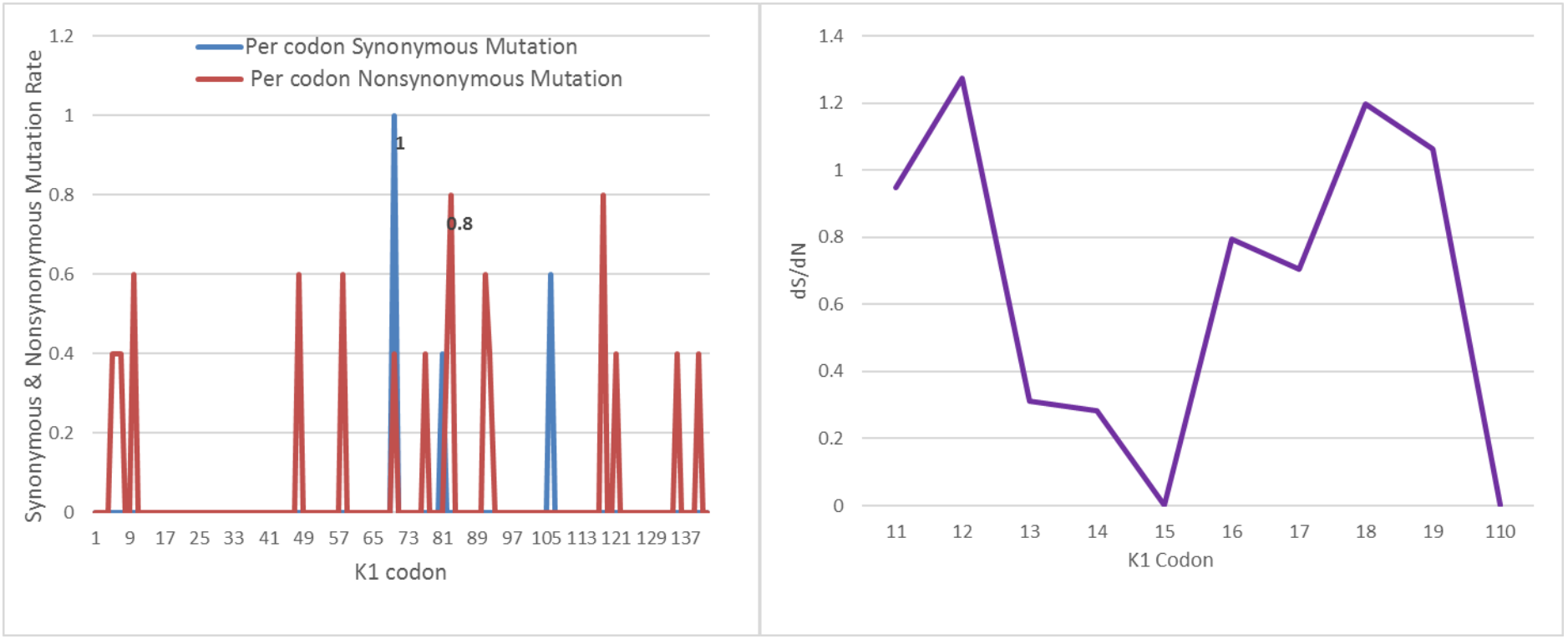
| Sample code | Positions on A5 prototype | Predicted amino acid change |
|---|---|---|
| | | |
| BM 585 | nt 115-117 (aa 39) | T→I (Threonine to Isoleucine) |
| All A5 subtypes | nt 127-129 (aa 43) | N→D (Asparagine to Aspartic acid) |
| BM 358 | nt 181-183 (aa 61) | T→K (Threonine to Lysine) |
| BM 483 | nt 187-189 (aa 63) | Q→P (Glutamine to Proline) |
| BM 115 | nt 196-198 (aa 66) | T→N (Threonine to Asparagine) |
| BM 18 | nt 205-207 (aa 69) | T→K (Threonine to Lysine) |
| All A5 subtypes | nt 208-210 (aa 70) | L→I (Leucine to isoleucine) |
| BM 463 | nt 271-273 (aa 91) | N→H (Asparagine to Histidine) |
| BM 388 | nt 376-378 (aa 126) | V→D (Valine to Aspartic acid) |
| | | |
| **Sample code** | **Positions on B prototype** | **Predicted amino acid change** |
| BM 408 | nt 19-21 (aa 7) | C→Y (Cysteine to Tyrosine) |
| All B subtypes | nt 166-168 (aa 56) | Q→R (Glutamine to Arginine) |
| All B subtypes | nt 265-267 (aa 89) | Q→E (Glutamine to Glutamic acid) |
| BM 189 | nt 271-273 (aa 91) | Y→F (Tyrosine to Phenyl alanine) |
| BM 125 | nt 352-354 (aa 118) | D→N (Aspartic acid to Asparagine) |

Key: nt- nucleotide, aa-amino acid.




(a) (b)

Figure 3.7 Graphical representation of samples designated as A5 subtype (a) synonymous and nonsynonymous mutation rates per codon (X axis/side) (b) synonymous to nonsynonymous mutation ratio (dS/dN) for the amplified region of K1 codon aa 1-142 (Y axis/bottom). Analysed with SNAP v2.1.1 by Korber, 2000 and graph are drawn to scale on Ms Excel, 2016. Nonsynonymous mutations and nonsynonymous mutation rates observed in A5 sequences is higher than those of the synonymous rates. The subtype A5 sequences obtained in this study have the highest dS/dN ratio of 1.0638 and median value of 0.5454.




(a) (b)

Figure 3.8 Graphical representation of samples designated as B subtype (a) synonymous and nonsynonymous mutation rates per codon (X axis/side) (b) synonymous to nonsynonymous mutation ratio (dS/dN) for the amplified region of K1 codon aa 1-142 (Y axis/bottom). Analysed with SNAP v2.1.1 by Korber, 2000 and graph are drawn to scale on Ms Excel, 2016. Synonymous mutations and synonymous mutation rates observed in B sequences are higher than those of the nonsynonymous rates. The subtype B sequences obtained in this study have the highest dS/dN ratio of 1.2724 and median value of 0.2979.

## 3.11 EVOLUTIONARY GENETIC DIVERGENCE BETWEEN SEQUENCES

The mean genetic distance among the isolates was used to determine the intra-genetic variability among the isolates based on the DNA sequences. The genetic distances were calculated using the JTT matrix-based model (Jones *et al.,* 1992) in MEGA6 (Tamura *et al.,* 2013). Tables 3.7, 3.8 and 3.9 contain the estimated evolutionary divergence among all the DNA sequences obtained, classified as subtype A and subtype B respectively.

**Table 3.7 Estimate of evolutionary divergence among isolates based on DNA sequences**

| Sample code | BM 18 | BM 115 | BM 358 | BM 388 | BM 444 | BM 463 | BM 483 | BM 585 | BM 547 | BM 125 | BM 189 | BM 273 | BM 378 | BM 408 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BM 18 | 0.000 | 0.023 | 0.015 | 0.020 | 0.007 | 0.036 | 0.036 | 0.039 | 0.055 | 0.206 | 0.210 | 0.213 | 0.205 | 0.209 |
| BM 115 | 0.023 | 0.000 | 0.018 | 0.023 | 0.015 | 0.047 | 0.047 | 0.050 | 0.068 | 0.210 | 0.214 | 0.217 | 0.209 | 0.212 |
| BM 358 | 0.015 | 0.018 | 0.000 | 0.010 | 0.007 | 0.039 | 0.039 | 0.041 | 0.057 | 0.203 | 0.208 | 0.211 | 0.203 | 0.206 |
| BM 388 | 0.020 | 0.023 | 0.010 | 0.000 | 0.013 | 0.044 | 0.044 | 0.047 | 0.063 | 0.210 | 0.215 | 0.218 | 0.210 | 0.213 |
| BM 444 | 0.007 | 0.015 | 0.007 | 0.013 | 0.000 | 0.033 | 0.033 | 0.036 | 0.052 | 0.200 | 0.204 | 0.207 | 0.199 | 0.202 |
| BM 463 | 0.036 | 0.047 | 0.039 | 0.044 | 0.033 | 0.000 | 0.000 | 0.003 | 0.052 | 0.207 | 0.208 | 0.211 | 0.207 | 0.210 |
| BM 483 | 0.036 | 0.047 | 0.039 | 0.044 | 0.033 | **0.000** | 0.000 | 0.003 | 0.052 | 0.207 | 0.208 | 0.211 | 0.207 | 0.210 |
| BM 585 | 0.039 | 0.050 | 0.041 | 0.047 | 0.036 | 0.003 | 0.003 | 0.000 | 0.055 | 0.211 | 0.211 | 0.215 | 0.210 | 0.214 |
| BM 547 | 0.055 | 0.068 | 0.057 | 0.063 | 0.052 | 0.052 | 0.052 | 0.055 | 0.000 | 0.223 | 0.220 | 0.223 | 0.219 | 0.222 |
| BM 125 | 0.206 | 0.210 | 0.203 | 0.210 | 0.200 | 0.207 | 0.207 | 0.211 | 0.223 | 0.000 | 0.033 | 0.036 | 0.028 | 0.028 |
| BM 189 | 0.210 | 0.214 | 0.208 | 0.215 | 0.204 | 0.208 | 0.208 | 0.211 | 0.220 | 0.033 | 0.000 | 0.002 | 0.025 | 0.025 |
| BM 273 | 0.213 | 0.217 | 0.211 | 0.218 | 0.207 | 0.211 | 0.211 | 0.215 | **0.223** | 0.036 | 0.002 | 0.000 | 0.028 | 0.028 |
| BM 378 | 0.205 | 0.209 | 0.203 | 0.210 | 0.199 | 0.207 | 0.207 | 0.210 | 0.219 | 0.028 | 0.025 | 0.028 | 0.000 | 0.010 |
| BM 408 | 0.209 | 0.212 | 0.206 | 0.213 | 0.202 | 0.210 | 0.210 | 0.214 | 0.222 | 0.028 | 0.025 | 0.028 | 0.010 | 0.000 |

The numbers of base substitutions per site from between sequences are shown on the lower left and as the mirror image on the upper right. Analyses were conducted using the JTT matrix-based model (Jones *et al.*, 1992) with bootstrap analysis on 1000 replicates. The rate variation among sites was modelled with a gamma distribution (shape parameter = 5). The analysis involved 14 nucleotide sequences. All positions containing gaps and missing data were eliminated. The evolutionary distance ranges from 0.000-0.223 i.e. from 77.7% similarity (between BM 547 and BM 273) to 100% similarity (between BM 463 and BM 483). There was a total of 413 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.,* 2013).

**Table 3.8 Estimate of evolutionary divergence among sequences belonging to A subtype**

| Sample code | BM 18 | BM 115 | BM 358 | BM 388 | BM 444 | BM 463 | BM 483 | BM 585 |
|---|---|---|---|---|---|---|---|---|
| BM 18 | 0.000 | 0.022 | 0.015 | 0.020 | 0.007 | 0.035 | 0.035 | 0.037 |
| BM 115 | 0.022 | 0.000 | 0.017 | 0.022 | 0.015 | 0.045 | 0.045 | 0.048 |
| BM 358 | 0.015 | 0.017 | 0.000 | 0.010 | 0.007 | 0.037 | 0.037 | 0.040 |
| BM 388 | 0.020 | 0.022 | 0.010 | 0.000 | 0.012 | 0.043 | 0.043 | 0.045 |
| BM 444 | 0.007 | 0.015 | 0.007 | 0.012 | 0.000 | 0.032 | 0.032 | 0.035 |
| BM 463 | 0.035 | 0.045 | 0.037 | 0.043 | 0.032 | 0.000 | 0.000 | 0.002 |
| BM 483 | 0.035 | 0.045 | 0.037 | 0.043 | 0.032 | **0.000** | 0.000 | 0.002 |
| BM 585 | 0.037 | **0.048** | 0.040 | 0.045 | 0.035 | 0.002 | 0.002 | 0.000 |

The numbers of base substitutions per site from between sequences are shown on the lower left and as the mirror image on the upper right. Analyses were conducted using JTT matrix-based model (Jones *et al.*, 1992) with bootstrap analysis on 1000 replicates. The rate variation among sites was modelled with a gamma distribution (shape parameter = 5). The analysis involved 9 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 426 positions in the final dataset. The evolutionary distance ranges from 0.000-0.048 i.e. from 95.2% similarity (between BM 585 and BM 115) to 100% similarity (between BM 463 and BM 483). Evolutionary analyses were conducted in MEGA6 (Tamura *et al.,* 2013).

**Table 3.9 Estimate of evolutionary divergence among sequences belonging to B subtype**

| Sample code | BM 125 | BM 189 | BM 273 | BM 378 | BM 408 |
|---|---|---|---|---|---|
| BM 125 | 0.000 | 0.032 | 0.035 | 0.027 | 0.030 |
| BM 189 | 0.032 | 0.000 | 0.002 | 0.024 | 0.027 |
| BM 273 | **0.035** | **0.002** | 0.000 | 0.027 | 0.029 |
| BM 378 | 0.027 | 0.024 | 0.027 | 0.000 | 0.012 |
| BM 408 | 0.030 | 0.027 | 0.029 | 0.012 | 0.000 |

The numbers of base substitutions per site from between sequences are shown on the lower left and as the mirror image on the upper right. Analyses were conducted using the using JTT matrix-based model (Jones *et al.*, 1992) with bootstrap analysis on 1000 replicates. The rate variation among sites was modelled with a gamma distribution (shape parameter = 5). The analysis involved 5 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 426 positions in the final dataset. The evolutionary distance ranges from 0.002-0.035 i.e. from 96.5% similarity (between BM 273 and BM 125) to 99.8% similarity (between BM 189 and BM 273). Evolutionary analyses were conducted in MEGA6 (Tamura *et al.,* 2013).

# CHAPTER FOUR: DISCUSSION, CONCLUSION AND RECOMMENDATIONS

## 4.1 DISCUSSION

This study was carried out with the intention of investigating HHV-8 infection in HIV/AIDS patients. Although there is variation in seroprevalence and genetic subtypes of HHV-8 among countries, little is known about HHV-8 in HIV-positive individuals of Cameroon. The study site is Baptist Health Mutengene (BHM), situated in the South-West region of Cameroon. The health centre receives patients from different towns of the South-West and Littoral regions. The HIV/AIDS care unit of the health centre provides treatment, counselling, home-based support, in addition to these; primary health care and doctor consultations are available. The study population was a cohort of HIV-positive outpatients of both sexes and age 2-68 years.

### 4.1.1 SEROPREVALENCE OF HHV-8

This study utilized HHV-8 whole virus ELISA kit (ABI) to investigate the prevalence of HHV-8 in HIV-infected subjects in a laboratory-based retrospective approach. Immunoglobulin-G (IgG) antibodies to HHV-8 antigens were found in 79.1% (321/406) of the studied population, this implies immunologic response in form of presence of anti-HHV-8 antibodies. Although the immune response does not imply the presence of the virus or viremia in these patients, however, it is an indication of exposure to and acquisition of HHV-8 infection.

Furthermore, the high seroprevalence rate suggests that HHV-8 infection is endemic in both South-West and Littoral regions of Cameroon. This observation may be due to similar environmental or geographical factors predisposing the study population to HHV-8 infection and/or KS, these factors include exposure to helminth parasites, phorbol ester plants and volcanic soils (Wakeham *et al.,* 2011; Whitby *et al.,* 2007). The observed HHV-8 prevalence is slightly higher than the 70% (236/336) reported by Mbondji-Wonje and colleagues (2013), in HIV-1 infected individuals of urban areas of Cameroon and the 78.6% (11/14) reported in HIV-infected patients without KS at the General Hospital of Yaounde, Cameroon (Jacky *et al.,* 2015). The observed difference in seroprevalence of HHV-8 in this study and in other studies conducted in Cameroon might be due to differences in risk factors participants are exposed to and it could also be due to the deterioration of humoral immune responses due to HIV/AIDS (Stolka *et al.,* 2014). Similarly, a high prevalence of 65.6% (164/250) was reported in HIV-positive blood donors in Ghana (Adjei *et al.,* 2008) while in Nigeria, a prevalence of 62% (44/71) was reported in HIV-positive adults without KS (Ogoina *et al.,* 2011). The prevalence of HHV-8 observed in this study is similar to that observed in previous studies conducted on HIV-infected population of Cameroon and other African countries, however, these other studies focused on relatively small samples (Jacky *et al.,* 2015; Mbondji-Wonje *et al.,* 2013; Ukonu *et al.,* 2011;

Adjei *et al.,* 2008). The association between HHV-8 seropositivity and some variables, which include region, gender, age, HIV clinical stages, CD4+ cell count, use of HAART, marital status and socio-economic factors was assessed and the following were observed.

Although the number of females in this current study is about three times of the males, a similar prevalence was observed in both groups. This observation is in concordance with previous studies conducted in Ntem region of southern Cameroon and in HIV-1 infected individuals of northern Nigeria (Pedergnana *et al*., 2012; Ogoina *et al*., 2011).

The association found between HHV-8 seropositivity and age revealed a clear increase in the prevalence of anti-HHV-8 antibodies with age. This is similar to observations in the general population and HIV/AIDS population of other African countries (Betsem *et al.,* 2014; Shebl *et al.,* 2011; Tornesello *et al.,* 2010) but not observed in other studies (Jacky *et al.,* 2015; Hladik *et al.,* 2003, Wawer *et al.,* 2001). The high prevalence of HHV-8 observed among children (≤14 years old) in this current study was 43.2% (16/37), which could be due to either vertical transmission during pregnancy (Melser *et al.,* 2015; Brayfield *et al.,* 2003) or horizontal transmission from infected adults or from children to children through salivary contact (Phipps *et al.,* 2014; Hladik *et al.,* 2012; Plancoulaine *et al.,* 2000). The high prevalence observed in this age group with other co-factors necessary for the development of KS suggests the risk of AIDS-KS in these children.

In African population, evidence for sexual transmission of HHV-8 remains unclear, even among high risk groups in HHV-8/KS endemic and non-endemic regions. However, in this study, the high prevalence (>60%) observed in adults of reproductive age groups suggests an ongoing transmission possibly through sexual activities that involve salivary contact, nonetheless, the exact mode or act of transmission needs to be explored. Studies conducted in Kenya and Nigeria on adult commercial sex workers suggested that sexual transmission of HHV-8 occurs (Eltom *et al.,* 2002, Lavreys *et al.,* 2003), while another study suggested an ongoing sexual transmission during adulthood (Baeten *et al.,* 2002). Furthermore, the significant increase in seroprevalence of HHV-8 from childhood to adulthood was also observed in this study. The high prevalence of HHV-8 (100%) found in older women (≥60 years old) suggests early acquisition of the virus probably during childhood (Mbulaiteye *et al.,* 2006; Mbulaiteye *et al.,* 2004), however, the possibility of exposure while carrying out their motherly duties to their infected children cannot be ignored.

Despite the slight difference observed in seroprevalence of HHV-8 in WHO HIV clinical stages, there was no significant association with either stage 1 and 2 (early stage) and stage 3 and 4

(late stage). This finding contrasts that of a cross-sectional study conducted in Zaria, Nigeria on HIV-1 infected patients without KS (Ogoina *et al.,* 2011).

In contrast to the observation by Ogoina and colleagues (2011), this current study found a significant correlation between CD4+ cell count and HHV-8 seropositivity. HIV-infected patients with CD4+ cell counts less than 500 cell/$mm^3$ were found to have a higher seroprevalence. It is an established fact that CD4+ T cells play important roles in protection against intracellular pathogens including HHV-8, and CD4+ cell count is a marker of the immune status of an individual (Zhu and Paul, 2008). The correlation found is an indication of the relationship between immunosuppression and HHV-8/or its progression to AIDS-KS. This finding is consistent with a cross-sectional study on HIV-infected patients in China (Zhang *et al.,* 2012). The observed higher prevalence of HHV-8 in ART-naïve patients suggests a higher risk of acquiring HHV-8 and/or developing KS. In a broader context, data from this study suggest that initiation of ART and prevention of immune suppression reduce the risk of acquiring HHV-8 among HIV-infected patients of South-West and Littoral regions of Cameroon.

Similar to observation by Munawwar and colleagues (2014), a statistically significant increase in HHV-8 seroprevalence among the married compared with other marital status was observed. This suggests that married HIV/HHV-8 co-infected population, either HIV/HHV-8 concordant or serodiscordant couples are at increased risk of HHV-8 infection/KS. This is consistent with findings in a Tanzanian study which investigated intra-familial HHV-8 patterns and found out that HHV-8 seropositive spouses (women) are more likely infected by their partners (husbands) (Mbulaiteye *et al.,* 2003). On a broader view, this supports the possibility of sexual transmission of HHV-8 among the married category.

No significant association was found with almost all the socio-economic variables explored in this study except for the use or ownership of a transistor radio set, which was found to be inversely associated with an increase in HHV-8 seroprevalence. There may truly be no association between seropositivity of HHV-8 and ownership of a transistor radio set, as this might be a chance finding, nevertheless, the association might be due to yet an unknown factor which requires further analysis.

The differences observed in this study and other studies might be due to differences in population group involved in studies which include demographic, immunologic, ethnic, geographic, type of assay, sensitivity and specificity of diagnostic assays used and cofactors influencing HHV-8 transmission, control and progression to KS. To date, routine screening of healthy population for HHV-8 is not recommended or performed in any country. Nonetheless, it

is paramount that this should be considered in countries termed “HHV-8/KS belt” and for populations at high risks of infection, especially HIV/AIDS population.

### 4.1.2 AMPLIFICATION OF HHV-8 DNA FROM TOTAL CELLS

The decision to screen HIV-infected individuals shown to have anti-HHV-8 antibodies after screening with ABI ELISA kit was stimulated by previous reports of HIV-infected individuals who are also HHV-8 seropositive and are at higher risk of developing KS (Pediatric AIDS-Defining Cancer Project Working Group, 2016; Bohlius *et al.,* 2011).

The detection of HHV-8 DNA in some and not all seropositive patients in this study supports findings from previous studies carried out in HIV/HHV-8 endemic or AIDS-KS populations (Isaacs *et al.,* 2016; Olp *et al.,* 2015; Betsem *et al.,* 2014). The presence of HHV-8 DNA in the blood of HHV-8 seropositive and/or AIDS-KS patient is an indication of viremia, which is an important event in the pathogenesis of KS, however, its presence in seropositive but non-KS patient implies a higher risk of developing KS than patients who are just seropositive. We cannot state precisely that the prevalence of HHV-8 DNA observed in this study is high or low because the HHV-8 disease status of the studied population is unknown and cannot delineate that HHV-8 DNA is frequently found in total cells than PBMCs since PBMCs were not included in this study.

Alagiozoglou and colleagues (2000) reported 14/334 (4.2%) of HIV-positive patients with ORF 26 gene amplified in PBMCs while 70/300 (23.3%) with ORF 26 gene in total cells was amplified in this study. This suggests that a high prevalence of HHV-8 DNA could be obtained in total cells than PBMCs of HHV-8/HIV co-infected individuals. Also, to the best of our knowledge, this is the first report of HHV-8 amplification using DNA from total cells as template. This suggests DNA from total cells as a better alternative for amplification of both K1 and ORF 26 gene, in the absence of PBMCs or biopsies. The high failure rate (76.7%) of ORF 26 gene amplification might be due absence of the HHV-8 DNA in total cells of participants, the quality and concentration of the DNAs, or perhaps, most of the participants are only HHV-8 seropositive. The low number of samples detected to contain HHV-8 DNA in the K1 region amplified may be due to the hypervariability of the gene, primer mismatches, point mutations, presence of recombinant strains or the low viral load of HHV-8 in total cells.

### 4.1.3 GENETIC VARIABILITY OF K1 GENE

Despite the small number of samples sequenced, phylogenetically, evidence for evolutionary differences in A5 subtype of K1 and geographic differences in B subtype of K1 in HIV-infected patients of South-West, Cameroon was observed. The clustering pattern observed between

Central African; Cameroonian sequences (from this study) and Southern African; Zambian and South African sequences (reference sequences) suggested human migration, the existence of contacts and viral exchange which could represent a distinct viral subtype. The approximate 100% identity observed in both nucleotides and amino acid sequences of BM 585, BM 463 and BM 483 could not attributed to PCR incorporation, however, might be due to genetic makeup of patients and/or infection of the patients by a single isolate of HHV-8. The possibility of certain motifs been associated with regions or restricted to geographical areas could not be ignored (Di Alberti *et al.,* 1997).

In some of the sequences (BM 115, BM 585, BM 463, BM 483 and BM 547) in this study, triple mutations were observed within the VR * loop of K1 gene amplified. These could have some effect on the gene structure and functions, *in vivo* / *in vitro* or promote positive biological selection. Most of the mutations in K1 gene amplified in this study were nonsynonymous.

Although nonsynonymous mutations in K1 subtype A5 viruses occur more in VR1 but not limited to the remaining part of the extracellular domain amplified. In contrast, nonsynonymous mutations were observed in the more conserved connecting region (signal region among others) and occurred throughout the amplified area in the extracellular domain of K1 in subtype B viruses. In general, across the conserved regions (C1 and C2) analysed, low level variability was observed while in VR1, higher variability was observed in subtype A5 viruses than subtype B viruses. This observation suggests a powerful biological selection acting on A5 viruses (Hayward and Zong, 2007; Zong *et al.,* 1999) with the advantage of escaping surveillance of the immune system, prolonging shelf-life of infected cells and establishing persistent infection (Lusso *et al.,* 1995) or it might enhance the virulence and pathogenicity of HHV-8 (Isaacs *et al.,* 2016). Hesla and colleagues (2013) postulated an evolutionary advantage on the human host due to infection with EBV and HHV-6, similarly, these nonsynonymous mutations observed in A5 viruses might have yet an unknown evolutionary advantage on the human host.

In the current study, the intra-subtype nucleotide variability of K1 was lower (6.6 and 3.5%) for A and B subtypes, respectively compared to 22.3% inter-variability (the subtypes combined). This implies a positive selection acting on K1 gene (Cook *et al.,* 1999). Similarly, a lower dS/dN ratio was observed in A5 viruses compared to B viruses, therefore, a greater positive selection acting on K1-A5 gene and greater genetic diversity in K1-A5 gene is suggested.

All but one of the patients in this study harboured virus that did not group with any of the known subtypes. Interestingly, “BM 547” grouped with an AIDS-KS sequence (KS11) of a black 21 years old female, classified as unclustered (Isaacs *et al.,* 2016). Similarly, a B subtype sample,

BM 125 lies between the subgroup B1 prototype, B4 reference sequence from Zimbabwe and a sequence classified as unclustered (KS92) from South Africa. With these observations, BM 125 is assumed to be located on the outgroup of B branch as an early/late variant of all set of B strains while BM 547 located on the outgroup of A5 branch is assumed to be an early/late variant of all A strains. This could be due to either recombination events between two existing subtypes/subgroups or the development of new evolving strains. Amplification of complete K1 gene is required for BM 547 and BM 125 to corroborate these suggestions.

Several polymorphisms such as A44T, W58L, D62G amongst others were observed in this study. These polymorphisms had been reported previously to be under positive selection pressure (Cordiali-Fei *et al.,* 2015). Interestingly, all the 12 predicted codon sites under positive selection were found in subtype A5 but only 8 of subtype B sequences obtained in this study. This further strengthens the possibility of higher selection pressure acting on K1-A5 viruses of Cameroon. Another interesting observation from results obtained is an amino acid change in one conserved NXS/T motifs (position 64/66 which is **S**F**T** in A5 prototype) between the conserved Cys bridge residues of VR * but in BM 115, this motif is replaced with SNL, immediately followed by another substitution V67L, reasons and impacts of these changes either in the structure or functions of K1 gene requires further studies. Furthermore, amino acid substitution of C for Y (C7Y) was observed in BM 408, an observation similar to K1-57/Eti of French Guiana (Lacoste *et al*., 2000), this substitution of a conserved cysteine residue for tyrosine may have the structural or functional effect on K1 protein. Several studies on structure of K1 protein have revealed that the overlapping Ig domain and C2 (aa 92-125) in the extracellular domain are involved in inducing an increase in intracellular calcium concentration and tyrosine phosphorylation (Lee *et al.,* 2003), substitutions observed in these regions may cause this protein to lose its capability. Also, some amino acid substitutions in the extracellular domain of K1 might affect the signalling ability of the protein, this, however, requires further investigation.

Indeed, the prevalence of subtype A5 in this study (57%) is higher than that observed in studies on KS and HIV-infected populations of Africa: 49% of 86 KS patients in South Africa (Isaacs *et al.,* 2016), 45% of 65 KS patients in Zimbabwe (White *et al.,* 2008), 53% of 31 KS patients in Uganda (Kajumbula *et al*., 2006) and 38% of 21 KS patients in West and Central African countries (Lacoste *et al.*, 2000). The distribution of K1 subtype A5 in Africa is hypothesised by Hayward and Zong (2007) as a result of an A5 prototype introduced into African populations through a “very rapid and recent aggressive spread” augmented by a selective advantage of the A5 allele. The prevalence of B subtype observed in this study (36%) doubles that reported by Betsem and colleagues (2014) in pygmy and Bantu populations in Cameroon but both A5 and B

sequences in this study were closely related to each other than to reference sequences from distant areas of Africa with little evidence of genetic diversity.

## 4.2 STRENGTHS AND LIMITATIONS OF THE STUDY

This study has several strengths that include- its large sample size; its diversity, since patients came from various locations and children were also part of the study; demographical, immunological and socio-economic data, which were assessed as risk factors for HHV-8 infection among HIV-infected patients of Cameroon. Despite these strengths, it also has several limitations which include lack of samples such as mouth wash, saliva and/or biopsies which might reveal a higher prevalence of HHV-8 DNA in HHV-8 seropositive patients. Lack of information such as the ethnicity, sexual orientation, family members, history of STIs/blood transfusion, substance abuse, the number of sexual partners, KS status/stage of the participants and HIV/HHV-8 viral load which could help in correlation and interpretation of observations. Since the study is retrospective and samples are of HIV and not HHV-8 cohort, there was no control over sample and data collection. However, it is recommended that AIDS-KS patients and HIV-negative patients be involved in future studies to act as control units for better comparison.

## 4.3 CONCLUSION

This study presents the image of HHV-8 infection in an HIV-infected population from Cameroon. This study also reveals that HHV-8 infection is endemic in the study population, with subtypes A5 and B as the most important epidemiological genetic variants. In addition, targeting the ORF 26 region by PCR could be an approach to detect replicating virus in individuals. This study provides basic information for future studies of HHV-8 in Cameroon and contributes data to the HIV/HHV-8 co-infection landscape in the study area and in Africa at large.

## 4.4 RECOMMENDATIONS

Since there are few studies on HHV-8 in Cameroon, further studies on complete K1 gene and ORF 26 gene of these study participants could give more detailed information on the genetic variability of HHV-8 in Cameroon. Studies on impacts of HHV-8 strains on its epidemiology in relation to environmental and host factors should be elucidated. Also, the possibility of association between its routes of transmission and circulating strains should be considered to give more insight on the viral epidemiology. On the molecular aspect, further studies on K1 protein will enhance better understanding of its functions in both transformation and carcinogenesis.

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