GENETIC DIVERSITY OF HUMAN HERPESVIRUS TYPE 8 IN NORTHERN SOUTH AFRICA

By

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DECLARATION

I, Mulalo Raphalalani (16008874) hereby declare that this dissertation for an award of Master of Science degree in Microbiology at the University of Venda has not been submitted by anyone for any degree examination at this or any other University. It is my work and all reference materials contained have been fully acknowledged.

Signature:  Date: 20 February 2024

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- To God Almighty, Thank you.

DEDICATION

This Master of Science dissertation is dedicated to my loving big brother, Raphalalani Rabelani and my late grandmother Maria Nemugumoni.

ABSTRACT

Background: Human herpesvirus type 8 (HHV-8), is an oncogenic virus responsible for causing all forms of Kaposi`s sarcoma (KS). HHV-8 prevalence varies globally, however, it is more prevalent in African countries, with South Africa having over 50% of HHV-8 infections. HHV-8 encodes a highly diverse open reading frame (ORF) K1 gene, which has led to the identification of seven major genotypes (A-F and Z) that are heterogeneously distributed across the world. The viral genetic landscape of any geographical area is of paramount importance in vaccine development and diagnostics. However, data on HHV-8 genotypes is scarce in northern South Africa. Therefore, this study will provide genetic diversity of HHV-8 in northern South Africa, and this may aid in the selection of genes for vaccine development.

Objective: The main objective of the study was to describe the genetic diversity of human herpesvirus type 8 in northern South Africa.

Methodology: Deoxyribonucleic acid (DNA) was extracted from 115 archived mouthwash samples collected from five healthcare facilities in northern South Africa. The partial open reading frame (ORF) K1 gene (~840bp) was amplified in a two round conventional PCR using JumpStart REDTaq master mix. The band of interest was extracted by phenol-freeze protocol and enriched using conventional PCR. Enriched amplicons were purified and sequenced in an Illumina MiniSeq platform. K1 genotypes were inferred using an online BioAfrica HHV-8 subtyping tool and confirmed by computing a phylogenetic tree. Intra-genetic diversity among HHV-8 genotypes was described by aligning study sequences with their respective prototype strains. Synonymous and nonsynonymous mutation rates were computed by the online SNAP tool.

Results: K1 gene was successfully amplified in 61.7% (71/115) samples, along with unspecified DNA bands. The band of interest was successfully recovered in 67 amplicons (94.4%). Sixty-five gel extracted products (65/67; 97%) were successfully enriched and purified using magnetic beads. Of the 65 purified samples, 63 were sequenced using Illumina MiniSeq platform. Thirty-seven sequences had an acceptable nucleotide base call. The prevalence of HHV-8 in the study sequences was 94% (35/37) and majority of the sequences (24/35;68%) had sequence reads that span partial or complete K1 gene.

Two major genotypes were detected (A and B); genotype B (19/24;79%) had a higher prevalence than genotype A (5/24; 21%). All sequences which grouped with genotype A were further classified as subtype A5. Interestingly, all sequences that were classified as genotype B did not cluster to any of the B subtypes. A higher genetic drift was observed among the

study sequences reaching up to 33.7% at the amino acid level. Genotypes A and B exhibited 16.67% and 7.41% intra-genetic diversity at the amino acid level, respectively. Several amino acid polymorphisms were observed at the ITAM region of genotype A sequences (OUHC 013 and ODF 029), while the ITAM region of the B sequence was conserved.

Conclusion: In this study, a predominance of HHV-8 genotype B was observed in northern South Africa. Additionally, there was a high degree of evolutionary divergence among the studied sequences. A higher frequency of nonsynonymous mutations was detected at the ITAM region of A5 sequences and these mutations may potentially affect the functionality of ITAM.

Keywords: HHV-8; Genetic diversity, K1 gene; Kaposi`s sarcoma; South Africa.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
AIDS-KS	Acquired Immune Deficiency Syndrome associated Kaposi's sarcoma
bp	Base pair
C1	Conserved region 1
C2	Conserved region 2
DNA	Deoxyribonucleic acid
dNTP	deoxyribose nucleotide triphosphate
EBV	Epstein–Barr virus
G-C	Guanine-Cytosine
HHV-8	Human Herpes Virus type 8
HIV-1	Human immunodeficiency Virus type 1
HSV	Herpes simplex virus
HSV	Herpesvirus Saimiri
IARC	International agency of research on cancer
ITAM	Immunoreceptor tyrosine-based activation motif
Kb	Kilobase pair
KICS	Kaposi sarcoma associated inflammatory cytokine syndrome
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated Herpes Virus
LANA	Latency associated nuclear antigen
LTR	Long terminal repeats
LUR	Long unique region
MCD	Multicentric cattleman disease
MSM	Men who have sex with men
Nef	Negative regulatory factor
ng	Nanogram
NGS	Next generation sequencing
ORF	Open reading frame
PCR	Polymerase chain reaction
PEL	Primary effusion lymphoma
PS	Peptide sequence
RRV	Rhesus macaque rhadinovirus
RTA	Replication and transcription activator
STD	Sexual transmitted disease

Tat	Transcriptional transactivator
TM	Transmembrane
TPA	Tetradecanoyl phorbol myristyl acetate
US	United States
vGPCR	Viral G coupled receptors
vIL	viral interleukin
VIP	Variable ITAM-containing protein
Vpr	Viral protein R
VR1	Variable region 1
VR2	Variable region 2
VZV	varicella-zostervir

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Human herpesvirus type 8 (HHV-8), also known as Kaposi`s sarcoma associated herpesvirus (KSHV), is the causative agent of all forms of Kaposi`s sarcoma (KS), characterized by excessive proliferation of cells lining the blood and lymphatic vessels (Chang et al., 1994). There are four clinical forms of KS: epidemic KS, endemic KS, iatrogenic KS, and classic KS (Kaposi, 1872; Gallo et al., 1998; Friedman-Kien and Saltzman, 1990). Ever since its discovery in 1994, it is estimated to have infected millions of people worldwide, with about 34,000 cases of KS and 15,000 KS related deaths (Chang et al., 1994; Bray et al., 2018). The seroprevalence of HHV-8 varies globally according to regions and populations. It is highly endemic in African countries followed by the Mediterranean basin, north-western part of China, and the Amerindians. The Southeast Asian countries, the Caribbean, and northern European nations experience a lower incidence of HHV-8 infection (Zhang et al., 2012; Gonçalves, Uldrick, and Yarchoan, 2017; Cesarman et al., 2019). A systematic review conducted in 2018 reported that Southern Africa has a seroprevalence rate ranging between 14 and 90%, with South Africa having over 50% of HHV-8 infections (Etta et al., 2018).

HHV-8 has also been described as the etiological agent of three other malignancies, namely, primary effusion lymphoma (PEL), multicentric Castleman`s disease (MCD), and Kaposi`s sarcoma-associated inflammatory cytokine syndrome (KICS) Caserman et al., 1995; Soulier at el.,1995). In sub-Saharan Africa, infection commonly occurs in children, and feeding practices facilitate it (Borges et al., 2012; Crabtree et al., 2014). Infection with HHV-8 commonly occurs via saliva (Dittmer and Damania, 2016). Most people infected with HHV-8 are asymptomatic, and those who are immunocompromised may develop fever, maculopapular rash, lymphadenopathy, and bone marrow failure (Andreoni et al., 2002). Though necessary for KS development, HHV-8 infection does not always cause KS, suggesting that there are other key role factors in the pathogenesis of HHV-8, such as co-infection with HIV or malaria (Ye et al., 2011; Newton et al., 2018; Iftode et al., 2020).

HHV-8 is characterized by a highly conserved genome; however, two open reading frames (ORF) located at either end of the genome harbours a high level of genetic variation. The hypervariability within these genes (ORF-K1 and ORF-K15) has led to the emergence of various distinct genotypes and variants that are heterogeneously distributed worldwide (Olp et al., 2015; Issacs et al., 2016; de Oliveira Lopes et al., 2021). Numerous studies have demonstrated that HHV-8 genotypes are distributed among human populations based on their

ethnic and geographic origin. The most widely used gene for HHV-8 molecular epidemiological studies is ORF-K1 due to its high genetic variability (44%) compared to ORF-K15 (30%) (Hayward and Zong, 2007). Phylogenetic analysis based on the K1 gene has led to the identification of seven genotypes (A, B, C, D, E, F, and Z) and 13 variants. The most prevalent genotypes in Sub-Saharan Africa are genotypes A5 and B (Etta et al., 2018; Mamimandjiami et al., 2021).

1.2 LITERATURE REVIEW

1.2.1 DISCOVERY OF HHV-8

Kaposi`s sarcoma associated herpesvirus was named after the tumor it causes, Kaposi`s sarcoma. It was first reported by a physician and dermatologist, Moritz Kaposi, in 1872. He described the tumor's appearance as multifocal pigmented sarcoma of the skin, which is now known as classic Kaposi sarcoma (Kaposi, 1872). Several years later, other forms of KS were documented, including endemic and iatrogenic KS (Montpellier and Mussini-Montpellier, 1947; D'Oliveira and Torres., 1972). Initially, KS was regarded as a rare tumor commonly occurring in the Jewish and Mediterranean populations. This was before it gained public attention during the AIDS epidemic and became the most common tumor in sub-Saharan Africa (Rohner et al., 2016).

In the early 1980s, there was a resurgence of KS, which was mostly observed in AIDS patients. This led to the notation that KS is caused by a sexually transmitted virus that rarely causes tumors unless the host is immunocompromised (Beral et al., 1990). More than 20 infectious agents were proposed as the causative agents of KS, and this includes cytomegalovirus and HIV. It was only later in 1994 that the search for the etiological agent of KS led to the discovery of human herpesvirus type 8 (HHV-8) (Chang et al., 1994). A year later, HHV-8 was also detected in primary effusion lymphoma (PEL) and a lymphoproliferative syndrome, Multicentric Castleman's disease (MCD) (Cesarman et al., 1995; Soulier et al., 1995).

1.2.2 CLASSIFICATION OF HERPESVIRUS

Herpesviruses are a large family of linear, double-stranded DNA viruses that cause infection in both humans and animals. Members of this family are extremely adaptive and are believed to have co-evolved with their host for millions of years during speciation (Roizman et al.,1981; Wen and Damania, 2010; Jung and Speck, 2013). The name herpesvirus originated from the Greek word "herpain," which refers to latent and recurring infections exhibited by these viruses (Alsayed et al., 2014). To date, more than 170 herpesviruses have been isolated in a wide range of organisms, such as birds, fish, reptiles, amphibians, and mollusks. Of the 170 herpesviruses known, eight primarily cause infection in humans and have been designated as human herpesviruses (HHV). Human herpesviruses rarely cause severe diseases in an infected host unless the host`s immune system is impaired (Roizman and Pellet, 2001).

All members of the Herpesviridae family share common features such as biphasic life cycles and the architecture of the infectious particle (Roizman and Pellet, 2001; Carter et al., 2007). Based on their biological properties, such as host range, replication cycle, cell tropism, and growth characteristics, herpesviruses can further be divided into the alpha (α), beta (β), and gamma (y) subfamilies that arise approximately 180 to 220 million years ago (Roizman et al., 1981). The alpha subfamily are neurotropic viruses, and they include herpes simplex viruses 1 and 2 (HSV-1 and HSV-2), and varicella-zoster virus (VZV). These viruses are characterized by a short replicative cycle, a broad host range, and their ability to induce cytopathology in monolayer cell cultures (Wen and Damania, 2010; Blaskovicova and Labuda, 2014).

The beta herpesviruses include cytomegalovirus (CMV), also known as HHV-5, HHV-6 variants A and B, and HHV-7. Members of the beta herpesvirus can be distinguished from other herpesviruses by their ability to grow well in fibroblasts and by their slow replicative cycle. Gamma herpesviruses are classified as lymphotropic viruses, and they include Epstein-Barr virus (EBV), also known as HHV-4 and HHV-8. These two viruses can further be classified as lymphocrytoviruses (EBV) and rhadinoviruses (HHV-8). Unlike the alpha and beta herpesviruses, the gamma herpesvirus can induce malignancies in an infected host cell (Jung and Speck, 2013).

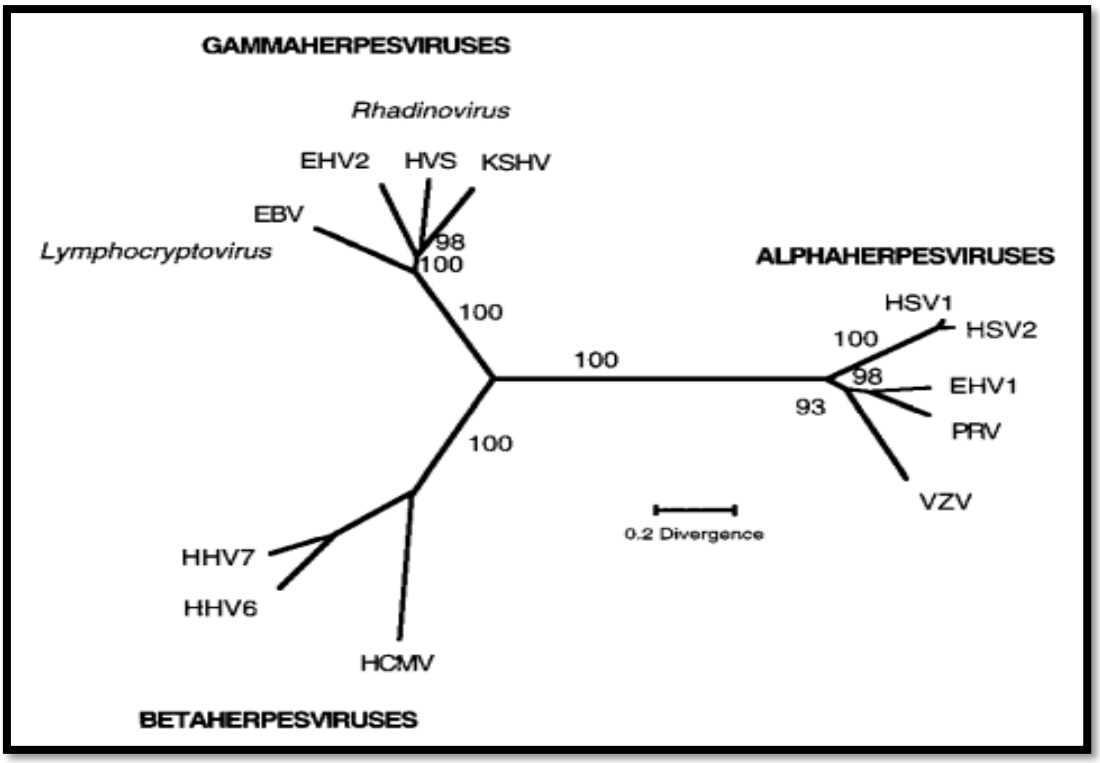

Figure 1.1: Neighbor-joining tree indicating the phylogenetic relationship of human herpesviruses. The tree was bootstrapped at 1000 replicates and drawn to scale (adapted from Moore et al.,1996a).

1.2.3 STRUCTURE AND GENOMIC ORGANIZATION OF HHV- 8

1.2.3.1 HHV-8 structure

HHV-8 is composed of four unique layered structures, namely, double-stranded DNA, capsid, tegument, and the lipid bilayer envelope. The virus contains a large double-stranded DNA that exists in different conformations depending on the life cycle of the virus (Renne et al., 1996; Neipel et al., 1998). The capsid is arranged in icosapetahedral symmetry and has 162 capsomeres. It is composed of major capsid protein, triplex components 1 and 2, small capsomer interacting protein, and scaffolding or assembly protein (Wu et al., 2000; Nealon et al., 2001; Zhu et al., 2005). The tegument consists of amorphous material and is distributed asymmetrically between the capsid and the envelope. It is composed of internal and external layers that are associated with proteins that aid in DNA replication (Sathish et al., 2012). The lipid bilayer envelope is derived from the host cell and consists of polyamines, lipids, and glycoproteins, which play a role in the attachment of the virus to the host cell (Akula et al., 2002).

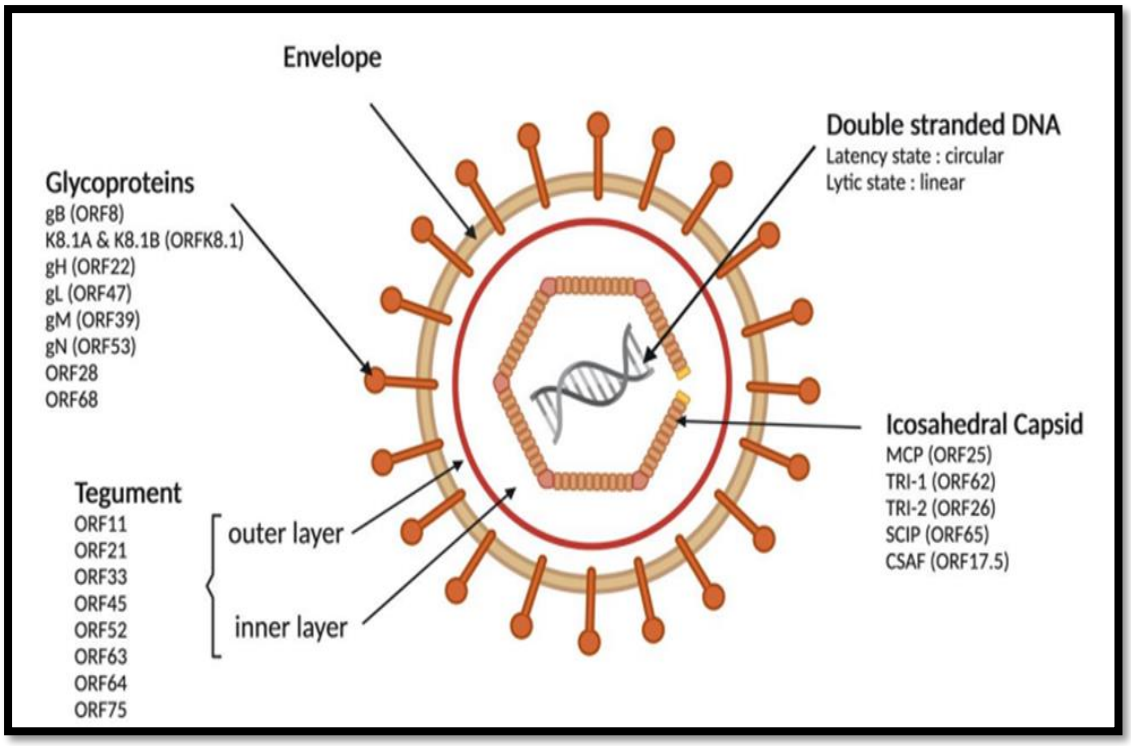

Figure 1.2: Structure of human herpesvirus type 8. Like all herpes viruses, the HHV-8 structure is composed of double-stranded DNA, tegument capsid, and the envelope (adapted from Jary et al., 2021).

1.2.3.2 Genomic organization of HHV-8

The genomic organization of HHV-8 is closely related to the herpesvirus saimiri (HSV) and rhesus macaque rhadinovirus (RRV) genomes (Swanton et al., 1997). HSV is the prototype of all gammaherpesvirus. HHV-8 is characterized by a large, conserved genome that is approximately 170 kb with a long unique region (LUR) of about 140 kb (Renne et al., 1996; Neipel et al., 1998). The long unique region contains 53.5% GC content and is flanked by numerous noncoding long terminal repeat (LTR) sequences. The LTR is composed of 801 bp nucleotides and is highly rich in GC content (Caserman et al.,1995; Russo et al., 1996; Duprez et al., 2007). The LUR houses over 90 open reading frames (ORFs) and 13 pre-microRNAs that encode 25 miRNAs. Most of the genes encoded by the HHV-8 genome are common throughout all herpesviruses (ORF4 to ORF75).

Genes that are unique to HHV-8 are denoted by the letter “K” with a respective number depending on their location, and they range from K1 to K15 (Russo et al., 1996; Zhu et al., 2005). Furthermore, HHV-8 also contains viral genes that have been pirated from their host genomes, and these genes are homologous to cellular genes (Swanton et al., 1997; Kwun and Damania, 2009). Moreover, genes encoded by LUR play a vital role in DNA replication and assembly of new virions, the transition from the lytic to the latent phase, and the prevention

of cellular innate responses such as apoptosis, cycle arrest, and subverting immunologic antiviral defenses while promoting proliferation in infected cells (Wu et al., 2000). LUR genes are arranged in four clusters and are distributed in a collinear position and orientation similar to other herpesviruses (Schulz et al., 2015).

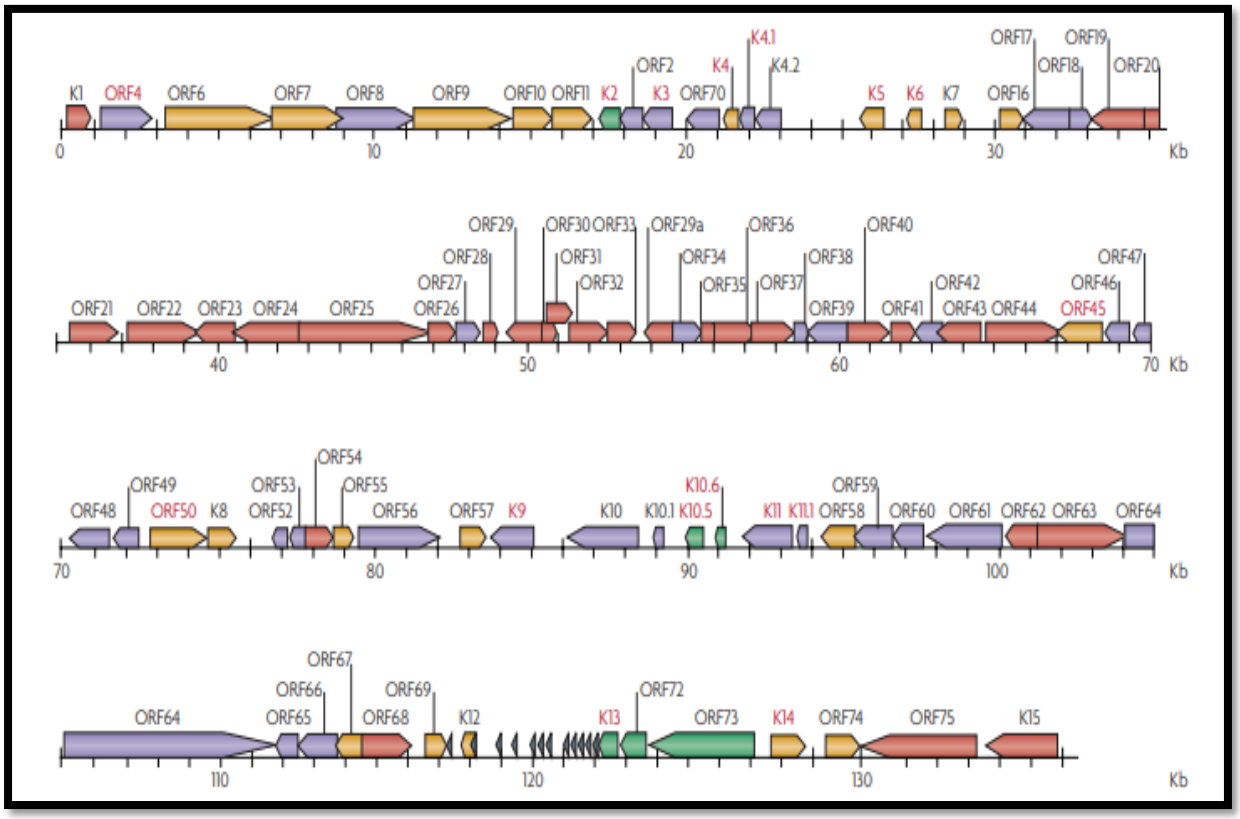

Figure 1.3: Genomic organization of HHV-8. The shaded colors indicate genes expressed during various stages of the HHV-8 life cycle. Yellow indicates genes expressed during early reactivation; orange indicates genes expressed during the intermediate phase while red indicates genes expressed during the late stage of reactivation. Green indicates genes expressed in the latent cycle and grey color indicates Micro RNAs (adapted from Coscoy, 2007).

1.2.4 HHV-8 "K" GENES AND THEIR FUNCTION

HHV-8 houses 15 ORF genes, which are denoted with the letter K and a respective number corresponding to their location. These genes play a vital role in both the latent and lytic life cycles of HHV-8. Their functions are explained below in **Table 1.1**.

Table 1.1: HHV-8 unique genes and their functions.

Gene	Alternative name	Function
K1	Variable ITAM-containing protein	Induces transformation, inhibits apoptosis, immune escape by the ITAM motif and B cell activation
K2	Viral interlukin-6	Aids in B cell proliferation, autocrine signaling, and interaction with cellular cycle
K3	Modulator of Immune Recognition 1	E3 ubiquitin ligase, immune evasion, mediate downregulation of several immunomodulatory proteins
K4	Viral macrophage inflammatory protein 2	Stimulate angiogenesis, inhibit naïve and active natural killer cells
K5	Modulator of Immune Recognition 2	E3 ubiquitin ligase and inhibit MHC class 1, B7, and ICAM expression
K6	Viral macrophage inflammatory protein 1	Stimulate angiogenesis, ability to bind to CCR5 and CCR8, and chemoattraction of T helper cells 2 cells and monocytes
K7	Viral inhibitor of apoptosis protein	Inhibit apoptosis and vGPCR expression and function
K8	Replication associated protein (RAP/K-ZIP)	Binds to RTA and represses its activity, stimulates P23 and P51, and promotes cell cycle arrest
K9	Viral interferon regulatory factor- 1 (vIRF)	Induces transformation, inhibits interferon signaling pathways, and mediates cell proliferation
K10	Viral interferon regulatory factor-4 (vIRF)	Serves as a coregulator for RTA
K11	Viral interferon regulatory factor-2 (vIRF)	Inhibit apoptosis and production of type 1 interferon
K12	Kaposin	Induces cell transformation, activate P18/MK2 pathways and stabilize cytokines expression
K13	Viral flice-inhibitory protein (vFLIP)	Inhibit apoptosis, induce cell transformation, and activate NF-kB pathways by interacting with NEMO
K14	vOX-2	Inhibit NF-kB pathways, regulate inflammatory cytokines such as IL-8, IFN-c, and IL-6
K15	LAMP	Activate intracellular signaling pathways

(Adapted from Wang et al., 2002; Manes et al., 2010; Wen et al., 2010; Jary et al., 2021).

1.2.5 REPLICATION OF HHV-8

1.2.5.1 Viral entry

HHV-8 is a lymphotropic virus that is primarily transmitted through saliva. It replicates in epithelial cells of the oropharynx and later targets B cells, which are the main viral reservoir. It also infects other cells, such as dendritic cells, monocytes, and fibroblasts (Bechtel et al., 2003; Chakraborty et al., 2012; Dittmer and Damania, 2016). To gain entry into the host cells, HHV-8 uses glycoproteins (gpK8.1, gB, gM-gN, or gH-gL), which are present on the viral envelope, to bind to the host cell receptors (Damania and Cesarman, 2013). Depending on the targeted cell, the virus will either bind to integrin (α3β1, αVβ5, and αVβ3) cellular receptors, the cysteine-glutamate transporter xCT, heparin sulfate, or the tyrosine-protein kinase receptor EPHA2 (Birkmann et al., 2001; Inoue et al., 2003; Kerur et al., 2010). This interaction results in the entry of the virus into the host cell cytoplasm via endocytosis (Damania and Cesarman, 2013). Once the virus enters the host cell, it undergoes the process of uncoating, wherein the capsid is traversed into the nucleus. In the nucleus, the virus can either enter a lytic state, where the virus replicates and releases infectious virions, or enter a latent state, where the virus remains dormant and tethered to the host genome (Cesarman et al., 2019; Jary et al., 2021).

1.2.5.2 Latent cycle

As with all herpesviruses, HHV-8 can establish a life-long infection, wherein the virus remains in a latent state. This state is characterized by a circular viral genome tethered to the host cell chromosome. The viral genome replicates together with the host cells as it undergoes cell division. However, the replication does not lead to the production of infectious virions or lysis of the host cells, but a minimal fraction of genes is expressed that helps maintain the latent state. (Wen and Damania, 2010; Caserman et al., 2019; Jary et al., 2021). Genes expressed during the latent phase include ORF-71, ORF-72, and ORF-73, which encode latency-associated nuclear antigen 1 (LANA-1), vCyclin, and viral FLICE inhibitory protein (vFLIP), respectively (Cai et al., 2005; Chandriani and Ganem, 2010). Some of the genes expressed include ORF-K1 and ORF-K15, which code for transmembrane proteins and viral IL-6; however, they are expressed in low quantities. Most of the genes expressed in the latent phase are thought to play a role in the development of HHV-8-associated malignancies (Abere et al., 2017; Cesarman et al., 2019).

1.2.5.3 Lytic cycle

The mechanism by which HHV-8 switches to the lytic phase is not yet fully understood. However, there are various factors that lead to the reactivation of HHV-8, and these include physiological stimuli such as oxidative stress, reactive oxygen species, hypoxia, or chemical substances such as TPA (tetradecanoyl phorbol myristyl acetate) and n-butyrate or co-infections. (Davis et al., 2000; Ye et al., 2011; Iftode et al., 2020). The lytic cycle is characterized by a replicative linear genome that produces infectious virions and expresses over 80 genes in a sequential order. The first to be expressed are the immediate-early (IE) genes, followed by the delayed-early (E) genes, and then the late genes (L) (Damania and Cesarman, 2013). There are three IE genes expressed during the lytic cycle, and one of those is ORF-50, which encodes the replication and transcription activator (RTA) protein that is responsible for initiating the lytic phase (Gradovile et al., 2000). The other two genes include ORF-K8 and ORF-45. All three of these genes play an important role in regulating the transcriptional cascade.

The early genes encode proteins that are responsible for the replication of HHV-8, and this includes ORF9, ORF21, ORF36, vIRF-1, and viral G-coupled receptors (v-GPCR) (Lei et al., 2010). The expression of late genes begins after 24 hours since lytic replication is activated. The L genes encode for structural and maturation proteins of the virion. The latter is responsible for the assembly of the new virions released during replication (Aneja and Yuan, 2017)

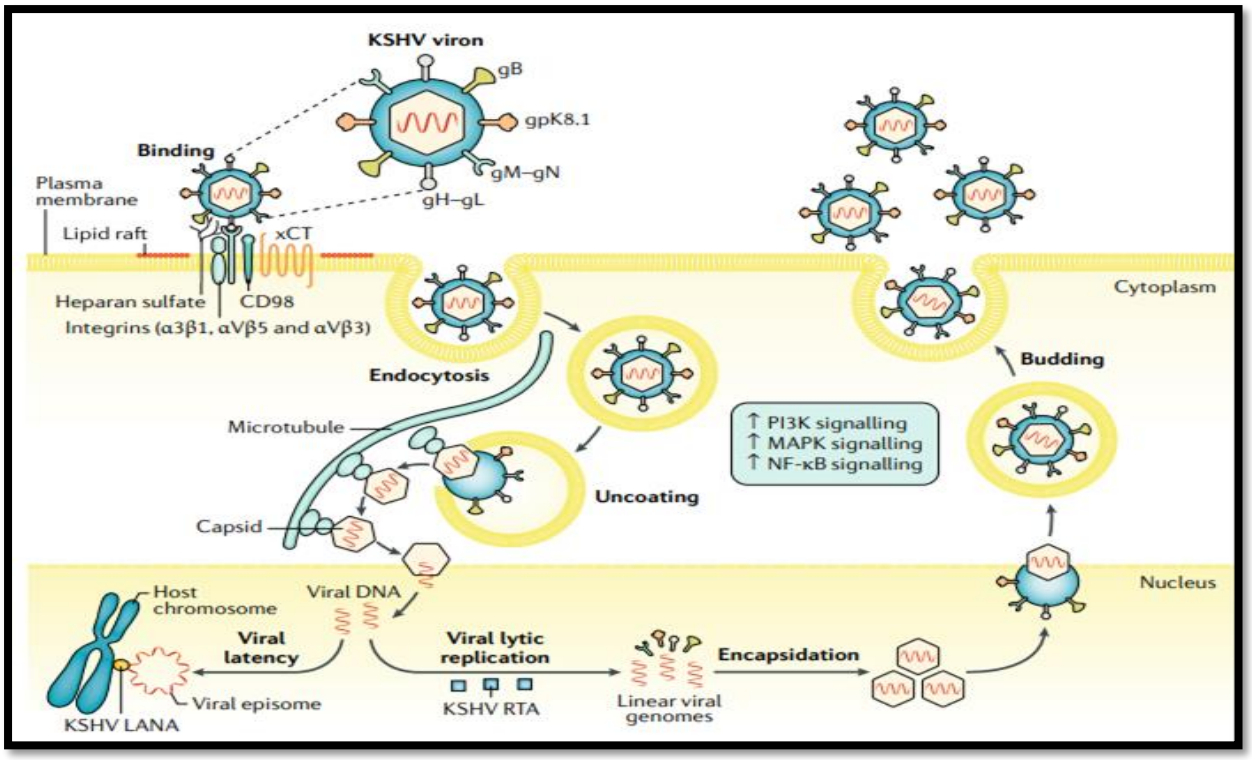

Figure 1.4: Life cycle of human herpesvirus type 8 (adapted from Cesarman et al., 2019).

1.2.6 TRANSMISSION AND PREVALENCE OF HHV-8

The manner in which HHV-8 is transmitted is not yet fully understood. However, HHV-8 can be detected in sources such as blood, saliva, and semen. Saliva exchange is documented as the principal spread of HHV-8 (Dedicoat et al., 2004; Pauk et al., 2007; Mamimandjiami et al., 2021). It is also the most common route of transmission in infants and children in sub-Saharan Africa, likely due to certain practices such as food pre-mastication or living with an infected family member (Ramano et al., 2010; Rohner et al., 2016). HHV-8 has also been reported to be sexually transmitted, and this route of transmission is associated with having multiple partners and sexually transmitted diseases (Casper et al., 2007; Martin et al., 2008; Mariggio et al., 2017). HHV-8 transmission can also occur via organ transplant and it is regarded as a clinically important route of HHV-8 infection. (Hladik et al., 2006; Riva et al., 2012; Lebbe et al., 2013). Various reports have reported transmission of HHV-8 through blood transfusion and injecting drug use (Cannon et al., 2001; Atkinson et al., 2003; Dollard et al., 2005; Hladik et al., 2006). Rare modes of HHV-8 transmission include maternal- to-fetal, perinatal, and breast milk. It has occasionally been detected in urine and anal sites (Cannon et al., 2003; Brayfiled et al., 2004).

The prevalence of HHV-8 infection varies geographically, and it reflects the incidence rate of KS (Whitby et al., 2004; Labo and Whitby, 2010). The HHV-8 infection rate is high in Sub-Saharan countries, with a seroprevalence rate above 50% followed by the Mediterranean basin and Eastern Europe with a seroprevelance between 10 and 30%(Dedicoat et al., 2013; Caserman et al., 2019). North America, Western Europe and Eastern Asia experience a lower incidence rate of HHV-8 infection, with less than 10% of the population infected with the virus (Chakraborty et al., 2012; Abere et al., 2020; Casper et al., 2022). The HHV-8 infection rate can still vary within the same region; such has been documented in China, where Hubei had almost doubled the incidence rate when compared to Xingiagm (Fu et al., 2009).

1.2.7 CLINICAL EPIDEMIOLOGICAL FORMS OF HHV-8

HHV-8 establishes a life-long infection, which can lead to the development of cancer in some individuals, especially those with compromised immunity. There are four clinical forms of KS, namely epidemic KS, endemic KS, iatrogenic KS, and classic KS (Kaposi, 1872; Gallo et al., 1998; Friedman-Kien and Saltzman, 1990). The first to be discovered was classic KS, also known as sporadic KS, which is a rare tumor mostly affecting the Mediterranean Basin and Eastern European people. It is restricted to lower limbs with few lesions, which is the opposite of epidemic KS, also known as AIDS (Kaposi, 1872; Iscovich et al., 1998). AIDS KS is the most aggressive and is not geographically restricted. Iatrogenic KS, also known as transplant KS, is mostly found in developed countries. It occurs following solid-organ allograft, and its lesions are usually localized; however, they may also involve organs (Jackson et al., 2016). Endemic KS mainly occurs in sub-Saharan Africa. It is aggressive, and infected children exhibit multiple lymph nodes with lymphedema, while adults exhibit lower-limb lesions that resemble those of classic KS (Cesarman et al., 2019).

The prevalence of endemic KS in children differs with respect to regions with an HIV- burden. Endemic KS was the most common cancer in children before the AIDS pandemic; its occurrence increased by 40-fold due to the emergence of the AIDS pandemic (Krown et al., 2006). These epidemiological forms of KS may differ in geographic distribution, localization, and aggressiveness; however, they share common clinical and histologically indistinguishable features. KS lesions are highly diverse, and the inflammatory infiltrate is surrounded by abnormal vessels. In the early stage of the infection, KS lesions appear as flat red patches, which then develop into plaques and later form macroscopically visible nodules in the late stage of the infection (Ciufo et al., 2001; Gramolelli and Thomas, 2015).

1.2.8 MANAGEMENT OF HHV-8 INFECTION AND ASSOCIATED MALIGNANCIES

To date, HHV-8 has no vaccine, but there are treatment options available for combating the infection. KS lesions are usually limited to one area; therefore, they can be treated by cryotherapy, radiation therapy, intralesional injections, or surgical resection (Bhutani et al., 2015). Another form of treatment is boosting the immune system in AIDS-KS patients through antiretroviral therapy with pegylated liposomal doxorubicin drugs. In iatrogenic KS patients, drugs such as calcineurins and rapamycin are used to reduce immunosuppression. There are inhibitors from other viral DNA polymerases such as cidofovir, ganciclovir, and foscarnet that can prevent HHV-8 lytic replication and hinder the development of KS. These drugs are used as prophylaxis and as treatment in combination with other chemotherapy drugs (Krell et al., 2014). Thus far, there is no HHV-8 inhibitor protein that has been clinically licensed.

1.2.9 MOLECULAR EPIDEMIOLOGY OF HHV-8

1.2.9.1 ORF-K1 gene

The K1 gene is composed of 289 amino acids, and it encodes a transmembrane glycoprotein that plays a vital role in viral reactivation and host immune recognition. The K1 gene is also known as VIP (variable ITAM-containing protein), which houses an immunoreceptor tyrosine-based activation motif (ITAM) that aids in lymphocyte activation signaling. The structural organization of K1 is made up of a long N-terminal extracellular domain, a transmembrane domain, and a C-terminal cytoplasmic tail (Zong et al., 1999; Lee et al., 1998; Lee et al., 2005). The transmembrane domain comprises a conserved region (C1 and C2) and two hypervariable regions (VR1 and VR2). Sequence variability of K1 is concentrated at VR1 and VR2, with up to 44% of amino acid divergence between HHV-8 isolates (Zong et al., 1999; Hayward and Zong, 2007; Tornesello et al., 2010; Jary et al., 2021).

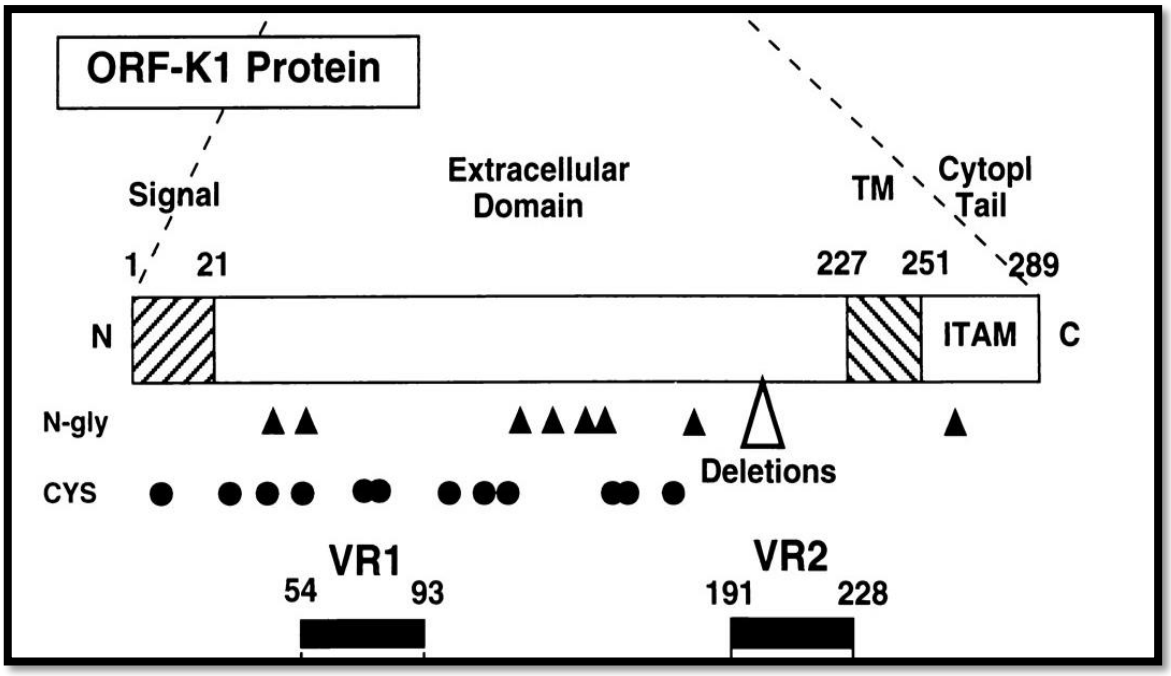

Figure 1.5:Structural organization and key features of the highly variable K1 gene. It is located at the left-hand side of HHV-8 genome between nucleotide position 105 and 974. K1 protein is composed of 289 amino acids and its key features includes signal peptide, transmembrane protein (TM) denoted by hatched bars, N- glycosylation sites denoted by small triangles, cysteine bridges residues denoted by dark circles and the VR1 and VR2 regions located between amino acids 54-96 and 191-228, respectively (adapted from Zong et al., 1999).

To date, seven genotypes and 13 sub variants (A-F and Z) have been identified based on phylogenetic analysis of the ORF-K1 gene (Whitby et al., 2004; Kajumbula et al., 2006; Etta et al., 2018; Mamimandjiami et al., 2021). Amino acid analysis of genotypes A and C shows that they differ from genotypes B and D by 30% and 24%, respectively. Whereas genotype A differs from genotype C by 15% (Zong et al., 1999). These genotypes are heterogeneously distributed across geographic and ethnic origins. Genotypes A and C are found in Europe, the United States, the Mediterranean basin, the Middle East, and Asia. Genotype D has been reported in individuals residing in the Pacific Rim, Japan, Taiwan, and Australia, and genotype E is predominately found in Brazilian Amerindians. Genotype B and A5 are predominately found in sub-Saharan Africa, whereas genotype F was first identified in Uganda and genotype Z in Zambian children (Kasolo et al., 1998; Kajumbula et al., 2006; Varmazyar et al., 2017; Jary et al., 2020).

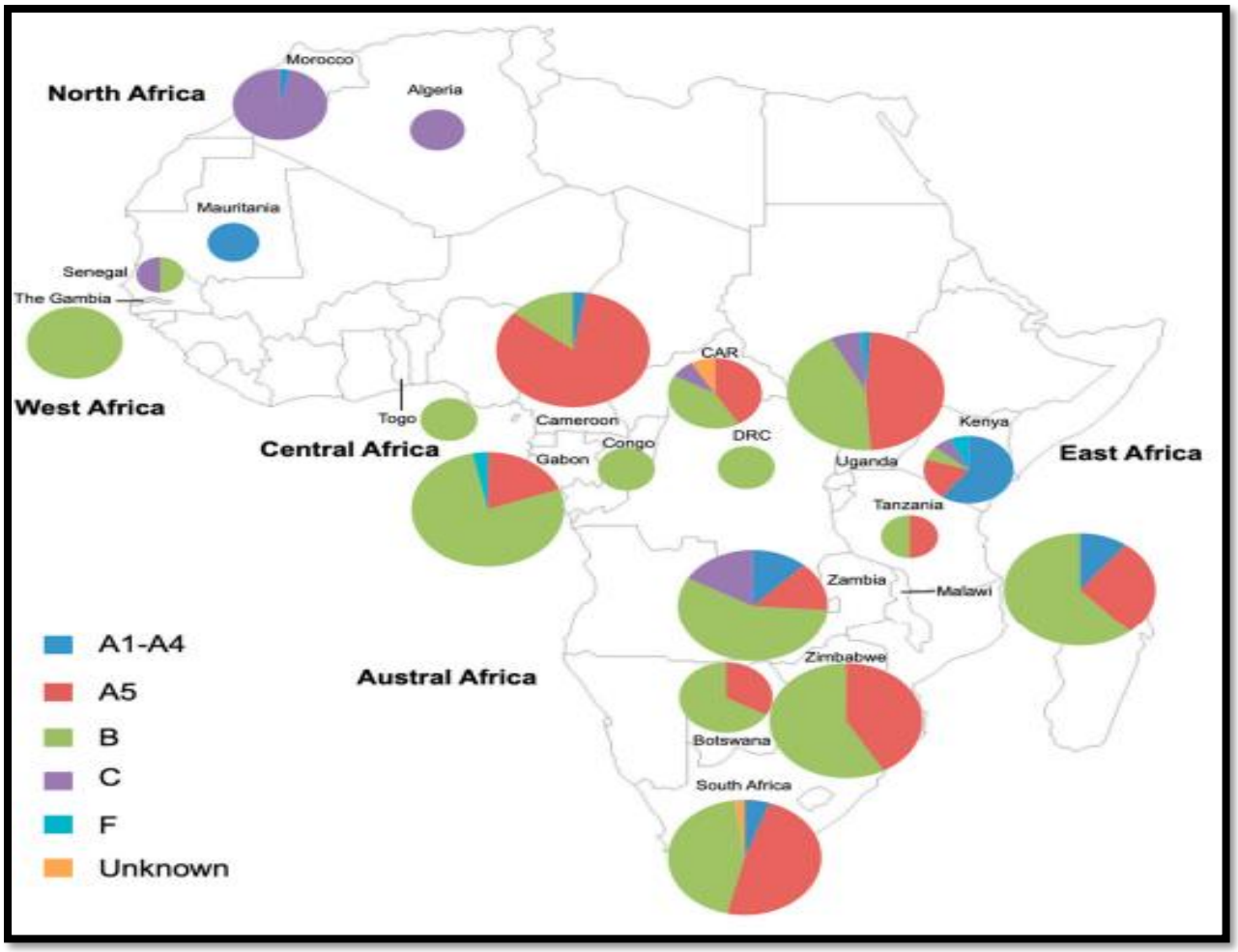

Figure 1.6: Geographical map of Africa indicating K1 genotypes and strains distributed across African countries. The analysis included 483 K1 sequences from 19 countries. The circle is proportional to the number of sequences characterized in each country (adapted from Mamimandjiami et al., 2021).

1.2.9.2 Other HHV-8 genes

In addition to the ORF-K1 gene, HHV-8 houses other multiple genes that have been used to characterize HHV-8. (ORF-K15) is a variable gene located on the right-hand side of the genome. The K15 gene encodes a transmembrane protein that plays a dynamic role in the activation of intracellular signaling pathways (Wen and Damania, 2010). Phylogenetic analysis of K15 has led to the identification of P, M, and N genotypes, and these genotypes share about 30% of amino acid identity (Hayward and Zong, 2007). Molecular characterization of HHV-8 based on a conserved ORF 26 gene has led to the identification of genotypes A/C, J, K/M, D/E, B, N, Q, and R (Hulaniuk et al., 2020). In addition to ORF26, there are other 8 loci within the center of the genome with low levels of sequence variation that have been used for genotyping; these include T0.7/K12, K2, K3, ORF18/19, K8, and ORF73. The most commonly used gene for genotyping is ORF-K1 due to its high degree of sequence variability. Overall,

HHV-8 has been classified into 12 principal genotypes based on the variability of ORF-K1 and ORF-K15 (Strahan et al., 2016).

1.2.10 K1 GENOTYPES AND DISEASE ASSOCIATION

Various studies looking at the diversity of K1 have suggested that genotypes have different pathogenic and tumorigenic properties, indicating that they might play a role in the progression of HHV-8 associated malignancies (Jary et al., 2020). The K1 gene plays a vital role in the oncogenesis of HHV-8, wherein it promotes cell proliferation by inhibiting apoptosis and stimulating tumor angiogenesis (Wang et al., 2014). VR1 and VR2 have been shown to contain viral genetic determinants that identify whether a patient is at risk of developing Kaposi sarcoma (KS) (Cordiali-Fei et al., 2015). A study done by Mancuso et al., (2008) reported that genotype A is associated with rapid progression of classic KS and a higher HHV-8 viral load. These results are in accordance with a study done by Jary and colleagues (2020), where they reported that HHV-8 viral loads were higher in genotype A than in genotype C, regardless of the immunovirological status of epidemic KS patients. Another study conducted by Isaac et al., (2016) in South Africa reported that genotype A5 is associated with the rapid progression of epidemic KS. According to Tozetto-Mendoza et al., (2016) genotype B is associated with a better prognosis for epidemic KS in Brazilian patients. Nevertheless, other studies did not find any association between K1 genotypes and HHV-8 pathogenicity, such as one study done by White and colleagues (2008).

1.2.11 NEXT GENERATION SEQUENCING (NGS)

Next generation sequencing (NGS), also known as massive parallel DNA sequencing, refers to several sequencing technologies that are capable of sequencing large number of clonally amplified DNA molecules or single DNA molecules in a flow cell (Voelkerding et al., 2016). There are a wide range of NGS platforms that have been developed, including Illumina, Roch 454, Helicos, and Ion Torrent. Illumina platforms are one of the major NGS technologies used, and this is because of their high-throughput and short run time (Razah and Ahmed, 2016).

The principle of Illumina NGS relies on sequencing by synthesis (SBS) and fluorescently labelled reversible dye terminators, which allow the identification of single bases as they are introduced into DNA strands (Mardis et al., 2016). The Illumina NGS workflow includes library preparation, cluster generation, and sequencing. Library preparation entails five steps: tagmentation, amplification, clean-up, normalization, and pooling of libraries. Cluster

generation and sequencing output depend on the Illumina platform used. The Illumina MiniSeq platform, in combination with the high output kit, generates 6.6–7.5 Gb of data, 44–50 million paired end reads of 150 bp, and a quality score (Q30) of over 80%. However, this can still vary depending on the MiniSeq Kit used (https://sapac.illumina.com/system.html).

Illumina NGS platforms offer a wide range of applications, including targeted sequencing and whole genome sequencing, which do not require intensive labour like traditional sequencing approaches. In addition, it also provides RNA sequencing, exome sequencing, chromatin immunoprecipitation sequencing (ChIP-Seq), and the study of human microbiome interactions (Raza and Ahmad, 2016). Several benefits afforded by NGS sequencing technologies include high throughput data, high sensitivity, reduced sequencing errors, and massively parallel sequencing, which reduces the cost per sample (Hu et al., 2021). Furthermore, due to its large data output per sequence, it allows the detection of variations that range from single nucleotide polymorphisms (SNP) to complex chromosomal rearrangements (Nekrutenko and Taylor, 2013).

1.3 STUDY RATIONALE

Initially, phylogenetic analysis of HHV-8 relied on the first two ORF genes that were discovered (ORF26 and ORF75), which led to the identification of genotypes A, B, and C. However, due to low sequence variability observed between these two fragments, this limited the study of genetic diversity (Zong et al., 1999). Currently, studies on the molecular epidemiology of HHV-8 have focused more on a hypervariable gene (ORF-K1) found between nucleotide coordinates 104 and 974 located at the 5' end of the genome (Strahan et al., 2016). K1 variability is concentrated in the VR1 and VR2 regions, with up to 85% of nucleotide polymorphisms that result in the alteration of the predicted amino acid sequence (Zong et al., 2007; Lascoste et al., 2000; White et al., 2008). Its high genetic variability makes it a good tool marker for genotyping and strain differentiation (Zhang et al., 2008; Gómez et al., 2022).

HHV-8 has been classified into A-F and Z genotypes based on the K1 gene (Issacs et al., 2016; Adegbidi et al., 2020; de Oliveira Lopes et al., 2021). Phylogenetic analysis has documented that K1 genotypes are heterogeneously distributed among geographical regions and ethnicity (Zong et al., 2002; Hayward and Zong, 2007; Hulaniuk et al., 2020). Genotypes A and C are predominately distributed across Europe in Caucasian patients, genotype D is found in the Pacific Islands, genotype E is found in Brazilian Indians, and genotype B is predominately found in Africa (Isaacs et al., 2016). Numerous studies done across the world have documented the presence of genotype A more often than genotype B. A study done by Mbulaiteye and colleagues (2006) suggested that it might be because genotype A is more easily transmissible than genotype B.

A systematic review with a focus on Africa reported that the most common genotypes based on ORF-K1 are A5 and B (Etta et al., 2018). According to an experimental investigation conducted in Cape Town, the most common genotypes in South Africa are A5 and B2, which are followed by B1, B3, A1, and A4 (Isaac et al., 2016). The high prevalence of genotype A in sub-Saharan Africa suggests that genotype A might have originated in Africa. Genotype B has been documented to have the highest rate of nonsynonymous mutations compared to genotype A. A study done by Cordiali-Fei et al., (2015) suggested that positive selection pressure due to high nonsynonymous mutation rate within genotype B might play a role in the development of KS.

Northern South Africa shares borders with Zimbabwe, Mozambique, and Botswana, making it an ideal portal of entry for immigrants into northern South Africa. It also houses one of the busiest borders between South Africa and Zimbabwe known as the Beitbridge border. It is well established that K1 genotypes cluster with the origin and ethnicity of infected individuals

(Hayward and Zong, 2007; Mamimandjiami et al., 2021; Marshal et al.,2022). Therefore, Immigrants from different countries might be introducing various K1 genotypes into northern South Africa. Hence, genotypes circulating in this area might be slightly different to K1 genotypes that have been reported in other parts of South Africa.

Several studies have proposed that different genotypes have different pathogenic and tumorigenic effects, suggesting that genotypes play a role in the progression of KS. Such a study reported that genotype A5 is associated with progressive disease (Zhang et al., 2008). Another study reported that genotype B is associated with a better prognosis for AIDS KS (Tozetto-Mendoza et al., 2016). However, other studies did not find any association between genotypes and HHV-8 pathogenesis (Tornesello et al., 2010; Kouri et al., 2012).

Genetic diversity of HHV-8 in South Africa though scanty, has been described in other parts of the country. However, data from northern South Africa is very limited. Genotyping of HHV-8 genes provides knowledge on the origin, genetic evolution, transmission, and disease association with the virus. Therefore, this study was aimed at determining the genetic diversity of HHV-8 in northern South Africa. This study will provide knowledge on the prevalence and diversity of HHV-8 genotypes in Limpopo. These findings will provide additional insights into the genetic diversity of HHV-8 and provide useful data for the selection of genes for the development of vaccines and diagnostics.

1.4 RESEARCH QUESTION

What are the predominant HHV-8 genotypes in northern South Africa?

1.5 HYPOTHESIS

Genotypes A5 and B are the most prevalent genotypes based on the K1 gene in Africa; therefore, the study hypothesized that genotypes A and B are the most dominant HHV-8 genotypes in northern South Africa.

1.6 STUDY OBJECTIVES

1.6.1 MAIN OBJECTIVE

The main objective of the study was to describe the genetic diversity of human herpesvirus type 8 in northern South Africa.

1.6 2 SPECIFIC OBJECTIVES

The specific objectives of the study were:

1) To determine HHV-8 genotypes in northern South Africa.
2) To investigate intra genetic diversity of HHV-8 genotypes in northern South Africa.

CHAPTER TWO: MATERIALS AND METHODS

2.1 ETHICAL CONSIDERATIONS

Ethical clearance to use archived mouthwash samples was obtained from the Human and Clinical Trial Research Ethics Committee of the University of Venda (SMNS/15/MBY/23/0710). No personal identifiable information was used in the study.

2.2 STUDY AREA AND STUDY POPULATION

Limpopo Province is the northernmost South African province. It is comprised of four districts, namely, Vhembe, Mopani, Sekhukhune, and Capricorn. It is also known as the portal of entry into Africa, bordering Zimbabwe on its north, Mozambique to its east, and Botswana on its west. One hundred and fifteen mouthwash samples obtained from a previously established cohort were used to investigate the objectives of the current study. The samples were obtained from five study sites, namely Donald Frazer Clinic (27), Thohoyandou Health Clinic (32), Univen Health Clinic (11) in Vhembe district, Rethabile Health Clinic (12), and Seshego Health Clinic (33) located in Capricorn district. Individuals sampled for human herpesvirus type 8 included HIV infected and HIV non-infected individuals. Demographic data collected from study participants included age, gender, marital status, employment status, and level of education.

2.3 TOTAL DNA EXTRACTION FROM MOUTHWASH SAMPLES

One hundred and fifteen supernatants from mouthwashes emanating from previous studies were centrifuged at 14000 rpm for 15 minutes at room temperature (25 °C). Pellets were re-suspended in 200 µl of Phosphate Buffered Saline (PBS). A Qiagen mini-DNA extraction kit (Qiagen Biosynthesis, Germany) was used according to the manufacturer's protocol to extract total DNA from suspended pellets.

2.4 AMPLIFICATION OF ORF-K1 REGION (~840)

A protocol by Cook et al., (1999) was optimized to amplify the partial ORF-K1 gene using JumpStart REDTaq Readymix reaction mix (Sigma-Aldrich) in a two round conventional polymerase chain reaction (PCR). The master mix is composed of 20 mM Tris-HCl, 100 mM KCl, 4 mM MgCl2, 0.002% gelatin, 0.4 mM each dNTP, inert dye, stabilizers, 0.1 mL Taq DNA Polymerase, and JumpStart Taq antibody.

Five microliters of total DNA were used as a starting template for the first reaction. The master mix was in a total volume of 25 µl and consisted of 15 µl of JumpStart REDTaq readymix, 1.25 µl of 10 mM forward and reverse primer, and 2.5 µl of nuclease free water. The second round reaction was in a total volume of 50 µl. Each PCR tube contained 30 µl of JumpStart REDTaq readymix, 2.5 µl of 10 mM forward and reverse primer, 5 µl of nuclease free water, and 10 µl of the first round PCR product. This primer set (**table 2.1**) and cycling condition (**table 2.2**) were used for both the first round and second round of the PCR.

Table 2. 1: Primer set for amplifying ORF-K1 gene.

Primer name	Primer sequence	Targeted fragment size
K1b-F	GTCTGCAGTCTGGCGGTTTGT	840bp
K1b-R	CTGGTTGCGTATAGTCTTCCG	

(Adapted from Cook et al.,1999).

Table 2. 2: Cycling conditions for amplifying ORF-K1 region.

Cycling step	Number of cycles	Temperature °C	Duration
Initial denaturation		96°C	1 minute 45 seconds
Denaturation	30	96°C	1 minute
Annealing		58°C	45 seconds
Extension		72°C	1 minute
Final extension		72°C	10 minutes
Hold		4°C	Infinity

(Adapted from Cook et al.,1991)

2.5 SIZE VERIFICATION OF ORF-K1 AMPLICONS

To verify the amplification of partial K1 gene, gel electrophoresis was conducted. One percent (1%) agarose gel was prepared by adding 1g of Top Vision agarose into a 200 ml bottle containing 100 ml of 1x TAE buffer. The mixture was slowly mixed and heated in a microwave for 3 minutes. The agarose solution was allowed to cool by placing it on the bench for 2 minutes. Once it cooled down, 3 µl of ethidium bromide was added into a 200 ml bottle. The solution was slowly mixed to allow ethidium bromide to dissolve and to prevent the formation of bubbles. The agarose solution was then poured onto a casting tray and left to solidify for 30 minutes. Once it solidified, it was placed in a gel chamber. Three microliters of second round amplicons were mixed with 6X loading dye on a parafilm. Amplicons were added to the agarose gel wells together with a 1kb DNA ladder and resolved by applying a current of 100 volts for 40 minutes. The gel was then viewed under a UV transilluminator.

2.6 AGAROSE GEL EXTRACTION USING PHENOL-FREEZE METHOD (in-house protocol)

To obtain the fragment of interest (~840 bp), agarose gel extraction was conducted using a phenol-freeze protocol. The protocol relies on the principle of liquid-liquid extraction of biomolecules. It uses three biomolecule reagents, namely chloroform, phenol, and isoamyl alcohol, to extract and purify the fragment of interest.

Forty-five microliters of second round PCR amplicons were loaded on a 2% agarose gel. The amplicons were resolved by applying a current of 100 V for 1 hour. The gel was then placed on the UV Transluminator box, and the band of interest was excised with a clean razor blade. The gel slice was chopped into slurry pieces on a gel casting tray and then transferred into 1.5 ml tubes that are free from DNAse, RNAse, human DNA, and pyrogens. 150–200 µl of phenol was added until the gel slices were wet. The mixture was vortexed for 3 minutes and then placed in a -80 freezer for 10 minutes. The mixture was centrifuged at 14000 rpm in an Eppendorf centrifuge for 15 minutes at room temperature (25°C). The aqueous phase was transferred into new 1.5 ml tubes. The phenol phase was then re-extracted with an equal volume of phenol added. The mixture was vortexed for 3 minutes, placed in a -80ºC freezer for 10 minutes, and centrifuged at room temperature at 14000 rpm for 15 minutes. The repetition of phenol extraction was done to increase the recovery of DNA.

The aqueous phase was removed and transferred to the first extraction. An equal volume of phenol was added to the aqueous phase, and the mixture was vortexed for 15 seconds and

centrifuged at 14000 rpm at room temperature for 2 minutes. The aqueous phase was extracted and transferred to a new tube. The aqueous phase was then extracted with an equal volume of CHISAM (chloroform and isoamyl alcohol) (24:1). The mixture was centrifuged for 2 minutes at room temperature, and the aqueous phase was transferred onto a new tube. The CHISAM extraction was repeated to enhance the recovery and purity of the DNA. The aqueous phase was transferred into a new 1.5ml tube, and ethanol precipitation was carried out as follows: 5M potassium acetate was added to the mixture at a final concentration of 0.25 mM, and two volumes of ethanol (200 proof) were added to the tube. The mixture was then placed in a -80°C freezer for 1 hour and centrifuged at 4°C for 20 minutes. The supernatant was discarded, and the pellet was resuspended in 20 µl of TE buffer (10 mM Tris pH8.0, 0.1 mM EDTA pH8.0).

To verify the extracted fragment size, 1-3 µl of the test sample was loaded in a 2% agarose gel together with a 100bp marker into their respective wells. The agarose gel was resolved at 100 V for 40 minutes and visualized under a UV transilluminator.

2.7 ENRICHMENT OF K1 GEL EXTRACTED PRODUCTS

Agarose gel extracted products were enriched using a conventional polymerase chain reaction (PCR). The enrichment step was done to increase the quantity of DNA so that the samples could have the acceptable DNA quality required for library preparation of NGS (DNA concentration 0.2 ng/µl). The total reaction volume was 25 µl, and it consisted of 15 µl of JumpStart REDTaq readymix, 1.25 µl of 10 mM forward and reverse primer, 2.5 µl nuclease free water, and 5 µl of agarose gel extracted product. The primer set and cycling conditions used were similar to those used in the amplification of the partial K1 gene (**tables 2.1** and **2.2**).

2.8 PURIFICATION AND QUANTIFICATION OF K1 AMPLICONS

Positive amplicons were purified using a Marchery Nagel NGS purification kit. This kit employs paramagnetic beads to selectively bind DNA based on the volume ratio of magnetic beads and DNA. The volume ratio is used to size-select DNA fragments during purification. A ratio of 1:1 was used to purify the amplicons according to the manufacturer`s protocol. Purified amplicons were quantified using a Qubit 3.0 double stranded DNA high sensitivity kit following the manufacturer`s protocol. Quantified K1 amplicons were diluted with elution buffer and normalized to 0.2 ng/µl.

2.9 NEXT GENERATION SEQUENCING (NGS)

K1 libraries were prepared using Nextera XT DNA library preparation kit according to the manufacturer's protocol. Library preparation entails five steps, which include tagmentation of the DNA, amplification, clean-up, normalization, and pooling of libraries.

Normalized K1 amplicons were randomly fragmented and tagged with adapter sequences by transposase enzyme. After tagmentation, a set of index adapters (i5 and i7) and sequence adapters required for sequencing and cluster generation were added to the libraries. Index adapters are used to differentiate the samples during the sequencing run. Following the addition of indexes, the libraries were subjected to polymerase chain reaction (PCR) using the NXT PCR protocol. After PCR, libraries were randomly selected and run on a 1% agarose gel for 30 minutes at 100 volts. This was done to verify the size of the libraries. The libraries were purified using magnetic beads and quantified using a DsDNA high sensitivity kit. Following quantification, the libraries were normalized to 1 nM and pooled into one tube by taking 5 µl per sample. This was done to ensure equal representation of the libraries during sequencing. Pooled libraries and Phix, which is used as a control, were denatured with 1.0 sodium hydroxide and diluted to 1.8 pM. To make a final volume of 500 µl, 495 µl of denatured and diluted libraries were combined with 5 µl of denatured and diluted phix control. The solution was then loaded into a 300 cycle high output cartridge and sequenced using a MiniSeq Illumina platform.

2.10 SEQUENCING ANALYSIS

The forward and reverse sequence of each test sample were analyzed as follows.

2.10.1 SEQUENCE QUALITY CONTROL AND VERIFICATION

Sequences were assessed for quality based on sequence quality score, base sequence quality, and length distribution using FastQC program embedded in Genome Detective Viral Tool version 2.11 (https://www.genomedetective.com). Sequences were imported to Geneious software version 2023.2.1 as paired end reads for further analysis. Reads were filtered and trimmed for poor base calls at an error probability rate of 0.01%.

To verify the sequenced amplicons and determine the prevalence of HHV-8 in the study sequences, reads were mapped to a previously described ORF-K1 sequence (AF178807) at a threshold frequency of 0%, which produces fewer ambiguities. The resultant contig was used to extract an 812-bp consensus sequence, which was then used for downstream analysis.

2.10.2 GENOTYPE ASSIGNMENT

Phylogenetic analysis has become an invaluable tool for examining the evolutionary relationships between organisms and discovering new strains. An online BioAfrica Oxford HHV-8 subtyping tool (https://www.genomedetective.com/app/typingtool/hhv8) embedded in Genome Detective Viral Tool was used to determine the subtypes of the study sequences. The subtypes were confirmed by computing a neighbor-joining phylogenetic tree in Geneious software. Previously described sequences of partial or complete K1 gene from several countries representing various K1 subtypes were retrieved from GenBank. The study consensus sequences were aligned together with previously described sequences using Clustal Omega embedded in Geneious software version 2023.2.1. The evolutionary relationship within the alignment was determined by constructing a Neighbor-Joining tree (Saitou and Nei, 1987). The reliability of the tree was bootstrapped 1000 times, and the evolutionary distance was computed using Tamura-Nei distance model.

2.10.3 INTRA-GENETIC DIVERSITY ANALYSIS

Single nucleotide polymorphism (SNP) is a substitution of a nucleotide base, which can result in either a transversion or transition mutation. Mutations are either synonymous mutations, which do not result in a change in the coded amino acid, or nonsynonymous mutations, which result in the alteration of a protein sequence. The study sequences were aligned using Clustal Omega tool in Geneious software at a frequency of 0% at both the nucleotide and amino acid levels. Study sequences were also aligned with their respective prototype strains to determine intra-genetic diversity within HHV-8 genotypes. The generated alignments were used to describe synonymous and non-synonymous mutations. An online SNAP tool incorporated into the Los Alamos HIV Database (http://www.hiv.lanl.gov/content/hivdb/mainpage.html) was used to analyze the rate of synonymous and non-synonymous mutations occurring at the partial K1 gene. Evolutionary divergence among the nucleotide study sequences was determined by computing the mean genetic diversity in Geneious software.

CHAPTER 3: RESULTS

3.1 DEMOGRAPHIC DATA OF STUDY PARTICIPANTS

The study comprised a total of 115 study participants who were visiting healthcare facilities in the Vhembe and Capricorn districts. Complete demographic data was available for 91% (105/115) of the study participants, of whom 44% (66/115) were HIV positive and 37.1% (39/115) were HIV negative. One HIV positive female participant (OTHC O61) from Vhembe district was diagnosed with Kaposi`s sarcoma. The age of the participants ranged from 2 to 75, with a median age of 37.

Table 3. 1: Demographic and clinical data of study participants

Characteristics	Distribution
District	
Vhembe	(68) 64.8%
Capricorn	(37) 35.2%
Gender	
Female	(72) 68.6%
Male	(33) 31.4%
Median age (2-75)	(37)
HIV status	
HIV positive	(66) 44%
HIV negative	(39) 37.1%
Marital status	
Single	(66) 62.9%
Married	(27) 25.7%
Widow/Widower	(8) 7.6%
Divorced	(4) 3.8%
Employment status	
Employed	(34) 32.4%
Unemployed	(71) 67.6%
Education level	
Grade 2-12	(81) 77.1%
Tertiary	(17) 16.2%
No schooling	(7) 6.7%
Smoking status	
Smokers	(84) 80%
Non-smokers	(16) 15.2%
Stopped smoking	(6) 5.7%
Comments on sample collection	
Participants who ate before mouth wash sample collection	(26) 24.8%
Participants who did not eat before mouth wash sample collection	(79) 75.2%
Participants diagnosed with KS	1

3.2 AMPLIFICATION OF ORF-K1 REGION

Open reading frame K1, a hypervariable gene, was amplified for the characterization of HHV-8 isolates. 115 mouthwash DNA samples were subjected to a two round PCR using JumpStart REDTaq ready mix. Of the 115 test samples, 71 (61.7%) successfully amplified the 840bp region, in addition to unspecified DNA bands.

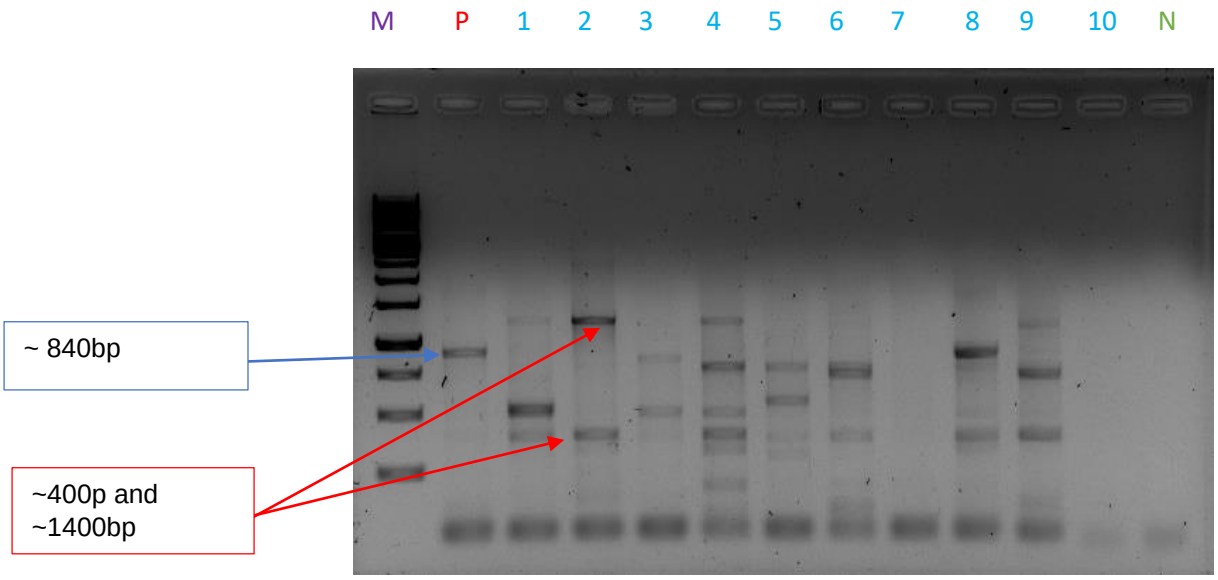

Figure 3.1: A 1% agarose gel representative of ORF-K1 amplicons visualized under UV transilluminator. The first Lane denoted M, indicate 1kb marker, lane P indicate positive control (a previously amplified sample), lanes 2-10 indicate test samples and lane N indicate negative control (nuclease free water). Lanes 3-6 and 7-8 indicate samples that successfully amplified ~840bp of K1 gene. The blue arrow indicates the targeted fragment size, and the red arrow indicates some of the multiple band sizes observed (400bp and 1400bp).

3.3 RECOVERY AND ENRICHMENT OF K1 FRAGMENTS FROM AGAROSE GEL EXTRACTION

ORF-K1 amplicons that were successfully amplified (71/115;61%) were subjected to agarose gel extraction using an in-house phenol-freeze protocol. The fragment size of interest (~840 bp) was successfully recovered in 67 amplicons (94.4%). K1 enrichment was achieved in 97% (65/67) of the gel extracted products.

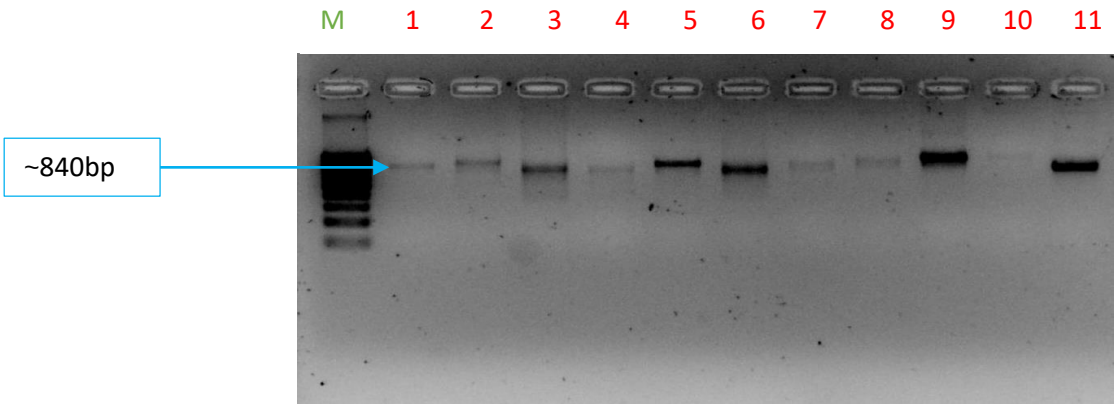

Figure 3.2: A 2% gel representative image of agarose gel extraction products. Lane M represents a 100bp maker and lanes 1-11 represent test samples. The blue arrow represents the expected band size.

3.4 SEQUENCE QUALITY EVALUATION

Sequences were evaluated for base call quality using FastQC program embedded in an online Genome Detective Viral Tool. A report detailing modules such as general statistics, base sequence quality, sequence quality score, and length distribution was generated. A per-base sequence quality score plot was used to assess the distribution of quality scores at each nucleotide position.

Of the 63 samples sequenced, 59% (37/63) sequences had an acceptable base call quality based on per base sequence quality plot. Sequences are classified as acceptable quality sequences when the median value of any nucleotide base is below 25, and this was observed at nucleotide positions 150–151. The length of the reads was 151 bp and poor base calls were observed from nucleotide positions 80 to 150. Majority of the sequences had sequence reads that ranged from 150 000 to 500 000. Poor base calls were filtered and trimmed using Geneious software at an error probability rate of 0.01%. After trimming, majority of sequences had sequence reads above 100 000 with good quality nucleotide bases.

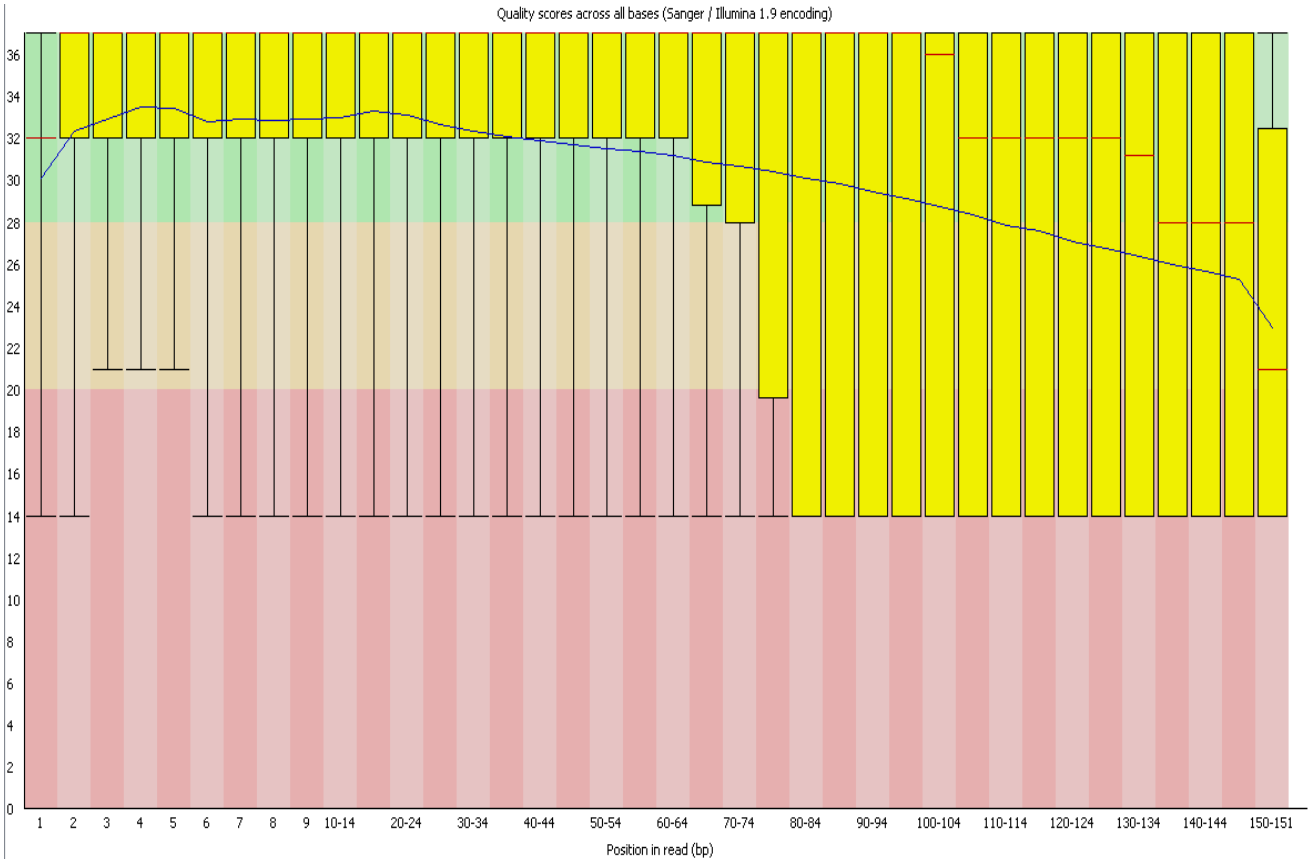

Figure 3.3: Per base sequence quality plot of ODF 114 (R1) indicating the "okay" quality base call. The green area indicates "good" quality, orange indicates "okay" quality, and red indicates "bad" quality base call. The quality of the reads starts to decrease as the yellow bars approaches the red area. Poor base calls of nucleotides were observed at position 80 to 151. The median value between nucleotide bases 150-151 was 21.

3.5 SEQUENCE MAPPING AND PREVALENCE OF HHV-8

Sequence reads (37) were mapped to a previously described sequence (AF178807) using Clustal Omega in Geneious software. A total of 35 sequences were mapped to the reference sequence. Twenty- four sequences had sequence reads that span full or partial K1 gene (**figure 3.4**). However, 18 of these sequences had gaps of approximately 50bp within the K1 region that were not covered by the reads. The remaining six sequences had reads that flanked the K1 region. Overall, the prevalence of HHV-8 in the study sequences was 94% (35/37).

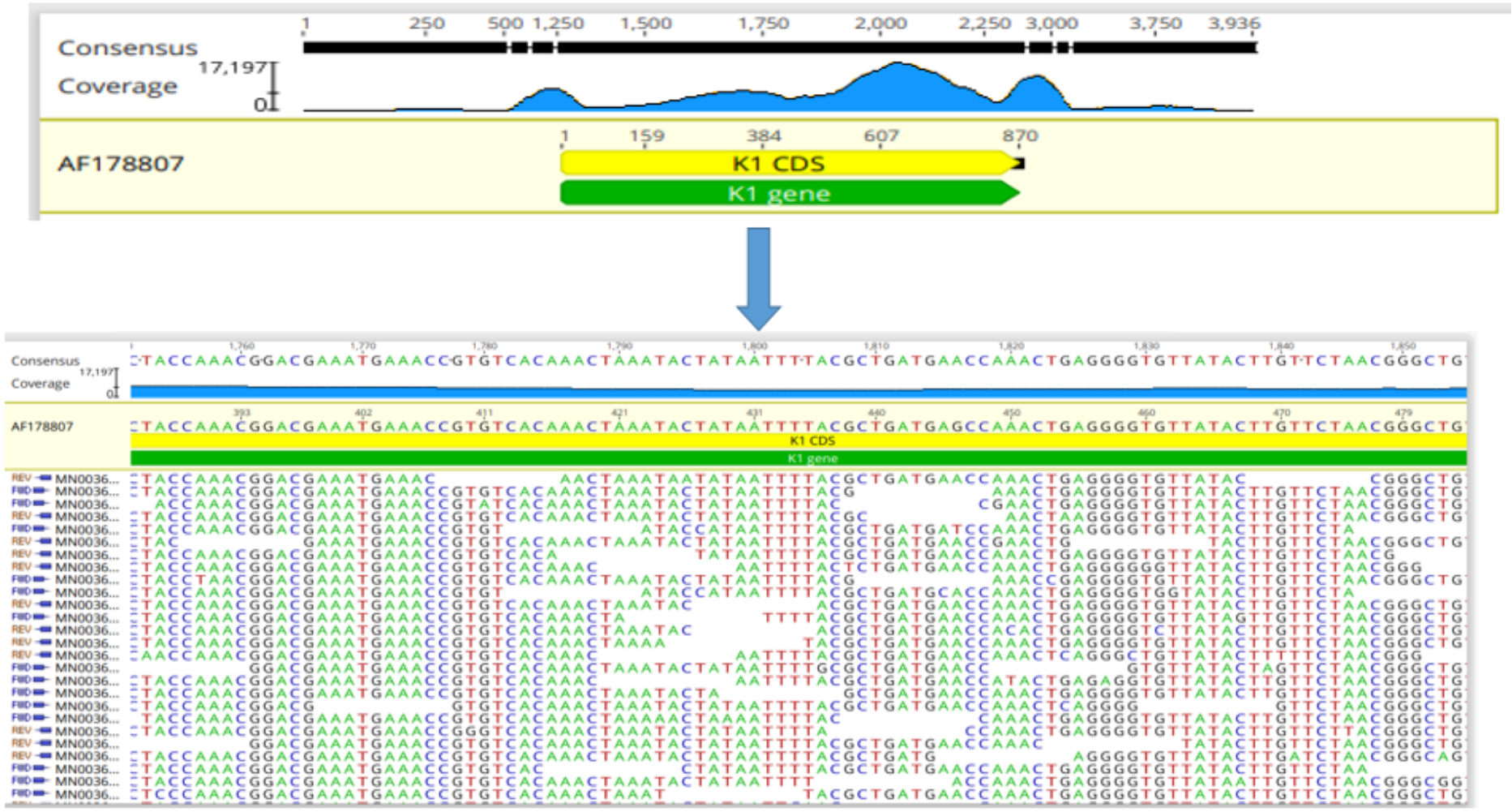

Figure 3.4 Contig representative of sequences that produced complete K1 coverage (ORHC 033). The contig was produced by mapping the reads to a reference sequence (AF178807) at a frequency of 0% which produces fewer ambiguities in Geneious software. ORHC033 had over 111 000 reads and over 88 000 of those reads spanned the entire K1 gene (~870) with a coverage of 101.15 fold.

3.6. GENOTYPE ASSIGNMENT OF PARTIAL ORF-K1 GENE

K1 genotype assignment was available in 68% (24/35) of the study sequences that mapped to K1 reference sequence (AF178807).

3.6.1 GENOTYPE ASSIGNMENT USING HHV-8 SUBTYPING TOOL

An online BioAfrica HHV-8 subtyping tool version 2.11 embedded in genome detective server was used to determine K1 genotypes. Genotype assignment was successful in six sequences (6/24; 25%); however, the remaining 18 sequences were not assigned genotypes due to the absence of sub-cluster reference sequences in the server. Of the six sequences, five were classified as genotype A (OTHC 014, OUHC 013, ODF 029, OTHC 032 and ORHC 033) and were further classified as subtype A5. The remaining sequence (AHDR R274) was classified as genotype B and was not further classified into any of the B subtypes.

3.6.2 GENOTYPE ASSIGNMENT USING PHYLOGENETIC ANALYSIS

To confirm K1 genotypes and subtypes detected by the BioAfrica HHV8 subtyping tool and assign genotypes for the 18 sequences, a Neighbor-joining phylogenetic tree was computed using Geneious software. Phylogenetic analysis of partial K1 gene was successful in 100% (24/24) of the study sequences. The phylogenetic tree was derived by aligning partial K1 study sequences together with previously described sequences obtained from GenBank. **Figure 3.5** shows the evolutionary relationship between study sequences and previously published sequences.

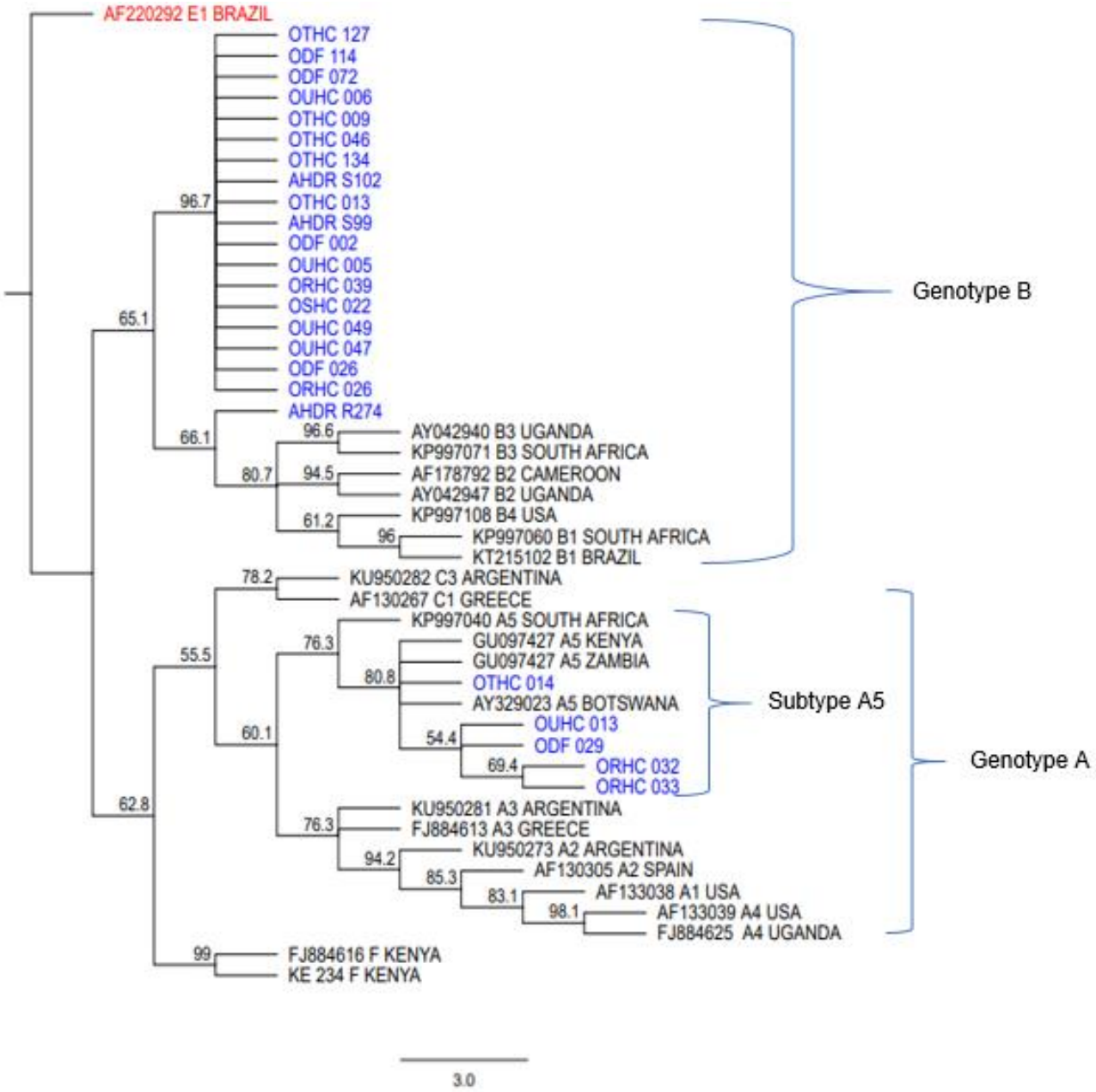

Figure 3.5: Figure 3. 5 Phylogenetic analysis of partial K1 gene. The phylogeny was performed by aligning 45 partial K1 sequences, which included 24 sequences (in blue) generated from this study and 23 previously described sequences from South Africa, United states of America (USA), Brazil, Cameroon, Greece, Argentina, Kenya, Spain, Botswana and Zambia. The tree was computed in Geneious software version 2023.2.1 using Tamura- Nei genetic distance model and was re-sampled 1000 times using bootstrap method. The nucleotide alignment indicated a 0.03 nucleotide substitution per site. AF220292, indicated in red was used as an outgroup.

3.6.3 PREVALENCE OF ORF-K1 GENOTYPES IN THE STUDY PARTICIPANTS

Two major genotypes (A and B) were detected from the analysis of partial K1 study sequences. Of the 24 study sequences, nineteen sequences (19/24; 79%) were classified into genotype B and the remaining five sequences (5/24; 21%) were classified as genotype A.

Table 3. 2: Demographic data of study participants and ORF-K1 genotypes

Sample code	District	Gender	Age	Marital status	HIV status	Genotype detected
AHDR R274	Capricorn	Female	49	Single	Positive	B
ODF 029	Vhembe	Female	43	Married	Positive	A
ORHC 032	Capricorn	Male	40	Married	Positive	A
ORHC 033	Capricorn	Male	50	Married	Positive	A
OTHC 014	Vhembe	Female	25	Single	Negative	A
OUHC 013	Vhembe	Female	26	Single	Negative	A
AHDR S102	Capricorn	Female	40	Married	Positive	B
AHDR S99	Capricorn	Female	26	Married	Positive	B
ODF 002	Vhembe	Male	32	Single	Positive	B
ODF 026	Vhembe	Male	52	Married	Positive	B
ODF 072	Vhembe	Female	43	Single	Positive	B
ODF 114	Vhembe	Female	35	Single	Positive	B
OTHC 009	Vhembe	Female	24	Single	Negative	B
OTHC 013	Vhembe	Female	20	Single	Negative	B
OTHC 046	Vhembe	DM	DM	DM	DM	B
OTHC 127	Vhembe	Female	20	Single	Negative	B
OTHC 134	Vhembe	Female	26	Single	Positive	B
OUHC 005	Vhembe	Female	22	Single	Negative	B
OUHC 006	Vhembe	Female	29	Single	Negative	B
OUHC 047	Vhembe	Female	20	Single	Negative	B
OUHC 049	Vhembe	Female	20	Single	Negative	B
ORHC 026	Capricorn	DM	DM	DM	DM	B
ORHC 039	Capricorn	Female	36	Single	Negative	B
OSHC 022	Capricorn	Female	50	Single	Positive	B

Key: DM= Data missing

3.7 INTRA GENETIC VARIABILITY OF PARTIAL K1 GENE

3.7.1 GENETIC VARIABILITY AMONG THE STUDY SEQUENCES

To describe the intra-genetic diversity of the K1 gene, only sequences spanning the near complete K1 gene (812 bp) and without gaps within the sequences were considered (6/24; 25%). A pairwise comparison of study sequences revealed 20.7% single nucleotide polymorphisms (SNPs) (**figure 3.6**) that led to 33.71% divergence at the amino acid level (**figure 3.7**). Over 98% of the observed SNPs were due to nucleotide substitutions, which were observed among the three sequences (AHDR R274, OUCH 013 and ODF 029). Common SNPs observed in genotype B sequence (AHDR R274) and Genotype A sequence (OUCH O13) include 2GA, C784G, and G786T. A codon deletion was observed in ODF 029, which led to a deletion of an amino acid at position 262. Amino acid variations were observed through the entire partial gene of the study sequences and were mostly concentrated at the sequence peptide and ITAM region. AHDR R 274 was the most hypervariable sequence, while ORHC 033 was the most conserved sequence at both the nucleotide and amino acid level.

< signal peptide >< conserved region 1 (C1) <

```
                1         10        20        30        40        50        60        70        80        90       100       110       120
Consensus       GAGGACTATT AAGCCTTTAT CTGCAATCGT CTCCAAATTT GTGCCCTGGA GTGATTTCAA CGCCTTACAC GTTGACCTGT CTGTCTGATG CATCCTTGCC AATATCCTGG TATTGCAACG
1. AHDR R274    C·AA····· G·····C·· ····C····· T····C·G·· .......... ........C· .......... .......... ·C····A·CA G····..... .......... ..........
2. OUHC 013     ··A·····A CGCAACCAGA TGT·TGCA·· ··GGCGG··· ·C·GA·S··· .........S ····GC···· .......... .......... .......... .......... ..........
3. ODF 029      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
4. OTHC 014     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
5. ORHC 032     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
6. ORHC 033     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
```

< variable loop (VR) > Variable region 1 (VR1) > <

```
                130       140       150       160       170       180       190       200       210       220       230       240
Consensus       ATACTCGGCT TTGGCGACTG ACGAACCCAT CATTCACTGT TGCCACCATT ACCTGCAATT TTACTTGTGT GGAACAATCT GGGCATCGAC AGAGCATTTG GATTACATGG AATGCACAAC
1. AHDR R274    ·G····.... ·CAC···A·A ···GCGT·TA ACC·A····· ·T·TT·GC·C .......... ........A· ·AC·AC···· ····C·AC·· ·C····.... ····GA···· T··A......
2. OUHC 013     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
3. ODF 029      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
4. OTHC 014     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
5. ORHC 032     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
6. ORHC 033     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
```

Conserved region 2 (C2)

```
                250       260       270       280       290       300       310       320       330       340       350       360
Consensus       CTGTCTTACA AACCTTGTGT GCACAGCCAT CAAACACAGT CACTTGTGGT CAGCATGTTA CTTTGTATTG TTCTACCTCT GGAAATAATG TTACCGTTTG GCATCTACCA AACGGACGAA
1. AHDR R274    .......... .......... .......... .......... GT........ ····C····· .......... ·GA····... TC........ .....A···· ··G·····A· .......AC·
2. OUHC 013     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
3. ODF 029      .......... .......... .......... ····T····· .......... .......... .......... .......... .......... .......... .......... ..........
4. OTHC 014     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
5. ORHC 032     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
6. ORHC 033     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
```

Conserved region 2 (C2)

```
                370       380       390       400       410       420       430       440       450       460       470       480
Consensus       ATGAAACCGT GTCACAAACT AAATACTATA ATTTTACGCT GATGAACCAA ACTGAGGGGT GTTATACTTG TTCTAACGGG CTGTCGTCTC GCCTGTCAAA TCGTATATGT TTTTGGGCGC
1. AHDR R274    ··C····... .......... .......... .......... .......... ···C...... .......... .......... .......... .......... .......... .........T
2. OUHC 013     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
3. ODF 029      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
4. OTHC 014     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
5. ORHC 032     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
6. ORHC 033     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
```

Figure 3.6: Nucleotide alignment of partial K1 study sequences. The alignment was computed by aligning the study sequences using Clustal Omega tool embedded in Geneious software. The K1 structural features are denoted by different colors. Dots (.) indicate agreement of study sequences with the consensus sequence and dashes (-) indicate nucleotide deletions. The study sequences varied from the consensus sequences at 159 nucleotide positions. Majority of the SNPs were observed in AHDR R274, OUCH 013 and ODF 029 and this includes nucleotide position G2C, C28A, T66G, respectively. ORHC 032 and ORHC 033 shared 100% nucleotide similarity with the consensus sequence.

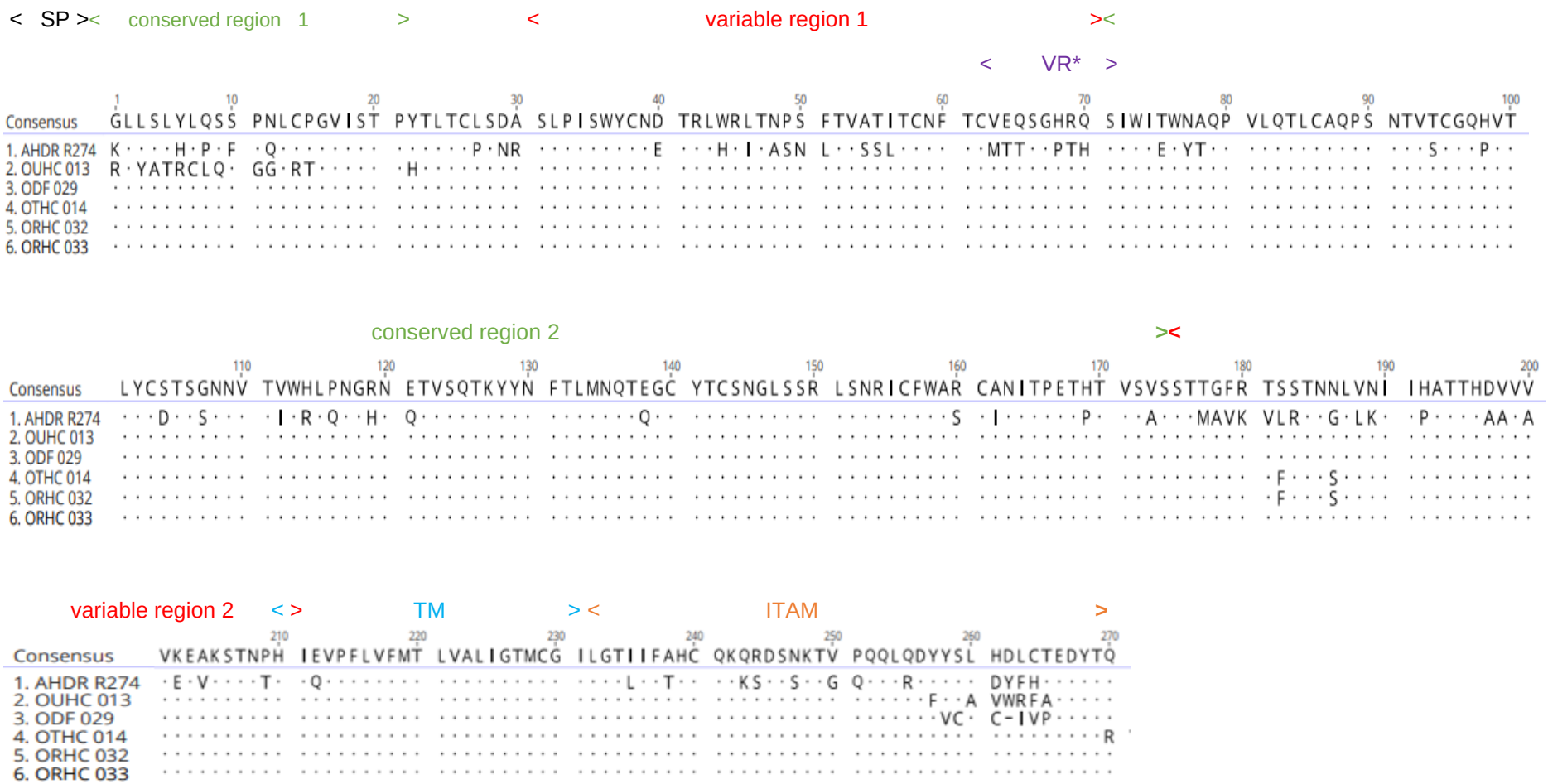

Figure 3. 7: Predicted amino acid alignment of partial K1 study sequences. The alignment was generated using Clustal Omega in Geneious software version 2023.2.1. The K1 structural features are denoted by different colors. The dots (.) indicate agreement with the consensus sequence and dashes (-) indicates deletion of amino acid. The study sequences varied from the consensus sequence at 93 amino acid positions and this include a deletion in position 262 in ODF 029. The most variable sequence was AHDR 274, while ORHC 033 shares 100% similarity with the consensus sequence.

3.7.2 GENETIC VARIABILITY OF GENOTYPE A AND B STUDY SEQUENCES

Genotype A study consensus sequence varied from genotype A prototype strain at 45 amino acid positions, resulting in over 16.67% amino acid divergence among the sequences (**figure 3.8**). These variations were observed throughout the features of partial K1 protein and mainly concentrated at the C1, VR1, and VR2. Over 20 amino acid variations occurred in all subtype A5 sequences at similar positions and this includes N29D, N40D, R50S S136N, F208P and P216L. The conserved cysteine residues (1,26,38,58,62,86,102,140,143,156,161,229 and 240) remained conserved in all sequences. Among the 8 glycosylation sites observed (30,40,60,110,131,136 and 238), two glycosylation sites changed the coded amino acid in all A5 sequences (N40D and S163N). OUHC 013 was the most hypervariable sequence with over 40 amino acid position that varies from the prototype strain.

A pairwise comparison of genotype B alignment revealed a 7.41% variation at the amino acid level (**figure 3.9**). AHDR R274 diverged from the prototype strain at 20 amino acid positions. These variations were concentrated at the signal peptide and conserved region 2 which accounts for 50% of the variation that were observed. The N glycosylation were conserved throughout the entire partial K1. From the 14 conserved cysteine bridges observed, two residues (F13G and V156T) varied position the prototype sequence.

From these alignments, Genotype A sequences displayed a higher genetic variations varying from genotype B by 9.6%. Multiple variations were observed in the ITAM region of A sequences and these also include a deletion at amino acid position 262, while it was conserved in the B sequence. A great degree of variation was observed at the conserved region 2 of AHDR R274 compared to A sequences. A similar amino acid variation was observed at peptide signal (amino acid position 5) wherein the coded amino acid changed from lysine and Valine to tyrosine in both the A sequence (OUHC 013) and B sequence (AHDR R274), respectively.

< SP > < conserved region1 > < variable region 1 ><

< VR* >

```
                                 1         10        20        30        40        50        60        70        80        90       100
1. AF178807                      GLLSLSLLSS PNLCPGVIST PYTLTCLSNA SLPISWYCNN TRLLRLTERR VILDTIACNF TCVEQSGHRQ SIWITWRAQP VLQTLCAQPS NTVTCGQHVT
2. consensus                     .....Y.Q.. .......... ........D. .........D ...W...NPS FTVA..T... .......... ......N... .......... ..........
3. OUHC 013                      R.YATRC.Q. GG.RT..... .H......D. .........D ...W...NPS FTVA..T... .......... ......N... .......... ..........
4. ODF 029                       .....Y.Q.. .......... ........D. .........D ...W...NPS FTVA..T... .......... ......N... .......... ..........
5. OTHC 014                      .....Y.Q.. .......... ........D. .........D ...W...NPS FTVA..T... .......... ......N... .......... ..........
6. ORHC 033                      .....Y.Q.. .......... ........D. .........D ...W...NPS FTVA..T... .......... ......N... .......... ..........
7. ORHC 032                      .....Y.Q.. .......... ........D. .........D ...W...NPS FTVA..T... .......... ......N... .......... ..........
```

conserved region 2 ><

```
                                 110       120       130       140       150       160       170       180       190       200
1. AF178807                      LYCSTSGNNV TVWHLPNGRN ETVSQTKYYN FTLMSQTEGC YTCSNGLSSR LSNRICFWAR CANITPETHT VSVSSTTGFR TLSTNSLVKI IHATTRDVVV
2. consensus                     .......... .......... .......... ....N..... .......... .......... .......... .......... .S...N..N. .....H....
3. OUHC 013                      .......... .......... .......... ....N..... .......... .......... .......... .......... .S...N..N. .....H....
4. ODF 029                       .......... .......... .......... ....N..... .......... .......... .......... .......... .S...N..N. .....H....
5. OTHC 014                      .......... .......... .......... ....N..... .......... .......... .......... .......... .F.....N. .....H....
6. ORHC 033                      .......... .......... .......... ....N..... .......... .......... .......... .......... .S...N..N. .....H....
7. ORHC 032                      .......... .......... .......... ....N..... .......... .......... .......... .......... .F.....N. .....H....
```

variable region 2 < > TM > < ITAM >

```
                                 210       220       230       240       250       260       270
1. AF178807                      VKEAKSTHFH IEVHFPVFMT LVALIGTMCG ILGTIIFAHC QKQRDSNKTV PQQLQDYYSL HDLCTEDYTQ
2. consensus                     .......NP. ...P.L.... .......... .......... .......... .......... ..........
3. OUHC 013                      .......NP. ...P.L.... .......... .......... .......... .....F..A. VWRFA.....
4. ODF 029                       .......NP. ...P.L.... .......... .......... .......... .....VC.. C-IVP.....
5. OTHC 014                      .......NP. ...P.L.... .......... .......... .......... .......... .........R
6. ORHC 033                      .......NP. ...P.L.... .......... .......... .......... .......... ..........
7. ORHC 032                      .......NP. ...P.L.... .......... .......... .......... .......... ..........
```

Figure 3.8: Amino acid alignment of genotype A partial K1 study sequences with a prototype strain of genotype A (AF178807). The comparison was computed using Clustal Omega in Geneious software. Similarity of study sequences with the prototype is indicated by dots (.) and variations are indicated by different amino acids. The study consensus varied from the prototype at 50 amino acid positions.These variations were concentrated at VR1, VR2 and ITAM.

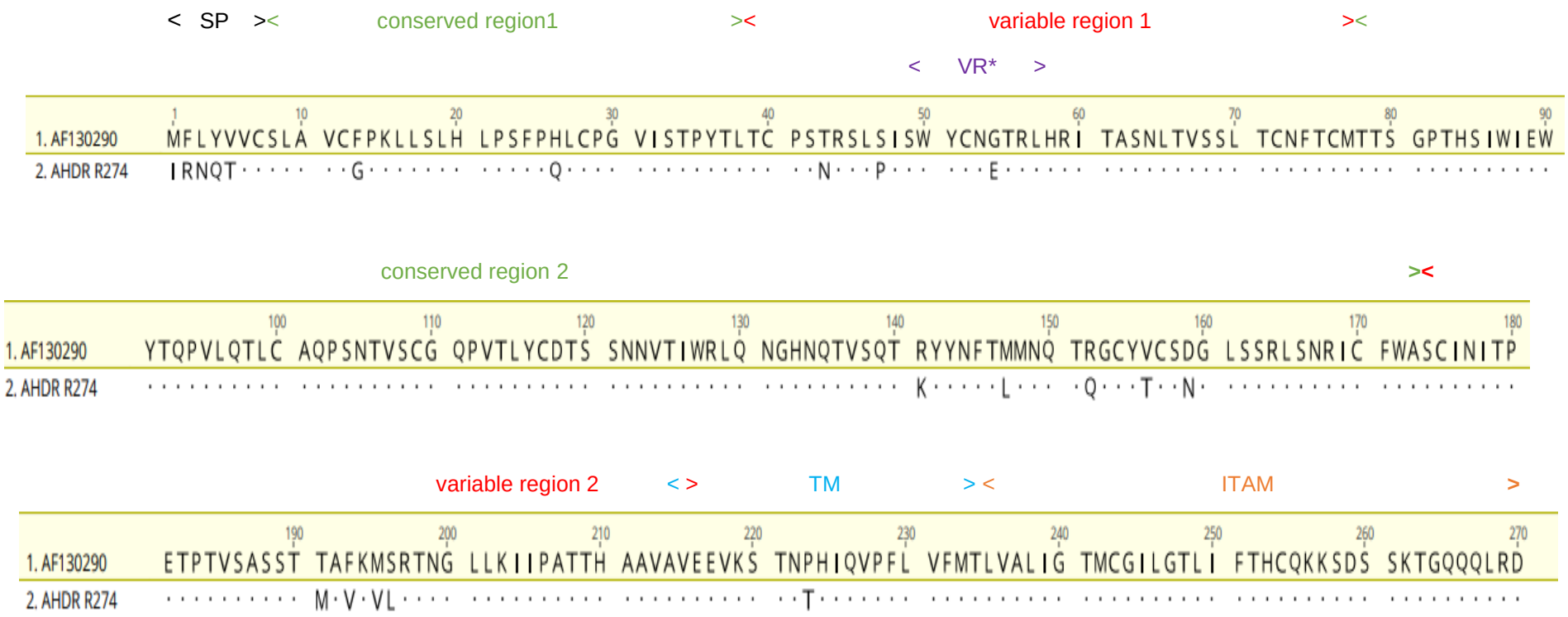

Figure 3.9: Amino acid alignment of gennotype B sequence and genotype B prototype sequence (AF130290). The alignment was generated by Clustal Omega in Geneious software version 2023.2.1. Dots indicate agrement of the study sequence with the protype sequece. The K1 features are highleted by various colors. AHDR R274 varied from the protype sequence at 20 amino acid positions and this include amino acid position 1-4 at signal peptide (PS) sequence and P223T in the tramsmembrane protein (TM).

3.7.3 SYNONYMOUS AND NON-SYNONYMOUS MUTATION

Pairwise comparison of the study sequences with their respective prototype strains were used to describe synonymous and nonsynonymous mutations occurring within the partial K1 gene. Nonsynonymous mutations were observed at a higher rate compared to synonymous mutation in both genotype A sequences and genotype B sequence. Synonymous mutation ranged from 0-0.33 in genotype A (**figure 3.10**) sequences and 0-1 in genotype B sequence (**figure 3.11**), while nonsynonymous mutation ranged from 0-2 and 0-2.67 in Genotype A and B sequences, respectively. Majority of synonymous mutations were observed within the peptide sequence (1-21), VR1 (38-78) and ITAM (256-266) in the A sequences and only in amino acid position 3 in the B sequence. Genotype A sequences had two amino acid positions (51 and 210) that exhibited 2.0 nonsynonymous mutation rate, while genotype B sequence had three amino acid positions (3,13 and 156). A higher nonsynonymous mutation rate was observed within the conserved region 2 (141-161) in genotype B sequence, while genotype A sequences remained conserved.

Table 3.3: Synonymous mutations observed within genotypes A study sequences.

Sequence name	Nucleotide position	Codon change	Predicted amino acid
OUHC 013	26	CT**A** > CT**G**	Leucine (L)
OUHC 013	65	CC**T** > CC**G**	Proline (P)
All sequences	126	A**G**G > A**C**G	Arginine (R)
ODF 029	275	AA**C** > AA**T**	Asparagine (N)
OUCH 013	779	TC**C** > TC**T**	Tyrosine (Y)
ODF 029	782	CT**A** > CT**C**	Serine (S)

Key: A-adenine, C-cytosine, G-guanine, T-thymine

Table 3.4:Nonsynonymous mutation observed in genotype A partial K1 study sequences

Sequence name	**Amino acid position**	**Codon change**	**Amino acid change**
OUHC 013	1	**G**GA > **A**GA	Glycine (G) to Arginine (R)
ODF 029	258	T**A**T > G**T**T	Tyrosine (Y) to Valine (V)
ODF 029	264	**TGC** > **CCG**	Cysteine (C) to Proline (P)
OTHC 014	812	C**AA** > C**GC**	Glutamine (Q) to Arginine (R)
All sequences	29	**A**AT > **G**AT	Asparagine (N) to Aspartic acid (D)
All sequences	77	**CG**T > **AA**T	Arginine (R) to Asparagine (N)
All sequences	135	A**G**C > A**A**C	Serine (S) to Asparagine (N)
All sequences	196	C**G**T > C**A**T	Arginine (R) to Histidine (H)

Key: A-adenine, C-cytosine, G-guanine, T-thymine

Table 3.5: Nonsynonymous mutations observed in genotype B partial K1 sequence

Genotype B sequence (AHDR R274)		
Amino acid positon	Codon change	Amino acid change
12	CA**T** > CA**G**	Histidine (H) to Glutamine (Q)
29	A**C**C > A**A**C	Threonine (T) to Asparagine (N)
33	**T**CA > **C**CA	Serine (S) to Proline (P)
40	G**G**G > G**A**G	Glycine (G) to Glutamic acid (E)
127	A**G**A > A**A**A	Arginine (R) to Lysine (K)
181	**A**T**G** > **G**T**A**	Methionine (M) to Valine (V)

Key: A-adenine, C-cytosine, G-guanine, T-thymine

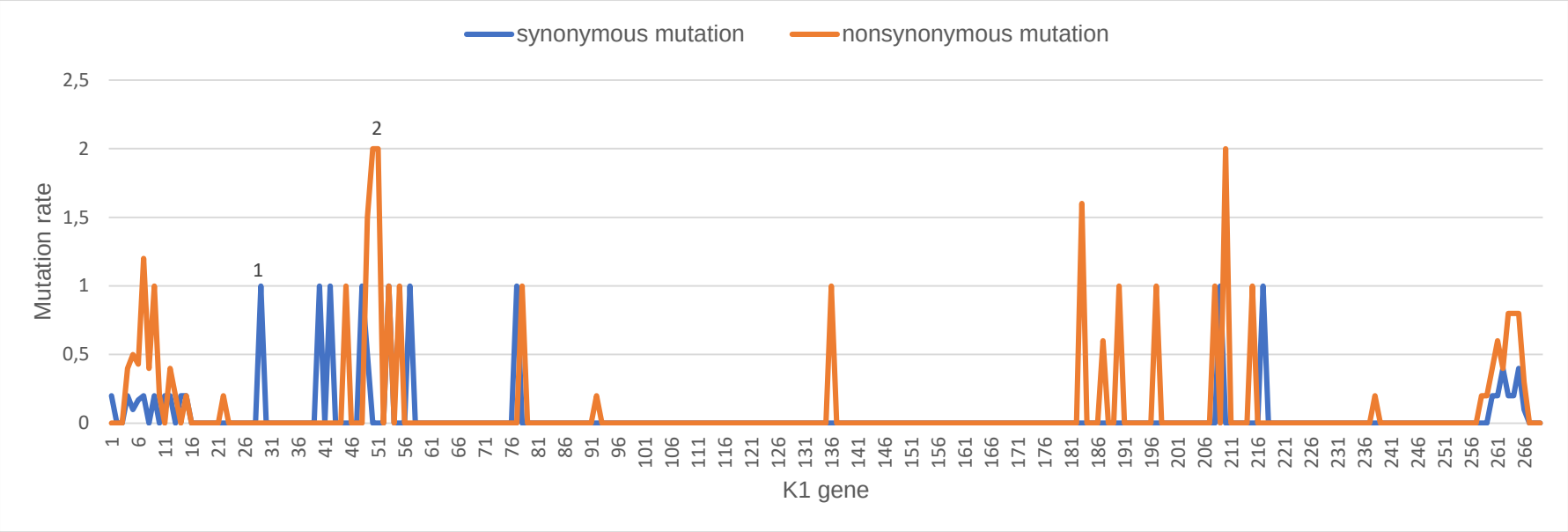

Figure 3.10: Graphical representation of per codon synonymous and nonsynonymous mutation rate of genotype A sequences. The analysis included five partial K1 sequences (812bp). Mutation rate were computed by SNAP tool embedded in in Los Alamos HIV Database and the line graph was generated in Microsoft Excel. Genotype A sequences displayed higher nonsynonymous rate (2) compared to synonymous mutation. Majority of these mutations were observed between amino acid position 1 -55 and 183 to 216.

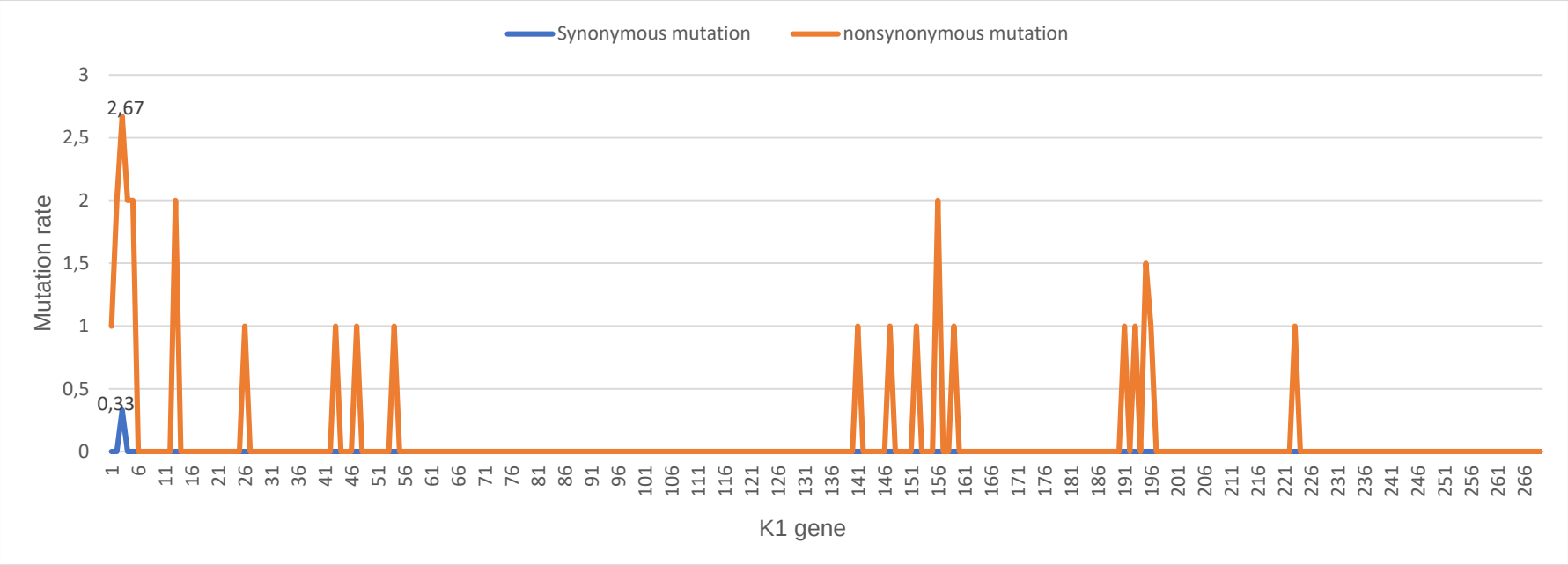

Figure 3.11: Graphical representation of per codon synonymous and nonsynonymous mutations rates of genotype B sequence. Mutation rates were calculated using SNAP tool embedded in in Los Alamos HIV Database. The line graph was drawn in scale using Microsoft Excel. Synonymous mutation ranged from 0 -0.33, while nonsynonymous mutations ranged from 0.0- 2.67. The highest rate of nonsynonymous mutations was observed in amino acid position 3. Non synonymous mutations were mostly concentrated at the conserved region 2 (141-161).

3.7.4 EVOLUTIONARY DIVERGENCE AMONG STUDY SEQUENCES

Nucleotide alignment of the study sequences was used to draw a Neighbor joining tree and infer evolutionary divergence among the sequences in Geneious software. The tree was bootstrapped by 1000 replicates and genetic distances were calculated using Tamura-Nei model in Geneious software version 2023. 2.1. The evolutionary genetic distances among the study sequences and genotype A sequences are shown below in **table 3.6** and **3.7**, respectively.

Table 3.6: Nucleotide divergence among partial K1 sequences

Sample code	**AHDR R274**	**OUHC 013**	**ODF 029**	**OTHC 014**	**ORHC 032**	**ORHC 033**
AHDR R274	0.0	0.201	0.168	0.148	0.146	0.147
OUHC 013	**0.201**	0.0	0.065	0.060	0.057	0.055
ODF 029	0.168	0.065	0.0	0.026	0.024	0.022
OTHC 014	0.148	0.060	0.026	0.0	0.003	0.006
ORHC 032	0.146	0.057	0.024	**0.003**	0.0	0.002
ORHC 033	0.147	0.055	0.022	0.006	**0.003**	0.0

Evolutionary genetic distance among the study sequences is shown on the lower left of the arrow and as a mirror image on the upper right of the arrow. Genetic distance indicates the number of nucleotide substitution per site. The evolutionary divergence range was between 0.003 to 0.201. A 99.7% nucleotide similarity was observed between OTHC 014, ORHC 032 and ORHC 033.

Table 3.7: Nucleotide divergence among genotype A sequences.

Sequence code	**OUHC 013**	**ODF 029**	**OTHC 014**	**ORHC 032**	**ORHC 033**
OUHCH 013	0.0	0.065	0.060	0.0.58	0.055
ODF 029	**0.065**	0.0	0.027	0.024	0.021
OTHC 014	0.060	0.027	0.0	0.003	0.005
ORHC 032	0.058	0.024	0**.003**	0.0	0.003
ORHC 033	0.055	0.021	0.005	**0.003**	0.0

Evolutionary genetic distance between genotype A sequences are shown on the lower left of the arrow and as a mirror image on the upper right of the arrow. Genetic distances indicate the number nucleotide substitution per site. Genetic distances ranged from 0.003 to 0. 065.The highest divergence rate was observed between ODF 029 and OUCH 013, which varied from each other at 143 nucleotide bases.

CHAPTER 4: DISCUSSION, STRENGTHS, LIMITATIONS AND CONCLUSION

4.1 DISCUSSION

Phylogenetic analysis of partial K1 isolates from this study resulted in a polyphyletic cluster, indicating the identification of two major genotypes (A and B). Majority of the isolates (19/24; 79%) were classified as B genotypes, and this was supported by a bootstrapping value of 65.1%. Interestingly, all the B genotype sequences did not cluster with any of the known subtypes, indicating a high genetic drift in genotype B sequences. Therefore, this could suggest the development of a new subtype, probably by recombination of the known B subtypes. These results are in accordance with a study done by Issacs et al., (2016) in a South African population, wherein they reported that one of the B isolates was not classified into any of the known B subtypes. The high variability of genotype B sequences might account for why the BioAfrica HHV-8 subtyping tool could not assign genotypes to 75% (18/24) of the study sequences and the phylogenetic tree could not assign subtypes to them.

The high prevalence of genotype B in the study sequences is consistent with a study done in a South African population by Treurnicht and colleagues (2002) and with a study done by White et al., (2008) in Zimbabwe. However, the findings from this study are in contrast with a study done by Isaacs et al., (2016) and by Hayward and Zong, (2007), where they reported that genotype A was the most prevalent among the studied population. Previous studies have demonstrated that genotype B might have originated in Africa and spread across the world during migration (Zong et al., 1999; Hayward et al., 1999). Furthermore, a systematic review conducted by Mamimandjiami et al., (2021) reported that genotype B accounts for 46.3% of all the seven K1 genotypes in the African population. This might account for its widespread diversity in the study population.

Five of the study sequences (5/24; 21%) were identified as subtype A5 using the BioAfrica HHV-8 subtyping tool and this was further confirmed by the phylogenetic tree with a bootstrap value of 76.3%. The A5 sequences from this study were phylogenetically heterogeneous and significant subgroupings were observed, indicating that there is high evolutionary divergence among the A5 study sequences. These results are in contrast with a study done by White et al., (2008) that reported that no significant subgroupings were observed among the A5 sequences from Zimbabwe. Interestingly, of all the A subtypes (A1-A5), only A5 was detected and this is in accordance with other previous studies (Treurnicht et al., 2002; Mamimandjiami

et al., 2021; Malonga et al., 2021). In addition, majority of studies have reported that subtype A5 is dominant in Africa and suggests an African origin (Lacoste et al., 2000; Etta et al., 2018; de Oliveira Lopes et al., 2021).

Initially, it was believed that subtype A5 emerged due to the recombination of the African B, N, Q and R genotypes (Hayward and Zong, 2007). However, recent reports have indicated that it has arisen due to natural genetic drifts and divergence from the A1-A4 subtypes. This is further supported by the high similarity observed between the A5 and A1-A4 subtypes (Duprez et al., 2005; Betsem et al., 2014; Etta et al., 2018). It is further reported that the divergence between the A5 and A1-A4 subtypes occurred approximately 4000 years ago (Mbulaiteye et al., 2004). Hayward and Zong (2007) reported that the wide spread of subtype A5 into the African continent is attributed to the “rapid and recent aggressive spread” of subtype A5 which is aided by a selective advantage of the A5 allele. This could account for the detection of only subtype A5 in the study population.

Despite the number of sequences (6/24; 25%) that were analyzed for K1 genetic diversity, a significant variation was observed among the study sequences. Pairwise comparison of partial K1 study sequence resulted in 20.7% nucleotide polymorphism and 33.71% divergence at the amino acid level. This was further supported by the evolutionary distances, which ranged from 0.0 to 0.201. These results are closely related to a study done by Mamimandjiami and colleagues (2021) that reported an observation of nucleotide and amino acid variation at 17.9% and 34.1%, respectively. These variations were attributed to sequences belonging to subtypes A5 (24/31) and B1 (3/31), which are almost similar to the subtypes observed in this study. The high degree of variability observed in this study is supported by the fact that the K1 gene is a target of the immune system and is susceptible to nonsynonymous mutations (Stebbing et al., 2003).

Genotype A sequences revealed a higher genetic drift from its prototype strain (AF178807) compared to genotype B; this was supported by 16.67% and 7.41% variation occurring at the amino acid level, respectively. However, these results were in contrast with a recent study that indicated that genotype B (14%) was more hypervariable than genotype A (6.5%) at the amino acid level (Mamimandjiami et al., 2021). The high genetic polymorphisms observed among the study sequences might be because South African HHV-8 genomes are reported to be highly diverse (Hayward and Zong, 2007). The amino acid alignments with the prototype indicated that genotype A is more diverse than genotype B, which varies from the phylogenetic analysis. These discrepancies can be augmented by the fact that only one sequence (AHDR R274) was analyzed for genotypic diversity of B sequences.

Genotype B sequences had a lower rate of synonymous mutations compared to genotype A sequences, wherein it was only observed once at amino acid position 3 at a 0.33 rate. The high rate of nonsynonymous mutations in genotype B sequence might indicate evidence of positive selection. Cordiali-Fei et al., (2015) suggested that positive selection pressure within genotype B might play a role in the development of KS. These results indicate a similar pattern, which was observed in a study done by White et al. (2008). Majority of nonsynonymous mutations in genotype A sequences were concentrated at VR1 and VR2, which is similar to what has been established by previous studies (Zong et al.,1999). This was not the case for genotype B sequences, as the amino acid variations were concentrated at the signal peptide and conserved region 2.

Multiple nonsynonymous mutations were observed in the ITAM motif and N glycosylation site of genotype A sequences; however, these regions were conserved in genotype B sequence. The ITAM motif plays a major role in the tumorigenesis of HHV-8, and mutations observed within this region might influence the progression of KS malignancies (Tamanaha-Nakasone et al., 2019). More than ten amino acid variations were observed in the ITAM motif of genotype A, and this includes Y257F, L260V, and a deletion at amino acid position 262. This might be one of reasons various studies have reported that subtype A5 is associated with rapid progression of KS, while genotype B is associated with a better prognosis of KS (Mancuso et al., 2008; Isaacs et al., 2016; Tozetto-Mendoza et al., 2016).

It has been established that HHV-8 is mostly detected in saliva (de Oliveira Lopes et al., 2021). Therefore, this study employed total DNA from mouthwash samples for the amplification of K1 gene. Over 60% amplification rate was achieved, however the fragment of interest (~840bp) was observed with other multiple bands, this was also observed by Whitby et al., (2004). This might be because of the sensitivity and specificity of the protocol used or mismatches of the primers with the test isolates since K1 is a highly variable gene.

4.2 STRENGTHS AND LIMITATIONS OF THE STUDY

The strengths of this study included the following **(1)** the geographical spread of the samples used. Samples were sourced from five clinical sites in two districts of the Limpopo province. **(2)** The use of mouthwash samples which enhanced the chance of amplifying the targeted gene. **(3)** The use of a near-complete sequence (270 aa) which is 93% of the K1 gene. *(4)* The use of high-throughput NGS platform (Illumina MiniSeq) that delivers high quality data that allows for the identification of minor variants and novel strains. Nevertheless, the study also had limitations. These include the lack of demographic and clinical data such as ethnicity, HIV status, HHV-8 viral loads, and CD4 cell counts, which would have been used in correlation analyses with viral genetics data generated.

4.3 CONCLUSION

In conclusion, this study showed that HHV-8 genotype B is likely predominant in northern South Africa. The high genetic drift observed in genotype B sequences compared to genotype A sequences supports the hypothesis that genotype B is an ancient virus and A5 has just been recently introduced. The synonymous mutations that were observed in the ITAM region of subtype A5 sequences might negatively impact the ITAM`s functions. These results provide insights on the genetic diversity of HHV-8 K1 genotypes in Northern South Africa which can further be used to draw inferences in selecting genotypes to be considered in designing vaccines.

Further studies on the complete genome of genotype B sequences are required to understand their evolutionary relationship, recombination, transmissibility and association with HHV-8 related malignancies. In addition, ITAM mutations associated with subtype A5 sequences needs to be further investigated in order to unravel their impact on the pathogenesis of HHV-8. Future studies should also examine treatment options available for HHV-8, and examine the determinants of HHV-8 transmission such as viral loads, comorbidities, host genetics, behavioral factors, age, gender, geographic, and environmental factors.

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