Molecular Prevalence of HSV1/2 from HIV-1 Positive and HIV-1 Negative sera collected from North-West and KwaZulu-Natal Provinces

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Dissertation submitted in fulfilment of the requirements for the degree *Master of Science in Biology (Medical Virology)* at the North West University

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DECLARATION

I, the undersigned researcher, make this declaration that this work is my original work and I have never at any time submitted it to any institution for another degree or qualification other than North-West University.

Name in Full: OBISESAN OLUWAFEMI SAMUEL

Signature: Date:

DEDICATION

I will like to sincerely dedicate this dissertation to my father, the supreme God who in His infinite mercy gave me the wisdom and showered me with strength to carry out this research. I will also like to dedicate this dissertation to my parents, Mr & Mrs Obisesan, my brothers and sisters for their continuous support, words of encouragement throughout the course of this degree. Words would fail me if I begin to express my gratitude for all you done. I am eternally grateful and I pray that God bless and enrich you all (Amen).

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I will be ungrateful if I fail to appreciate North-West University (NWU) bursary team for their financial support all through my study period at the university because there is an adage that says “to whom much is given, much is expected”. Your support is a contributing factor towards the completion of this degree and to God be honour and glory! Without Him I can do nothing.

ABSTRACT

Background

Herpes simplex virus (HSV) is a highly infectious virus that is found almost everywhere. It belongs to the alphaherpesvirinae sub-family which is further classified into two specie (Human alphaherpesvirus type 1 and Human alphaherpesvirus type 2). These herpetic viruses are highly pervasive and can be transmitted unconsciously from persons to persons sexually or through contact. Most sexually transmitted herpes infection are caused by type 2 herpes while HSV-1 is acquired through oral transmission. A strong synergistic interaction between HSV and HIV may speed up the progression of HIV and increase its infectiousness which may heighten sexual transmission of HIV and increase morbidity and mortality rate. The high prevalence of Herpes Simplex Virus in Africa (20-80% in women, 10-50% in men) and (49.7% in women, 50.3% in men) for HSV-2 and HSV-1 respectively makes it a pertinent problem as there is no active vaccine against it.

Aim and Objective

To determine the molecular prevalence of herpes simplex virus in sera collected from HIV positive and HIV negative patients from the North-West and KwaZulu-Natal Provinces and to check for an association between these herpetic viruses and human immunodeficiency virus.

Methods

A total number of forty-four sera samples were donated randomly from the two provinces. Twenty (20) from North-West and twenty-four (24) from KwaZulu-Natal. The samples were screened for both HSV and HIV using Enzyme-Linked Immunosorbent Assay (ELISA) kits and characterized using polymerase chain reaction and four samples were sequenced using both Sanger and Next generation sequencing (NGS) methods. Further analysis was also done using Statistical Package for Social Sciences (SPSS) software version 25 to check for an association between HSV and HIV.

Results

From the forty-four samples, thirty-six (81.8%) were positive for HIV-1 while thirty-four (77.3%) were positive for HSV when screened with ELISA kits. The samples were also confirmed with polymerase Chain Reaction (PCR) using type specific primers, and the result showed four (9.1%) out of the samples to be specific for HSV-1 while thirty (68.2%) were specific for HSV-2. Data analysis done on SPSS to check for a relationship between herpes simplex virus and human immunodeficiency virus showed that a strong association between HSV-2 and HIV-1 existed with a statistical significant P value (0.000^{*}), X^2 (1) = 20.952, P < 0.05.

Conclusion

The findings from this study revealed high HSV/HIV-1 co-infections suggesting that HSV plays a significant role in the transmission of HIV. It also showed that as Herpes Simplex Virus type 2 increases in the study population, the rate of HIV-1 acquisition also increased.

LIST OF ABBREVIATIONS

AIDS	Acquired Immune deficiency syndrome
CD4+	Cluster of differentiation 4
CE	Capillary electrophoresis
CCR5	C-C chemokines receptor type 5
CXCR4	C-X-C chemokines receptor type 4
CMI	Cell-mediated immunity
DCs	dendritic cells
DNA	Deoxyribonucliec acid
ddNTPs	dideoxynucleotides triphosphates
dsDNA	double stranded DNA
env	codes for envelope glycoprotein
gag	group specific antigen
gG	Glycoprotein G
GUD	Genital ulcer disease
HSV	Herpes Simplex Virus
HSV-1	Herpes Simplex Virus type 1
HSV-2	Herpes Simplex Virus type 2
HIV-1	Human Immunodeficiency Virus type 1
HSE	Herpes Simplex Encephalitis
HVEM	Herpes virus entry mediator
HCF	host cell factor
HVEM	herpes virus entry mediator
IE	immediate early
IL	Interleukins
ICP47	infected cell protein 47
IRF-7	inhibition of interferon regulatory factor 7
IFN	interferon
KZN	KwaZulu-Natal
MSM	Men sleeping with men
MHC	Major Histocompatibility Complex

Nef	Negative regulatory factor
NGS	Next Generation Sequencing
pDCs	plasmacytoid dendritic cells
PRRs	pathogen recognition receptors
PAMPs	pathogen-associated molecular patterns
pol	DNA polymerase
pDCs	plasmacytoid dendritic cells
RNA	Ribonucleic acid
RTCs	reverse transcription complex
Rev	Regulator of virion
ssRNA	single stranded RNA
STI	Sexually Transmitted Infection
SNV	single nucleotide variation
Tat	Trans-activator of transcription
TLRs	toll-like receptors
UL	Unique length
Us	Unique short
Vpr	Viral protein, regulatory gene
Vpu	Viral protein, unknown gene
VP16	virion protein 16
3-O-S-HS	3-O-sulfated heparan sulfate

DEFINITIONS OF TERMS

DNA is a nucleic acid responsible for transporting genetic information in cells and viruses which consists of two twisted long chains of nucleotides into a double helix structure and linked by hydrogen bond between complementary bases.

RNA is a polymeric molecule responsible for biological roles of coding, decoding, regulation and gene expression.

CD4+ is a glycoprotein found on the surface of immune cells with a sole responsibility of sending signal types of immune cells which then destroys the infectious particles.

PCR is a quick technique in molecular genetics used in amplifying small quantities of DNA to produce millions of copies of DNA molecules.

Sequencing is the precise order of nucleotide detection in post amplification analysis which can be used to discover the entire genome of an amplified DNA.

CHAPTER 1

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Herpes simplex virus (HSV) is an extremely communicable virus that is transmitted from one individual to another either via the parenteral route, through contact (oral-oral) or coitus (sexually transmitted) (Looker et al., 2015) and travel to the nerve tissues where they persist in a dormant stage. HSV is seen in different parts of the body but when symptoms appear, it's mostly on the mouth and the genitals.

Most HSV infections do not present with visible signs and symptoms but when there is an indication of the disease, it is presented in the form of sores at the infection site. The virus persist a lifetime in its host with a characteristic latent and periodic subclinical reactivity and viral shedding **(**Woestenberg et al., 2016). Herpes simplex virus is of two types. HSV-1, a neurotropic virus that occurs very early in childhood is responsible for herpes infection on the mouth and is transmitted through the touch on the lips or the use of the same drinking glasses with an infected person. It infects the adjacent sensory neuron endings during the primary infection and reaches the sensory ganglia where it becomes dormant. Occasionally, the virus is reactivated and travels down to the entry site causing tissue damage (Krug et al., 2004) while HSV-2, on the other hand, spread through sexual contact, infecting the genital tract (Vyse et al., 2000) and is regarded as the principal cause of ulcers in the genitals. However, HSV-1 genital herpes emergence in some populations has a significant impact on transmission of HSV infection in pregnancy which may cause severe concerns like foetal damage, miscarriage, or hereditary problems to the foetus or neonate (Ficarra et al., 2009).

HSV infection affects a large population regardless of the age, sex, or race because the risk of infection is almost entirely based on exposure to the infection. Therefore, risk factors associated with HSV are biological or behavioural which are markers to population subgroups that are significantly at risk of contracting the infection. The foremost factors responsible for HSV-2 seropositivity are female gender, black race, previous STI history, too many sexual partners, coitus with sex workers and poverty (Daniel et al., 2016).

Herpes simplex virus is regarded as the most common cause of genital ulcer disease (GUD). Genital herpes caused by these two herpetic viruses became a global burden with an estimation of 544 million individual (15% prevalence) infected with genital herpes within the age of 15-49 years. Africa and America had been reported to harbour a high prevalence of genital herpes (>19%) with the prevalence of genital HSV-1 estimated to be >9% in America and approximately zero in Africa but Africa has a higher HSV-2 prevalence (Feng et al., 2013). As a result of this high prevalence and lifelong infection, HSV has a detrimental effect on human health globally. In HIV uninfected individual, genital herpes causes genital ulceration and mucosal disruption thus providing a port of entry for other infections transmitted through sexual debut.

The transmission of herpes simplex type 2 through sexual means is the source of several genital herpes occurrences (Looker et al., 2008). It is also contracted during child delivery from an infected pregnant woman to her foetus. Neonatal infection with HSV-2 can be fatal and is the cause of 80% infant death when they are not treated properly (Domercant et al., 2017).

In Sub-Saharan Africa, there is a high infection rate of HSV-2 with a prevalence of 10-50% in men and 30-80% in women. However, this increase has been linked as a cause of the steady rise in HIV-1 as indicated in Moodley et al. (2003) and in this study.

1.1.1 Research Aim and Objectives

1.1.1.1 Research Aim

The aim of this study was to determine the Molecular prevalence of HSV1/2 from HIV-1 positive and HIV-1 negative sera collected from North-West and KwaZulu-Natal Provinces.

1.1.1.2 Research Objectives

The objectives of this study were:

- To screen for the presence of HSV-1/HSV-2 from both HIV positive samples and HIV negative samples and to type HSV using Enzyme Linked Immunoassay kits (ELISA)
- To determine the HIV status of the participants' sera included in this study.

- To amplify HSV-1 and HSV-2 monoinfected samples as well as HSV-1/2 and HIV co-infected samples using Polymerase Chain reaction (PCR)

- To sequence PCR products of co-infected samples using Sangers and Next Generation Sequencing (NGS) methods.
- To evaluate for an association between HSV and HIV-1
- To determine the effect of Age on HSV-1, HSV-2 and HIV-1

1.1.2 Problem Statement

Herpes simplex virus is a major threat that may double the chances of HIV acquisition and other sexually transmitted infections (STI`s), accelerating disease deterioration and increase the infectiousness of HIV resulting in high morbidity and mortality rate (Freeman et al., 2006; Moodley et al., 2003). This sis true given the scourge of HIV in South Africa that can be promoted through the availability of HSV as well as the HIV/HSV co-existence that may be prevalent among South Africans. Currently, unavailability of active vaccine against herpes simplex virus intensifies the need to evaluate the problem that this infection might pose so as to appreciate the scale of an epidemic which might stimulate the interest of the government and other stakeholders towards the management or effective treatment of co-infections (Anzivino et al., 2009; Smith et al., 2002).

1.1.3 Significance of the Research Study

The significance of this study is that the findings thereof might be of help in understanding and identifying the possible risk factors present and this process may help stimulate possible direct intervention aimed at reducing the transmission and acquisition of HSV. It is also anticipated that findings of this study may support ideas and data that will help in monitoring HSV seroprevalence which may help prevent HSV/HIV co-infection and this can have a positive impact in reducing the spread of HIV infection hence, reducing HIV morbidity rate.

1.2 Literature Review of Herpes Simplex Viruses (HSV)

1.2.1 Introduction

Human population infected with HSV originated from East Asia where antibodies produced were used progressively to combat herpes viruses. Herpes simplex virus was first accepted in Greece which is why Greek scholars used the word ‘‘herpes’’, which means creeping or crawling used in describing the binge of lesions (Whitley et al., 1998). However, herpes simplex virus which is the most common transmissible infection of human is a double-stranded DNA virus, present almost everywhere and can cause series of infections or illnesses on the skin, mucous membrane and eyes of man initiating herpes labialis, genitalis, keratitis and encephalitis.

1.2.2 Virology of Herpes Simplex Viruses

1.2.2.1 Classification of HSV

Classification of viruses was thoroughly conducted by a popular biologist, David Baltimore and he based viral classification on how messenger RNA is formed during virus replicative cycle. In his classification of viruses into groups, Herpesviruses were classified into Group 1 (Double stranded DNA (dsDNA)) while HIV was grouped into Group 6, Single stranded RNA with reverse transcriptase (ssRNA-RT) as described by Murphy et al., (2012).

In 2017, International Committee on Taxonomy of Viruses (ICTV) as illustrated in figure 1.1 groups herpesviruses into order herpesvirales which has three families (Alloherpesviridae, herpesviridae and malacoherpesviridae). Herpesviruses belong to the family Herpesviridae consisting of more than 200 species causing diseases in animals, including man. It has three sub-families; alphaherpesvirinae, betaherpesvirinae and gammaherpesvirinae. The life cycle of alphaherpesvirinae are relatively short, reproduce rapidly and exhibit quiescence mainly in the sensory ganglia. This sub-family of herpesvirus is further classified into six genera (lltovirus, mardivirus, scutavirus, simplexvirus, varicellovirus and one unassigned genus). Simplexvirus genus has twelve species out of which is human alphaherpesvirus1 and human alphaherpesvirus 2 known as HSV-1 and HSV-2 respectively which are the species of choice in this study (Kukhanova et al., 2014). HSV has a tendency of blighting tissues with a typical lytic and latent cycle and infects a wide host range (Wagner, 2012).

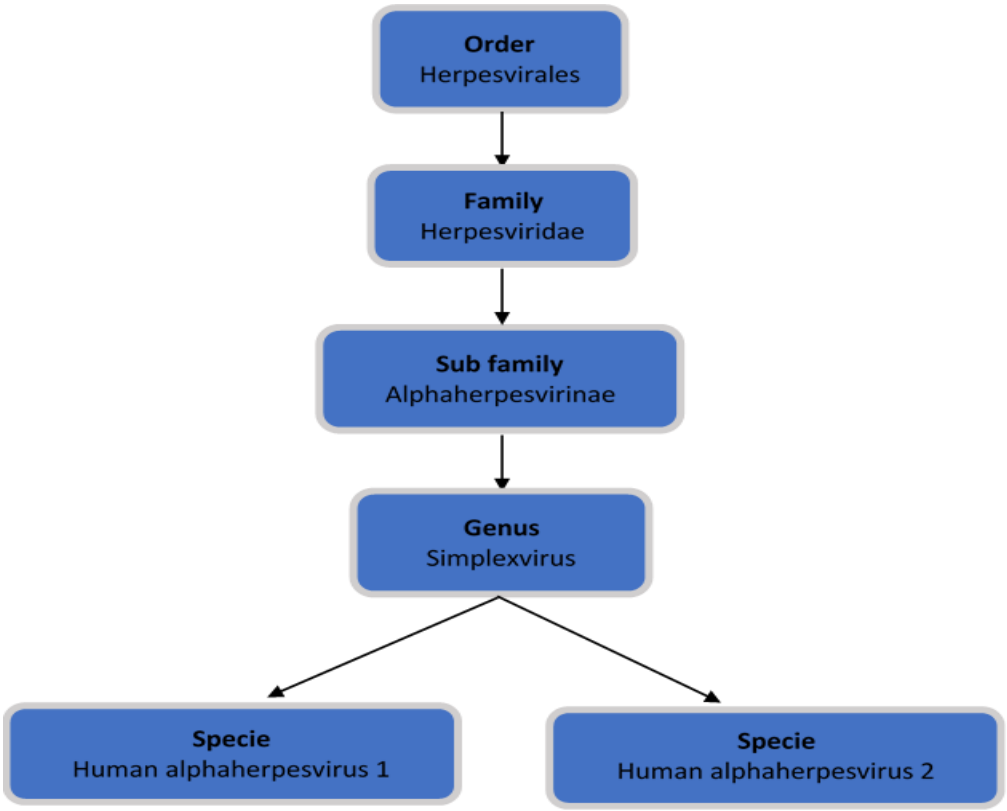

Figure 1.1: Herpesviruses classification adopted from International Committee on Taxonomy of viruses (ICTV, 2017)

1.2.2.2 Morphology and Genomic Organisation of Herpes Simplex Virus

HSV virion is a large double-stranded, linear DNA, sheathed inside a protein cage (capsid). It is made up of a core that is densely filled with electrons, an icosadeltahedral capsid around the core, a shapeless tegument around the capsid, and an outer envelope inclosing glycoprotein spikes. There are inconsistencies in the structure of HSV virion which is as a result of the difference in the structure of the tegument and the condition of the envelope (Roizman et al., 1974).

The capsid is wrapped within the lipid bilayer called envelop as shown in figure 1.2. It is the protein shell that encloses the nucleic acid and when it is with the enclosed nucleic acid, it is called nucleocapsid. This shell comprises a protein, organized into smaller units called capsomers which is about 15nm thickness and 125nm in length. It has an icosahedral configuration of 162 capsomeres (150 hexons and 12 pentons.). Capsid, when isolated from infected cells, has three categories; procapsids or A-capsids is deficient of scaffold proteins and viral DNA, B-capsids contain scaffold protein with the absence of viral DNA while C-

capsids holds the viral genome. Capsid prevents ingestion of nucleic acids through enzymes, has specific regions on its surface that allows the virion to attach to the host cells and also offers protein which to introduces infectious nucleic acid into host cells cytoplasm (Kukhanova et al., 2014; Laine et al., 2015).

Tegument is the space amid the envelope and the capsid. It comprises of 26 proteins involved in the start of replication. UL36, UL37, ICP0 carry capsid to the nucleus and other organelles. Viral inoculation of DNA into the nucleus is performed by VP1-2, UL36, activation of early genes transcription VP16, encoded by UL48 gene and suppression of cellular protein biosynthesis, and mRNA degradation VHS, UL41 (Laine et al., 2015).

An envelope is an outgrowth from inner membrane altered when glycoprotein is inserted. It has lipid bilayers mingled with protein molecules (lipoprotein bilayer) formed by cell membrane through endocytosis and may contain membrane material of a host cell in addition to the viral origin. However, the exterior of an envelope virion consists of two lipid layers and 11 glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gK, membrane gL, and gM) with the minimum of two unglycosylated membrane proteins, UL20 and US9 (Laine et al., 2015).

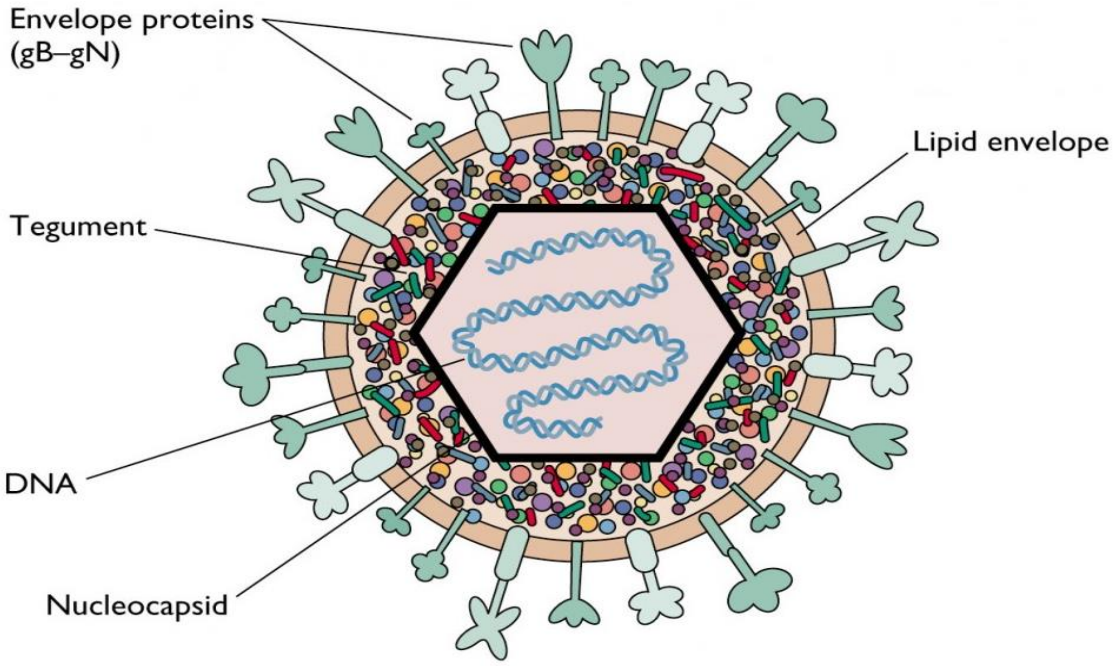

Figure 1.2: The uneven location of HSV capsid containing DNA inside the virion surrounded by a protein layer, tegument which is bounded by envelop that is covered with distinct glycoprotein spikes Gaurab (2018).

1.2.2.3 Genomic organisation of Herpes simplex virus

The genomes of HSV encodes about 80 genes (Jiang et al., 1998). The structure of the genome as illustrated in figure 1.3 has two important and distinct regions (long and short) joined together by a covalent bond. These regions consist of unique sequence that is lined by inverted repeat sequences (McGeoch et al., 1986). Occurrence of inverted repeats will make the unique long and unique short sequences of the genome to relatively upturn one another, yielding four linear isomers (Kukhanova et al., 2014).

The variation in the genomic size of herpesviruses range from 120 to 250 kbp with a bent-on size of HSV-1 at 152kbp and that of HSV-2 at 155kbp (McGeoch et al., 1988). The weight of herpes simplex virus is around 100 x 10^6 with a base structure of 67 and 69 G+C mole % respectively. The base pair sequence of HSV-1 and HSV-2 DNA conform by 50% but vary in many restriction enzyme cleavage site (Roizman et al., 1979). HSV genome is classified into six (6) important regions which are the; the unique long region (UL), unique short region (US), origin of replication (OriL), "a" sequence, long repeats(RL) and short repeats(RS). The inversion b'a and ca of UL and Us having being flanked by the sequence ab and a'c' is around 9kbp and 6.5kbp respectively. The a sequences which are targets for endonuclease G enclose the packaging sites. There is variation in the sequence size with a chief role in viral DNA circularization and may be present in single or multiple copies next to the b′a′ or c sequence (Roizman et al., 1996).

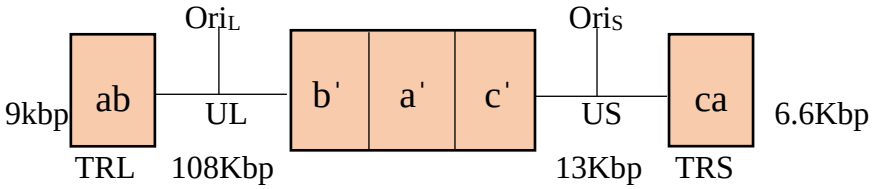

Figure 1.3: Genome arrangement showing repeats in the long unique sequence and short unique sequence labelled as ab, b′a′ and a′c′, ca respectively with two origins of replication (oriL, oriS). The repeat long, unique long and short repeats have 9kbp, 108kbp and 6.6kbp respectively (Elbadawy et al., 2012).

1.3 Life Cycle and Pathogenesis of Herpes Simplex Virus

1.3.1 Life cycle of Herpes Simplex Virus

Herpes simplex virus gains access into a host cells when the virion envelope merges with the cellular membrane aimed at releasing capsid and tegument into the cytoplasm. The few basic phases that HSV viral cycle undergo before the cycle is complete are, entry into the host cell, expression of viral genes, replication, virion assembly, and egress of the new generation of viral particles (Kukhanova et al., 2014).

1.3.1.1 Viral entry

The medium through which an incoming viral particle gains access to the cell host is referred to as viral entry (Mercer et al., 2010). HSV enters the cell through the attachment of viral envelope with the membrane cells which allows capsid and tegument proteins to be deposited in the cytoplasm. This is the first and very important stage in viral pathogenesis. However, different entry pathways have been identified depending on their cell type: direct attachment with the plasma membrane, endocytosis accompanied with acidic endosome fusion and phagocytosis-like uptake. Micropinocytosis is another means through which HSV enters the cells most especially in special cells (Akhtar et al., 2009; Campadelli-Fiume et al., 2012; Nygårdas, 2013).

There are three basic steps through which herpes simplex virus enters the host cell; attachment, stabilization of the attachment, and penetration. Viral attachment is an essential stage of viral entry which requires the contact of viral glycoproteins with specific receptor cells. Glycoproteins are implicated in viral entry process and the most implicated ones in this process are gB, gD, gH and gL (Akhtar et al., 2009). Glycoprotein C (gC) and (gB) facilitates the connexion between the virion and the cell surface by interacting with glycosaminoglycans (heparan sulfate) (Kukhanova et al., 2014).

The attachment is stabilized through exclusive binding of gD to one of the herpesvirus receptors like nectin-1, HVEM and 3-O-sulfated heparan sulfate (3-O-S-HS) which also causes membrane fusion when it muddles with gB and gH/gL complex. After the viral particle penetration, the viral tegument and capsid are moved to the nuclear pores which are then conveyed into the nucleus using the cytoskeleton proteins of the infected cell. As the capsid

gets to the nucleus, the linear viral DNA is inserted through the capsid portal to the nucleus of the diseased cell) (Kukhanova et al. 2014).

1.3.1.2 Gene expression

During infection, three groups of virus-specific polypeptides are produced in a sequential fashion which is in tandem with HSV viral arrangements. The group-specific polypeptides designated α, immediate early (IE), β early and γ late gene (Weir, 2001). The reproductive cycle begins when the immediate-early (α) genes transcribed in the absence of de novo protein synthesis. α- genes codes for viral regulatory proteins involved in the transcriptional control of early gene (Gruffat et al., 2016). As soon as HSV infection is initiated, HSV IE genes are stimulated resulting in the emergence of many protein complexes. The formed complexes consist of two cellular proteins that are present in the host (Oct-1 and HCF) and virion protein 16 (VP16). HCF muddles with VP16 to ease a stable relationship with Oct-1 and could be the cause for nuclear import of VP16 in the early stage of infection. From the five immediate early protein, four serve a crucial role in gene expression management. As ICP4 and ICP27 are used expediently in protein regulation in vitro and in vivo, so is ICP0 and ICP22 important in the control of the viral gene (Weir, 2001).

The main function of an early gene is to stop the yields of immediate early gene and trigger how late genes are revealed. Early gene products are essential in the way the virus duplicates its genes, late genes are revealed or communicated and the build-up of early and late mRNAs. Early gene expression diminishes as the late gene is transcribed during DNA replication, causing the assembly and release of viral agents (Gruffat et al., 2016). Activation of late gene expression is necessitated by ICP4 and facilitated through TATA element. True HSV late genes (γ late gene) require DNA replication for accumulated mRNAs but some late genes are expressed in the absence of replication and they are referred to as the leaky-late gene (Weir, 2001).

1.3.1.3 Replication

The way and manner in which herpes simplex virus duplicates itself involves three (3) important stages: viral gene transcription, viral assembly in the nucleus, and budding through the nuclear membrane. Before viral DNA replication can take place, the host cells

are suppressed by a protein, UL 41 gene product protein. This process of host cell suppression is the early shutoff or virion associated host shutoff (VHS) (Gallaher, 2013). Transcription and viral genome in addition to the gathering of new capsid occur inside the nucleus. Entry of the viral DNA into the nucleus initiates a change in viral DNA from linear to circular without protein synthesis, hence, permits for origin dependent replication. HSV makes use of three sites on its genome for its replication which are oriL and two different duplicates of oriS that are proximate to both ends of US (Boehmer et al., 1997).

After replication, DNA is sliced and re-organised into a new capsid entailing preserved proteins (UL6, UL18, UL19, UL35, and UL38). The formation of capsid goes through several stages ranging from partial capsids to closed spherical capsids through the evolution of closed circular capsids into polyhedral capsids. Immediately the capsid is formed, it migrates towards the inner membrane before the envelope is formed, exiting the nucleus which is eased by microfilament actin protein. Also, the interaction of the capsid with the nuclear envelope is aided by UL31 and UL34 proteins (Mettenleiter et al., 2006). The capsid leaves the nucleus and receives its tegument, formed at two sites (capsid and envelope sites) and secondary envelope. At the capsid site, teguments are either assembled by UL36 and UL37 protein or UL25 and US3 protein while teguments formed at the envelope sites are assembled with glycoproteins. Glycoprotein M (gM) plays an important role in the future envelopment in a mature virion by gathering other glycoproteins and the target genes towards the location of the envelope. Fusion of the various viral assemblies forms a mature virion enclosed in a cellular vesicle which later transfers to the membrane cells, attaching itself to release the fully developed virion (Gallaher, 2013).

1.3.1.4 Latency and Immune Evasion

Herpes simplex virus has the ability to show quiescent in the host cell. It is able to do this by evading the immune system subsequent to viral entry. The virus bypass the immune system by either mimicking the molecular homologs of cellular interleukins (IL), chemokine receptors or reduce staging of viral antigens via the major histocompatibility complex (MHC) of infected cells by inhibiting the display of both MHC class I and II molecule. For latency to begin, DNA episomal element is shaped from viral genome to occupy histones (Grinde, 2013). Latency is initiated when the nearby neuron is infected which begins by binding of the viral particle to the

receptor cells resulting in a membrane union event that allows the nucleocapsid access into the peripheral cytoplasm; the nucleocapsid is then transferred into the nucleus to insert the virus DNA (Brown, 2017).

Viral genome does not undergo amplification during latency because replication ceases (Boehmer et al., 2003). Most neurons with HSV genomes are engrossed in the sensory ganglion as they are commonest neurons innervating the oral and genital membranous tissue. Reactivation of latent infection occur if an infected person is exposed to ultraviolet light, emotional stress, tissue damage and immune suppression but latent infection does not present with symptoms (Brown, 2017).

1.3.2 HSV Pathogenesis

Herpes simplex virus can infect epithelial mucosa cells of individuals and animals but, only human show the symptom of the disease. The virus enters the skin of the host cells if there are external openings either in the oral mucosa, eyes or genitalia. Following primary infection, the virus moves to the nerve axon where latency begins within the dorsal root ganglia. In most cases, primary infection does not advance beyond latency with seeming symptoms in infants or immune deficient persons. The viral DNA relics in the gangliocytes deceptively after symptoms are reduced and reactivated by tension or common cold. Replication of the virus starts through viral reactivation which can present as blisters, papules or sometimes manifests as asymptomatic shedding (Barnabas et al., 2012). The choice of disease that herpes simplex virus presents with is as a result of the viral strain and the route of infection. Some of which are stromal keratitis, encephalitis, neonatal ophthalmic, and neurologic complications as seen in infants (Thiry et al., 1986; Smith et al., 2002).

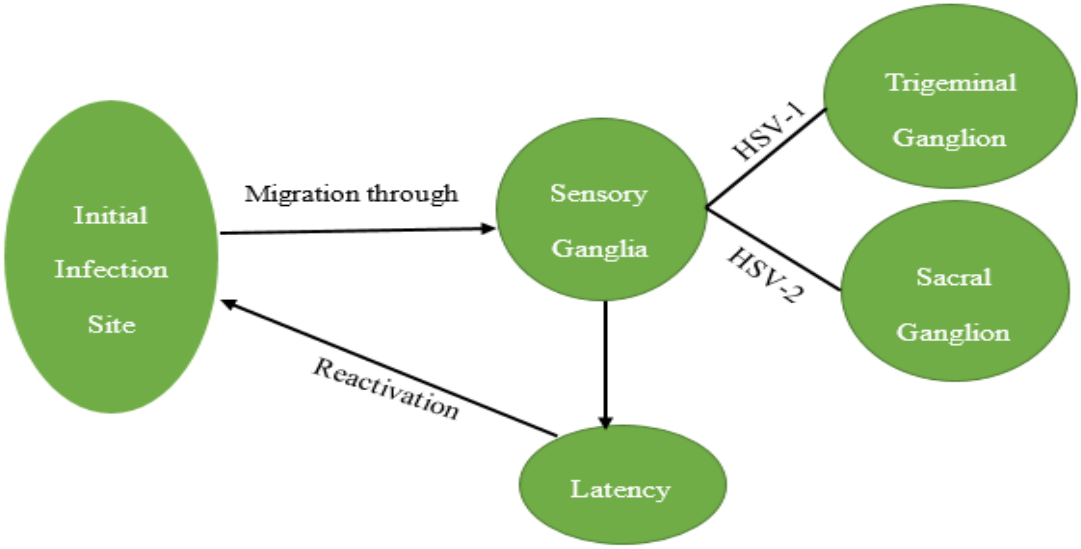

Figure 1.4**:** The pathway of Herpes Simplex Virus pathogenesis

1.3.2.1 Immune Response to HSV

Herpes simplex virus (HSV) is an infection causing virus in human capable of causing a resolving ailment that can be devastating in an individual whose immunity has been compromised. In response to the infection, the host builds a defence upon viral entry known as an immune response which is dependent on a number of factors. The mechanisms involved in combating the infection are innate (non-specific) and adaptive (specific) immune mechanisms.

Innate immune mechanism

This is the host first line of defence generated within minutes or hours of infection that can either halt or lessen viral infection. It can also support the activation of host adaptive response (Piret et al., 2015). The response of innate immune system to HSV viral entry is key to influencing the outcome of HSV infection (Chew et al., 2009). This is done when the instinctive antibody response detects the virus as foreign from its cellular components by pathogen recognition receptors (PRRs). PRRs find pathogen-associated molecular patterns (PAMPs) as the cause of innate immune response and inflammation (Piret et al., 2015). However, natural killer (NK) cells and plasmacytoid dendritic cells (pDCs) contribute to innate immune response. The roles of natural killer cells in innate immunity are the production of cytokines, recognition and killing of infected cells while (pDCs) is responsible for IFN type I production (Chew et al., 2009).

Natural killer (NK) cells have an inhibitory or activating receptors which the ligand binds with to decide the effector function of the cell. These receptors (inhibitory) can find MHC class I protein on healthy cells and foil the activation of a natural killer cell. Downregulation of MHC is a means by which the virus escapes the immune system. ICP47, an immediate early protein is responsible for the downregulation of MHC class I by binding the cellular antigen transporter TAP-1 which prevents MHC molecules from reaching the cell surface (Chew et al., 2009).

pDCs are separate section of dendritic cells (DCs) that are useful in the effector role of type I interferons assembly. The type I IFN signalling pathway starts by recognizing viral protein or nucleic acids and relatively exact IRF-7 gene tasked with IFN amplification (Chew et al., 2009).The major cytokines produced in the early hours of HSV infection are IFN-α/β of type-1 IFNs (Melchjorsen et al., 2009). IFN-α and -β has a significant impact in the protection against HSV-1 infection by inhibiting the herpes simplex virus type 1 replication (Cunningham et al. 2006; Melchjorsen et al., 2009). However, the innate immunity against HSV infection is mediated through the toll-like receptors (TLRs). HSV can interact with either TLR-2 or receptor 9. Communication between TLR- 2 and the two herpetic virus is superficial where HSV-2 interaction with TLR-9 is within the endosomes of the viral DNA preferably pDCs which is actively stimulating IFN-a production (Cunningham et al., 2006).

Adaptive immune mechanism

Adaptive immune responses against pathogens eliminate the pathogen and any other toxic substances it is presented with. These responses are always destructive responses against invading pathogen which can either be through humoral mediated B cells or cell-mediated T cells (Mitchell et al. 2009). Recent reviews showed the influence of adaptive immune response on latency, disease progression, as well as control of HSV disease (Chew et al., 2009).

Humoral immunity against HSV

HSV infection marks the onset of antibody stimulation that neutralizes the antigen. The goal of the stimulated antibody is to combat and control the spread of the pathogen and this process is referred to as a humoral response. The interaction of the antibody with the virus leads to the cell inhibition from infection using one of these steps; virus-cell surface fusion, viral

penetration into the cell, and uncoating of the virus inside the cell. There is a close association between HSV recurrence and immunoglobulin concentration because the response to the infection has a long-term effect on the patient as evident in new infection cases, although, no exact influence has been stated about humoral immunity towards regulating HSV infection (Chew et al., 2009).

Cellular immunity against HSV

Cell-mediated immunity (CMI) is an essential host defence mechanism against viral infections. It was supposedly believed that CMI was mediated solely by T lymphocytes but recent studies have shown that it is facilitated by different cell types or factors. At the initial stage of HSV-2 infection, CD8+ T cells are conscripted to infiltrate the lesional cells, contributing greatly to immune control and cytolysis (Chew et al., 2009). Several glycoproteins gB, gC, and gD are considered immunodominant due to T cell specific responses in HSV-2 infections (Franzen-Röhl et al., 2011).

Glycoprotein B (gB) is an immunodominant epitope against which CD8+ T cells are produced. Nonetheless, there is phenotypic similarities between the specific and the nonspecific gB-CD8+ T in their membrane markers, cytokine production, and lysis. In as much as CD8+ T has an important role to play in cell-mediating antiviral activity against HSV, CD4+ T cells can also act in a similar manner in controlling HSV infection. Hence, CD4+ and CD8+ T cells can protect the local mucosa from duplicating HSV-2 (Chew et al., 2009).

1.4 Epidemiology of Herpes Simplex Virus

Herpes simplex viruses are pervasive pathogens that cause series of diseases in humans, affecting 60-95% of the adult population worldwide (Marchi et al., 2017). HSV-1 is highly transmissible and is a very common infection that affects people at a tender age. Most people do not know they are infected with the virus because the clinical episodes do not show any symptoms. HSV-1 can be a major source of disease problems like encephalitis Looker et al. (2015) but it is not so common**.**

Internationally, close to 500 million people have contracted HSV-2 with about 20 million incidence occurring every year while 3.7 billion people within 0-49 years lives with type 1 herpes worldwide with new infection cases of 118 million in 2012. Africa has the highest population followed by South-East Asia and Western pacific owing to their large population density. There is an appreciable fluctuation in HSV-2 prevalence within countries as HSV-1 increased with age in all population which was at a peak in Africa (87%) and least in the Americas (40–50%) (Daniels et al., 2016; Looker et al., 2015).

In comparison, type 2 herpes is exclusively transmitted through coitus and closely associated with genital herpes though, HSV type 1 can also spread through oral mediated sex to infect the genitals. This change in the course of herpes transmission is liable to 30% new cases of genital herpes in USA. As a result of the unavailable statistics to show genital herpes influenced by HSV-1, it is assumed to be low in developing countries (Paz-Bailey et al., 2006). Despite the difficulty in severing genital infections, it was globally evaluated in 2012 that HSV-1 genital infection among 15–49 years' ranges from 140 to 239 million. The global distribution of Herpes simplex virus type 1 is illustrated in table 1.1.

In the United States, the incidence of HSV-2 increased from 16.4% to 21.7% among people who are older than twelve years from 1976 to 1994 when compared with unindustrialized countries where the incidence rate of HSV-2 in adults is between 20% to 80% in women and 10-50% in men especially in sub-Saharan Africa (Looker et al., 2015; Paz-Bailey et al., 2006). HSV-2 prevalence differs from region to region in the population of a nation as shown in figure 1.5 and increased with age in all geographical area but insignificant in individuals who are sexually inactive. Someone infected with HSV-2 may not necessarily show visible manifestations of the disease but shed the virus occasionally from the genitalia. According to surveys conducted on HSV-2 in different parts of the world, 10.4%, 7.6%, 20.7% and 10.8% expectant mothers in Sweden, Italy, Switzerland and China are carriers of the virus (Smith et al., 2002; Bochner et al., 2013). The seroprevalence in sub-Saharan Africa is among the highest in the world, which is about 80% in men and women between 35 years (Smith et al., 2002). In Uganda, the prevalence varies between different age groups in gender with men aged 15–19 years and 20–24 year showing 10% and 27% prevalence while women showed 35% prevalence among 15–19-year-old and 74 % prevalence in women aged 20–24 years (Rajagopal et al., 2014).

Genital herpes causes serious illnesses like meningitis and neonatal herpes. More than eighty-five percent (85%) of neonatal herpes infections acquired during childbirth was due to contact of the foetus with either HSV-1 or HSV-2 shedding before delivery. Neonatal infection with HSV during pregnancy can cause series of distressing problems. As rare as this is, it results in increased illness and death with 60% death rate when the infection is untreated**.** Maternal IgG antibodies in the mother reduce the threat of neonatal herpes because the antibodies gained access through the placenta initiating a protective response (immunity) against the neonate. The risk of neonatal herpes infection increased significantly in new cases of infection where the mother is close to labour due to virus release from the genitalia because maternal IgG antibodies are not formed (Looker et al., 2017).

According to Rajagopal et al. (2014), studies conducted in South Africa at KwaZulu-Natal (KZN) province showed 84% incidence among female commercial sex workers while women aged 15-26 had 31% prevalence of HSV-2 but there is no documented study about the seroprevalence of HSV in Mafikeng. Most unindustrialized nations especially African nations have over half its population infected with HSV-2 with females having a greater tendency of acquiring the infection (Kukhanova et al., 2014).

Table 1.1: Regional distribution of Herpes Simplex Virus type 1 prevalence among male and female cohort below the age 50 in 2012 (Adopted from WHO, 2015).

Region	**Age group**	**Male prevalence**	**Female prevalence**	**Total**
America	0-49	142 million (44.4%)	178 million (55.6%)	320 million
Africa	0-49	355 million (50.3%)	350 million (49.7%)	705 million
Eastern Mediterranean	0-49	202 million (51.8%)	188 million (48.2%)	390 million
Europe	0-49	187 million (47.5%)	207 million (52.5%)	394 million
South-East Asia	0-49	458 million (51.5%)	432 million (48.5%)	890 million
Western Pacific	0-49	521million (51.6%)	488 million (48.4%)	1009 million
Total		**1865million**	**1843million**	**3708 million**

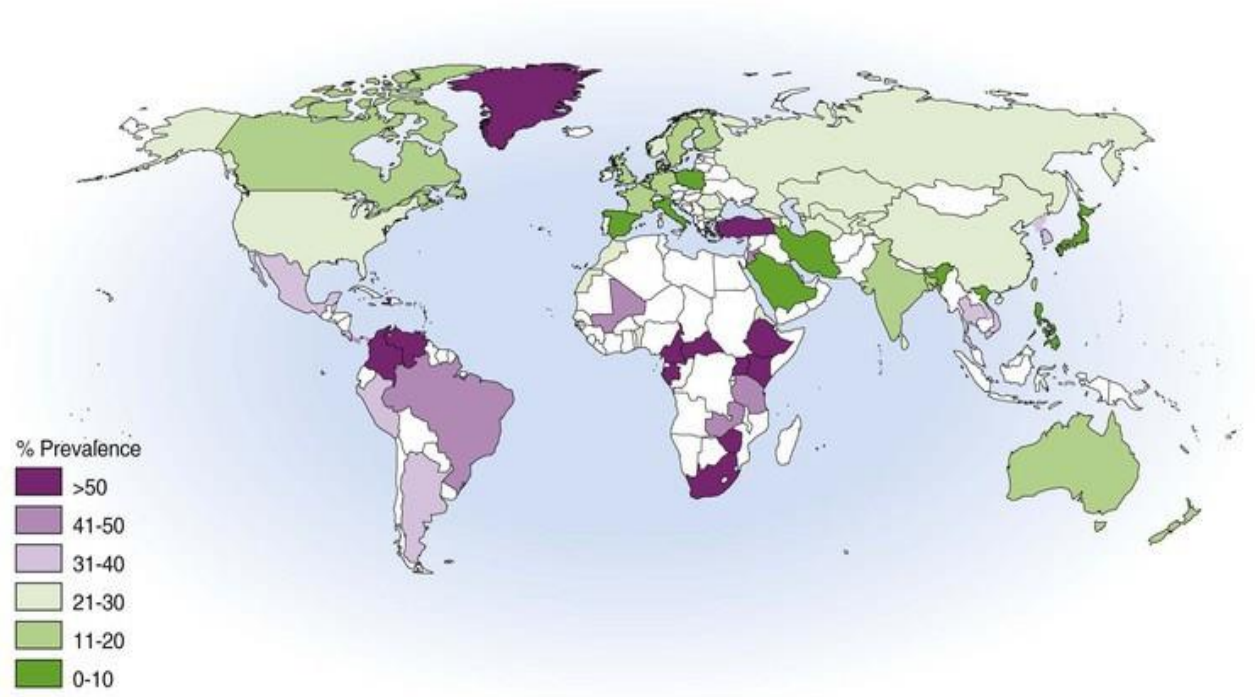

Figure 1.5: Global incidence of Herpes simplex virus type 2 (Joshua et al., 2017).

1.5 Transmission of Herpes Simplex Virus

The primary means of acquiring herpes simplex virus is through undeviating contact of the mucous or scuffed membrane with the mucosal fluids of a person who is a carrier of the virus (Fatahzadeh et al., 2007). Herpes simplex can be transmitted either by a symptomatic lesion or asymptomatic viral shedding through the respiratory droplets of an asymptomatic person. Transmission of herpes simplex virus is rampant in man than in woman because of the extent disease recurrence is greater in man. The asymptomatic mode of viral transmission makes it almost always under-diagnosed and increase disease transmission. This shedding (asymptomatic) in human is more easily detected in women from the cervix and vulva (Brugha et al., 1997). It is also commonly associated with infections that affect the anus and the genital region and is transmitted usually through genital fluids. Although HSV-2 can be transmitted via oral shedding of the virus and intimate nonsexual contact, the rate of transmission is somewhat minimal (Fatahzadeh et al., 2007).

1.6 Virus Infection and Diseases

1.6.1 Herpes Simplex Virus Infection

Herpes viruses are known for their ability to cause lytic and lysogenic infections with no exception to herpes simplex virus. The tendency of the virus to duplicate itself into several progeny virions within the host cell and cause lysis is termed lytic infection while lysogenic infection occur when the virus decides to enter quiescent state with little or no viral replication in the host cell which prevents the diseased cells from being damaged via immune response. More so, there is minimal expression of coded genes because of the little or no DNA copies existing in the diseased cell. Exposure of the host to factors like sunlight can cause the latent virus to reactivate and make copies of itself (Brown, 2017).

1.6.1.1 Lytic Infection

One principal function of the immune system is the ability to respond to foreign particles which can either be virus, bacteria etc. An immune response is stimulated in response to viral entry during lytic infection with the major role of clearing the virus from the host. More often than not, the virus is able to migrate from the route of entry during primary infection towards the sensory neurons in the trigeminal ganglion where it establishes latency. Reactivation of the virus will allow the virus to move from the neuron where it has been dormant to the initial area where it affects a second lytic infection. In HSV-1 infection, the virus attaches itself to the membrane receptors of the host. This attachment results in the deposition or transfer of the nucleocapsid into the host cytoplasmic cells. The nucleocapsid later migrates into the cell nucleus and berths at the nuclear pore where it injects its DNA into the nucleoplasm. The injected DNA when released from the capsid, enters into the nucleus where it is being replicated and synthesized to express mRNAs. Furthermore, viral assembly commences immediately adequate amount of DNA has been produced. Capsid is put together in the nucleus and packed with DNA which later exits the nucleus and obtain tegument and membrane layers. Mature progeny then exits the cell as the host cell is lysed (Brown, 2017).

1.6.1.2 Lysogenic Infection

Latency is the doggedness of viral genome within the host tissue where the infected viral particles, proteins or viral lytic transcript are not detectable but are rather dormant and have the

tendency of being reactivated (Thellman et al., 2017). Most latent infections are recognised because the mechanisms supporting the organised life cycle are not properly supported. In latent infection, infected cells are not easily detected by the immune system because the viral gene expression is relatively low (Thellman et al., 2017). This then makes the virus to survive immune responses enabling its recurrence in the spread of infection in an environment that is less inimical to antibody response (Brown, 2017). The infected tissue releases viral particles that enter the innervating sensory neuron axon, preserved in the neuronal nucleus and continues as DNA that is not incorporated into the genomes bearing heterochromatin marks at a lytic gene. During latency, viral transcription is blocked excluding non-coding RNA that has an important role in maintaining HSV latency after it is being spliced into stable intron (Thellman et al., 2017).

1.6.2 HSV Diseases

Herpes simplex virus often causes a persistent infection that can affect the skin, eyes, lips, mouth and genitals when it attacks a part of the body. It is known for its ability to attack and reproduce in the CNS and also undergo quiescent resulting in infrequent complications (Whitley et al., 1998). However, some of the common diseases caused by herpes simplex virus are herpes keratitis, neonatal herpes disease, a disease of the CNS among others.

1.6.2.1 Herpes Keratitis

Herpes keratitis is a disease of the cornea occurring when the mucous membranes of the host are introduced to infectious HSV particle. Immediately after the virus enters the host, it establishes latency in the sensory ganglia which can be stimulated via exogenous factors to enter its replicating or infectious cycle, through which the virus enters the cornea. It is accompanied by tearing, photophobia and edema of the eyelid. Recurrent infection of the cornea will damage the eye resulting in astigmatism, corneal scarring and in some cases blindness (Azher et al., 2017).

1.6.2.2 Neonatal Herpes

Neonatal herpes is not a very common disorder that presents itself daily but when it does, it is severe which can ultimately lead to death. It has a high morbidity and mortality ratio

in neonates despite progress in treatment and diagnosis. It was projected that out of every 3200 live births, one occurrence of neonatal herpes infection is observable and about 1500 cases of neonatal herpes was reported yearly in the United States (Mirchandani et al., 2017). Most neonatal herpes infection are transmitted at the delivery process from an infected mother to the neonate and can also be acquired postnatal via contact with a carrier. It is greatly spread in primary maternal infection, when the mother recently contracted herpes infection in the course of her pregnancy compared to mothers who had recurrent genital HSV infection. Neonatal herpes are manifested in various forms. It presents with fever, rash in the first few days of life (10–12 days), cause CNS infection in the second or third week of life or fulminant or disseminated infection involving multi-organ systems (Jones et al., 2014; Mirchandani et al., 2017).

1.6.2.3 Infection of the Central Nervous System (CNS)

Inflammation of the central nervous system due to HSV invasion is the basis of herpes simplex encephalitis. It is unique among HSV debilitating infections in the Western nations with a prevalence of 1-3 cases per million a year (Jaques et al., 2016). HSE affects both young and old. In some cases, it is a function of the host's immune response to the virus. Though the immune system may be strong enough to fight the virus and suppress it and the brain may be affected in the process. This type of infection is called post-infectious encephalitis. HSE has a remarkably poor outcome in spite of good antiviral therapy with a mortality of about 70% in untreated infection compared to 30% with adequate treatment (Jaques et al., 2016; Leib, 2012). Initially, it was a notion that HSV-1 was the sole cause of HSE while HSV-2 was responsible for aseptic meningitis but in recent times, HSV-2 has been the root cause of HSE and not meningitis (Jaques et al., 2016). Most appearances of HSE in affected person shows the part of the brain that is being affected as in the case of primary focal encephalitis linked with fever, altered consciousness and unusual behaviour (Whitley et al., 1998).

1.6.3 Laboratory Diagnosis of Herpes Simplex Virus

The diagnosis of viral infection in the laboratory is required for the demonstration of viruses in suspicious clinical samples or to estimate the infectivity of the virus, its risks of being transmitted sub-clinically and the patient's immune reactivity to the virus. The methods used

for detecting herpes simplex virus is classified into direct detection and indirect serological test. Direct tests used for HSV detection are summarized in Table 1.2.

Table 1.2: Direct detection methods of Herpes Simplex Virus Diagnosis

NUMBER	Direct methods	ROLE/SIGNIFICANCE
1	Viral isolation	Viruses need an active living host for replication which differs from bacteria that thrives on synthetic nutrient media. For decades, isolation of virus in cell cultures has been the basis of herpes simplex virus diagnosis. Most strains of HSV in infected cells when grown will replicate between 12-18 hours in cell lines and the cytopathic effect is observed within three days. Improper transportation of the samples from the site of collection to the laboratory can affect the result of the test. Although viral isolation seems cumbersome and takes a lot of time, it is advantageous in validating active infection in a sample and also helps with antiviral agents in treating the infection through susceptibility testing (Domeika et al., 2010; Ashley, 1993; Singh et al., 2005).
2	Cytology	This method of HSV diagnosis is less expensive and does not differentiate HSV-1 from HSV-2 as well as HSV from other herpesviruses. This test is helpful in emergency cases but it does not give a proper diagnosis of HSV because of the follow-up that is required when used as only 30-80% are sensitive for HSV from genital lesions (Ashley, 1993; Singh et al., 2005).
3	Electron microscopy	Electron microscopy (EM) is a new method of HSV diagnosis that requires the help of a well-trained staff. EM requires more copious viral particles before it can be easily identified which is why the method is less sensitive. Microscopic identification does not differentiate the virion from other herpes virions which is why the method was replaced with direct fluorescence antibody staining that can type HSV infection (Ashley, 1993; Singh et al., 2005).
4	Antigen detection	This direct detection technique makes use of antibody conjugated with fluorescent dye or enzymes to detect the presence of viral antigen in a clinical specimen. These methods are useful when improper sample handling or transportation conditions may inactivate the virus. DFA is a quick diagnostic test that allows the differentiation of genital herpes viruses into types. It is a highly sensitive test that can help a cell culture result (Domeika et al., 2010; Ashley, 1993; Singh et al., 2005).
5	Virus DNA detection	Polymerase chain reaction is a preferred method of choice in detecting viral DNA to hybridization technique because of its sensitivity and it requires less difficult procedures. The way antigen detection is made more sensitive by amplifying

		antigen through replication in cell culture, PCR can be used to amplify DNA in vitro. PCR can detect viral DNA for several days when there is no demonstrable infectious virus in the lesions, which makes viral culture less sensitive compared to nucleic acid amplification. However, the risk of false-positive result may arise but the initiation of real-time PCR has lessened the chance of a false-positive result because the reaction does not require any further amplification, although, it is relatively expensive, the use of a small reagent volume and minimal technical hands make it cost effective for most laboratories (Ashley, 1993; Singh et al., 2005).

1.6.3.1 Indirect Serological tests

This method of detecting herpes simplex virus when antibodies produced by an individual against HSV is detected using a known antigen that has the ability to stimulate HSV antibody. This is because antibodies are only produced in response to specific antigen stimulation. Indirect serologic tests can either be qualitative or quantitative. A qualitative indirect serological test determines the presence or absence of the antibody against HSV infection used mainly for screening purposes while quantitative indirect serological test is used to determine the amount or antibody titre of HSV present in the serum. Quantitative serological testing can be used to check the disease progression of HSV because high value titre indicates high infectivity of the patient which will help a clinician in administering drug to the patient. More so, identification of HSV antibody can be done using different kinds of test but none of them can differentiate HSV into types and no serological test has been able to distinguish oral HSV infection from genital infection. This is why serological assays that are not type-specific are inadequate because their clinical usefulness is limited (Singh et al., 2005). Some of the serological assay tests used in detecting herpes simplex virus are briefly explained in Table 1.3

Table 1.3: Indirect detection methods of Herpes Simplex Virus Diagnosis

NUMBER	Indirect techniques	ROLE/SIGNIFICANCE
1	Complement fixation (CF)	The technique is one of the out-dated test used in demonstrating HSV antibody in serum. This test can be used to detect HSV antibodies within the first two weeks after onset of infection but it is cumbersome and is not likely to be performed in the laboratory. The presence of anti-complement antibody in some sera will interfere with the test (Ashley, 1993).
2	Western blot ("immunoblot")	Immunoblot technique is a highly expensive, laborious and sensitive standard method used in the detection of HSV antibodies. The test can be used to categorize HSV into HSV type-1 and HSV type-2 with sera containing the antibody binding with blots from HSV infected cell lysates. The pattern of binding between the antibody and the infected cell shows a band that indicate the infection (Singh et al., 2005).
	Molecular methodologies	
3	Nucleic acid amplification	Nucleic acid amplification was introduced in late eighties to improve the sensitivity of viral detection. At present, some nucleic acid amplification technique can be used singly or in combination for post amplification analysis of viral particles for easy identification. Also, the problem of false negative result as seen in PCR, although preventable is due to inhibitors, poor extraction of viral template and incompetent amplification process while contamination is responsible for false positive result. Certain PCR machines that use real-time technique are able to reduce the turn-out time of result and possible contamination because the machine is able to accomplish the process from extraction of the samples through amplification of the viral template and quantification (Alshaikh et al., 2011; Malhotra et al., 2014).
4	Sequencing	Sequencing is well-thought-out to be a point of reference used in the discovery of known as well as indeterminate variants in the genomic DNA. It is the precise order of nucleotide detection in post amplification analysis which can be used to discover the entire genome of an amplified DNA. Several methods can be used in sequencing ranging from Sanger's method, de novo sequencing and next-generation sequencing technology (NGS). NGS have shown to be the most sensitive, scalable, flexible and highly efficient method of DNA

		sequencing since invention and they are used mainly in the research laboratories (Alshaikh et al., 2011; Bisht et al., 2014; Gasperskaja et al., 2017; Illumina, 2017).

1.6.4 Signs and Symptoms

Not everyone experiences visible signs and symptoms during herpes simplex virus outbreaks. Many a times, the body of an HSV infected patient sheds the virus without earlier signs and symptoms. This type of virus shedding is referred to as asymptomatic viral shedding. Although viral transmission during the asymptomatic phase is possible but its risk is low compared to when there is visible signs and symptoms. So, many people may show visible or clinical presentation of HSV but may be unaware of it because the signs sometimes seem so mild that it goes unnoticed. However, symptoms may appear four to five days after contact with the virus but the virus can be in some persons for months or even years before there will be any visible signs. Hence, appearance of symptoms does not necessarily mean that the infection has just been contracted as the case may be but it does not neglect the fact that proper treatment to relieve the pain caused should be pursued. Some of the signs and symptoms presented during the outbreaks of genital herpes infection are:

- The person feels unwell showing some symptoms of flu like headaches, tiredness, and pains in the back, thigh and groins.
- After the onset of flu-like symptoms, the patient shows prodromal signs like stinging, itching or tingling in the anal or genital area.
- Painful small fluid filled blisters are seen in the area affected which are painful when they burst leaving sores or ulcer in the area
- Dysuria is another sign caused by urine flowing over the sore during urination.

1.7 Factors Associated with HSV Infection

“Education, religion and socio-economic status are not associated with HSV-2. Although, certain study was conducted in Tanzania with a reported higher prevalence of HSV-2 among uneducated men”. These were the words of Mlaba, (2009).

There are several factors that influence herpes simplex virus infection. Recognising these factors will assist with health promotion programmes, with a focus on ways to primarily prevent the disease. The predominance of HSV infection is largely influenced by age, race, geographical region, socioeconomic status, sex, STD history, coitarche, lifetime numbers of sexual partners, and lack of condom use (Beydoun et al., 2010; Tideman et al., 2001). Sexual risk behaviour, that is, lack of condom use with someone infected before with genital herpes is a factor that influence HSV seropositivity. However, the use of condoms lessens the burden of HSV transmission (Woestenberg et al., 2016).

Age as an imperative factor in the transmission of STIs especially in adults who in one way engaged in sexual activity are predisposed to HSV seropositivity than adults who never engaged in such act. Studies conducted by Munjoma et al. (2010) presented that HSV-2 infected members as well as their spouses were considerably older than the HSV-2 uninfected members and their partners. This age difference is a key part in the epidemics of sexually transmitted diseases as older men are more exposed than the young women leading to unequal prevalence of STI between male and female with the female having the highest risks of contracting infection.

1.8 Human Immunodeficiency Virus (HIV)

1.8.1 Introduction

Over three decades, AIDS caused by HIV infection has been a challenge to public health globally (De Cock et al., 2011). HIV was discovered in the early 1980's and has infected and caused the death of millions of people around the globe because of the unavailability of active vaccine or a curative medication for the virus (De Cock et al. 2011; Kharsany et al., 2016). Those infected with the virus are at the risks of diseases and death emanating from nosocomial infections and neoplastic complications. However, HIV belongs to a family of lentivirus, a subgroup of retroviruses and it replicates through DNA intermediate (Klimas et al., 2008).

1.8.2 Epidemiology of HIV

According to Williams et al. (2017), one-fifth of the global population infected with HIV resides in South Africa. Epidemiology of HIV can be differentiated into two types globally, which can either be concentrated or generalized epidemic. In concentrated epidemic, the

transmission of HIV occurs in vulnerable people like men who sleep with men (MSM), drug addicts and commercial sex workers while generalized epidemics is the transmission of HIV that occur mainly through sexual activity in the population.

In 2015, the global estimate of HIV infected people were 36.7 million with 2.1 million different cases of HIV occurrence (Catie, 2015). HIV has contributed immensely to disease problems and is the principal cause of disability-adjusted life because most HIV infected persons have a greater chance of being affected with myocardial infarctions (MI) and also, HIV increase their chance of becoming co-infected with other viruses like hepatitis B which can cause liver disease (Maartens et al., 2014).

The widespread of HIV changed over three decades after its discovery in the early 1980s to as high as 3.7 million new infections in 1997 which declined throughout the 2000s. HIV incidence among young adults in sub-Saharan Africa in 2012 when compared, decreased to half its incidence in the year 2000 because of increased availability of antiretroviral drugs which has a profound impact on the incidence of HIV (Fettig et al., 2014). The development of new HIV infections within sub-Saharan African countries have declined greatly from 2.2 million in 2005 to 1.5 million in 2013 but it is still excessively high with newest cases of infection emanating from South Africa, Nigeria, Uganda, Mozambique and Kenya with a prevalence of 23%, 15%, 10%, 8% and 7% respectively (Kharsany et al., 2016). Also, reduced global HIV incidence in sub-Saharan Africa is as a result of a decline in heterosexual transmission, meanwhile, in western and central Europe and Americas where MSM is the route of disease transmission, the incidence is stable regardless of the access to antiretroviral therapy (Maartens et al., 2014).

Furthermore, 70·8% of the global prevalence of HIV originates from sub-Saharan Africa (Maartens et al., 2014). 12.2% of the South African population (6.4 million) were seropositive to HIV in 2012 with a vast variation in seropositivity within geographical locations and by province (Shisana et al., 2014). The prevalence rate of HIV in terms of the geographical region in South Africa is at its peak in KwaZulu-Natal and Mpumalanga and lowest in Northern and Western Cape (Department of Health, 2016).

The transmission of HIV in most geographical area of Africa is through unprotected sexual activity with a doubled risk of infection when an individual has many sex partners or when the

individual has been previously infected with sexually transmitted infections like herpes simplex type 2 (Fettig et al., 2014). This virus is responsible for the unyielding damage of the immune system and when the defence mechanism is been breached, the infection progresses into AIDS which has been an epidemic over the years resulting in the fatality of most of its victims (Maartens et al., 2014). The genetic variations in HIV-1 become clear due to the inconsistencies in the role played by the enzyme (reverse transcriptase) leading to a high rate of mutation while HIV-2, on the other hand, is restricted to West Africa and causing less transmissible illnesses comparable to that of HIV-1 (Maartens et al., 2014). Sexual interaction with an HIV-infected person is the sole cause of HIV infections acquired globally and more than 90% of the infections are acquired in sub-Saharan Africa. Women have a tendency of acquiring HIV through heterosexual contact with an infected person than their male counterpart in areas where the main route of HIV acquisition is heterosexual (Delpech et al., 2009).

1.8.3 HIV Transmission

The way HIV is transmitted is subjective to the load of the infectious viral particles in the body fluid and the degree of impact with the infected fluid (Levy, 1993). In Africa, most HIV infections are heterosexually transmitted as a result of contact with infected sexual partner meanwhile, MSM is one of the major means by which HIV is transmitted in Western Europe, North and South America (Delpech et al., 2009). The three main ways through which the infection is transmitted are through contact (blood or sexual) and mother to child transmission.

Sexual interaction (either heterosexual or MSM) with an HIV-infected person is accountable for most of HIV infections acquired globally with an estimate of over 90% of the infections in sub-Saharan Africa (Delpech et al. 2009). Generally, about 30% clinical specimen collected from the reproductive organ show virus-infected cells (Levy, 1993). The probability that women will contract HIV heterosexually is higher compared to men in most countries where heterosexuality is the mode of disease transmission. Homosexuality is another means by which HIV can be contracted as illustrated by Delpech et al. (2009) with a global estimate of about 3% and 20% of men have had sexual relationship at least once with their sex counterpart.

Behavioural factors such as having too many sexual mates, low contraceptive usage, sharing sharp injecting objects during intravenous drug use are factors responsible for HIV

transmission. Most HIV-infected men contract HIV through vulnerable anal intercourse with their male counterpart and are at a high risk of HIV transmission (Levy, 1993). The use of injecting drug materials that have been contaminated with HIV is responsible for 1 in 10 of HIV infections worldwide which is similar in proportion to HIV infections attributable to MSM. The influence of injecting drug use to HIV epidemics varies greatly from region to region and accounts for about 50-90% of HIV infection in Asia and Eastern Europe (Delpech et al., 2009). Hence, the risk of HIV transmission is lessened when the use of the same sharp objects is avoided and a sterile clean syringe is used by drug addicts.

1.8.4 HIV Pathogenesis

HIV pathogenesis and its disease development is dependent on the infecting viral particle and the host's immune system. Interaction between these two, the host's immune system and the virus will decide the effect of the infection. HIV cannot survive outside the body; it needs the blood to thrive which is why it is easily incapacitated when exposed to detergents or disinfectants. Hence, the spread of HIV is necessitated by exposition to infected blood, secretions or body fluids (Fanales-Belasio et al., 2010). Also, HIV pathogenesis targets CD4 T lymphocytes causing the depletion of CD4+ T-cell pool which eventually results into immunodeficiency (Lane, 2009; Maartens et al., 2014).

As the infectious agent gains access through the host body defenses, the infectious particle attaches itself to the required CD4 cells, the spikes of gp 120 fuses with the CD4 receptors and enable binding to the virus-infected cell. The envelope surrounding the virus is shed in the infected cell and its contents are freely dispersed into the cell's cytoplasm especially, the viral RNA and the enzyme. The likelihood of HIV infection is reliant on the load of the viral particles in the body fluids with which the host is exposed to or the quantity of CD4 receptor cells present at the point of entry (Sundquist et al., 2012).

1.8.5 Structure and Replication of HIV

HIV is of the family Retroviridae and Genus Lentivirus. It is grouped into two types which are HIV type 1 and HIV type 2. HIV-1 is a global agent for AIDS while HIV-2 is the causative agent for AIDS in Western and Central Africa. However, it does not mean that either of the causative agent for AIDS cannot be found in any part of the world but the prevalence are

somewhat different from region to region. The genome of the retrovirus is made up of two indistinguishable copies of RNA molecules containing structural genes (gag, pol and env). The virus types differ from each other in terms of their organisational genomic structure with both types inflicting an infection of the central nervous system but it is a lot common with an HIV-2 infection. Also, HIV-2 is less infectious in comparison with the disease development of HIV-1 to AIDS (Romani et al., 2010).

HIV is about 100-120nm in diameter and differs from other retroviruses in terms of its unevenly spherical structure. It comprises of an envelope that contains lipid bilayer. Although, the viral structure of HIV-1 is similar to that of HIV-2, the core of the viral particle is encircled by a macromolecule filled matrix containing glycoproteins and major surface antigens. The genes encoding structural proteins is the "gag" gene as seen in figure 1.6 whereas "pol" gene encodes for viral replication enzymes that reverse transcribe converting RNA into DNA and integrase that inserts the DNA copy of the virus into the DNA of the host as "env' codes for envelope glycoprotein. Also, HIV contains six accessory proteins that monitor the infectivity of the virus, the replication of new more virions and its disease progression (Fanales-Belasio et al., 2010). Nef (negative regulatory factor) inhibits the diseased cell from making quite a few proteins essential in cell defence (Romani et al., 2010).

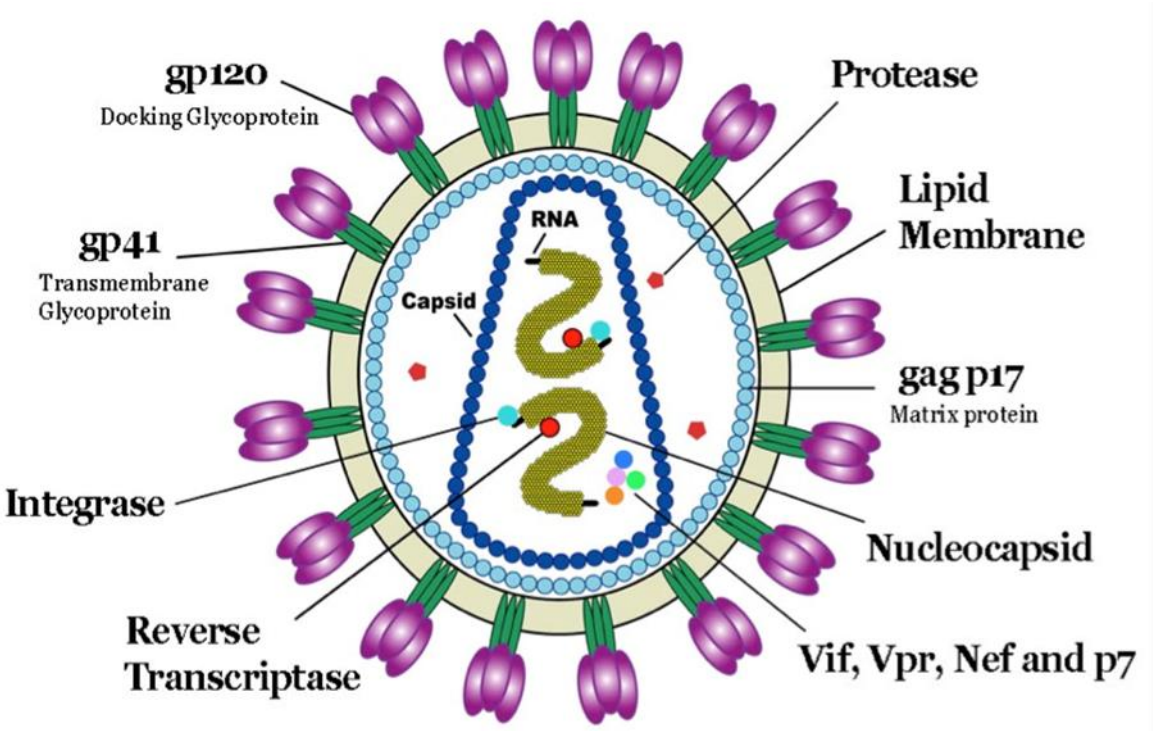

Figure 1.6: Viral structure of HIV capsid (Areetha, 2015).

1.8.6 HIV Replication Cycle

Viruses are obligate intracellular parasites that make a replica of itself only in an active host. Strategies and ideas can be developed to improve antiretroviral therapy against HIV if we know how the virus relates or interacts with its target cells for reproduction (Kirchhoff, 2013). There are six basic steps that HIV undergo during viral replication, namely; Binding and Fusion, Uncoating and Nuclear Entry, Reverse transcription, Integration, Assembly and Budding. HIV cycle begins when it enters the host cell through three major events: it binds with CD4+ T lymphocyte co-receptors, becomes activated then fuses with the host cell (Fanales-Belasio et al., 2010).

The process through which HIV enters its host is complex and requires several steps that either bind immediately or circuitously to its target. Interactions between glycoprotein and cellular receptors to induce variations in the env trimers is responsible for HIV infectiousness. CCR5 found during chronic infection is almost always sexually transmitted while CXCR4 is seen during the late stage or the progression of HIV to AIDS. Glycoprotein gp120 interacts by binding both with CD4 and a chemokine receptor at the same time that is responsible for a firm and protracted attachment of the virus and allows gp41 to enter the membrane of the cell

(Fanales-Belasio et al., 2010). Gp41 has a hydrophobic peptide which it uses to attach the membrane and cause the possible contact between the virus and its target. This interaction is responsible for the trimeric complex which brings into close proximity, the cellular and viral membrane thus allowing the virion to attach and deposit its contents into the host (Kirchhoff, 2013).

Uncoating is the process whereby the viral capsid is in disarray before ingress of the viral genome into the nucleus which sometimes can cause defective viral infectivity if the viral capsid are uncoated early. However, uncoating is closely linked with reverse transcription which is accompanied by the change of the reverse transcription complex (RTCs) to the pre-integration complex (PIC) capable of being incorporated into gene cells. Initially, vpr and integrase were used actively in transporting HIV-1 pre-integration complex through the nuclear pore because of its large size with capsid protein playing a major role in the process (Kirchhoff, 2013).

A process where single stranded RNA (ssRNA) is used to make copies of dsDNA with the help of reverse transcriptase enzyme is called reverse transcription. However, reverse transcription ensue after the viral particle attaches itself with the host cells and release its genetic material into the cells. The effective completion of linear dsDNA and its passage through membrane barriers cause the incorporation of the genomic particle of HIV into the cell host where they are being expressed. Integrase is used to incorporate this particle which makes the cell to be infected throughout its life span causing the provirus to be replicated with the host cell's DNA. Eradicating the virus becomes difficult because the provirus may remain inactive or latent for a long period of time which makes it difficult for the immune system to recognise them but when the provirus is activated, it uses the host enzyme, RNA polymerase to create short strands of messenger RNA (mRNA) which is used in making long HIV proteins and also make copies of the HIV genetic material that can destroy the immune system .The arrangement of HIV is an intricate and well organised method that make use of HIV enzyme called protease. Protease slices the extended protein chains into lesser individual proteins amassed with copies of HIV's RNA to form a new infectious particle. The new viral particle formed buds to release the progeny virion from the infected cell (host cell). However, the new virus still makes use of the outer membrane (glycoprotein) of its host cell for protection which it used to bind with the CD4 and co-receptors allowing its passage to other new cells (Kirchhoff, 2013).

1.9 HSV/ HIV Coinfection

Herpes simplex virus type 2 infection is the leading cause of ulcer disease in the genitals, it affects the acquisition and the course of HIV-1 in some ways (Aebi-Popp et al., 2016; Sheth et al., 2008). It is not only a factor in the HIV acquisition but it also fuels its transmission and disease deterioration. HSV-2/ HIV-1 coinfection increases HIV viral load compared with someone who is seronegative for HSV-2. This is because people infected with genital herpes are disposed to HIV-1 as a result of the genital micro-ulcerations presented during the infection, serving as a port of entry required for HIV transmission. Also, there is increase in the number of CD4+ lymphocytes seen in genital lesions which are target cells for HIV (Sheth et al., 2008). During latency, HSV-2 is capable of evading immune system destruction which can be reactivated after being triggered by external factors to produce longer and recurrent infection in HIV coinfection (Aebi-Popp et al., 2016).

Most of the sexual HSV infection are asymptomatically transmitted because the infected persons are not aware of the virus shedding (Jacob et al., 2015). Herpes is reactivated regularly in someone infected with HIV-1 as HIV-1 is seen in herpes lesions but often in larger quantity responsible for easy transmission to vulnerable individuals (Gupta et al., 2007). HSV-2 can upsurge the chances of HIV acquisition by two to threefold and about six fold for female prostitutes (Jacob et al., 2015; Sheth et al., 2008).

In sub-Saharan Africa, HIV is epidemic with more than 60% of the population infected with genital herpes caused by HSV-2 with an estimated half of HIV infection attributed to HSV-2 infection (Sheth et al., 2008). According to Celum et al. (2004), research conducted in Carletonville South Africa between the age group 14-24 in the mining district showed that there is a robust relationship between HSV-2 and HIV because the seroprevalence of HSV-2 is almost two times as high as that of HIV. Nine percent (9%) of men and 34% of the women were seropositive for HIV while 17% of the men and 53% of the women were seropositive for HSV-2. The coinfection of HSV-2 with HIV is greater both in men and women with the prevalence of 65% and 91% respectively Celum et al. (2004), with a similar greater rates of coinfection as identified by Jacob et al. (2015) in other countries like UK (55%) and Uganda (86%).

1.9.1 Epidemiological Relationship of HSV and HIV

It is not certain if it is HSV that is first transmitted or HIV but each is responsible for the disease deterioration of the other. The epidemiological interaction between HIV and HSV has become encumbrance because genital herpes has a three-fold tendency of converting a seronegative person to seropositive per sexual début and a fourfold tendency of transmitting HIV (Bradley et al., 2013). It is also anticipated that people with HSV/HIV coinfection have a greater tendency of transmitting the disease to others (Looker et al. 2015). However, contrasting opinion from the report of the research conducted in Tehran, Iran showed that HSV-2 coinfection with HIV has no role in quickening HIV infection (Mohraz et al., 2017).

1.10 Treatment of HSV

At present, there has been no licensed vaccine for Herpes simplex virus infections and most experimental vaccines for HSV-1 and HSV-2 are in various stages of their clinical trial (Dropulic et al., 2012). Observing the established hygiene practises, avoiding direct contact with mucocutaneous lesions, use of hand gloves by physicians, nurses and laboratory personnel when handling infected tissues and fluids, avoidance of oral sex and the use of condoms during sexual intercourse are some of the ways to prevent the spread of HSV infections.

Management of herpes simplex virus varies with the course of infection as well as the site of infection which are used in prescribing the dosage and frequency of medication. When there is a new clinical presentation of signs and symptoms of genital herpes accompanied by prolonged disease course, therapy for ten days is usually recommended. Counselling, as well as methods that will help reduce disease transmissions such as condom usage, abstinence and antiviral therapy, are engaged in managing the disease. Antiviral drugs, though not able to eliminate the virus, are the choice of drugs needed in the management of HSV and other natural remedies that have been proposed to help in treating the disease most of which are not well substantiated. Antiviral to some extent control the clinical presentations of HSV and reduce the frequent episodes or outbreak of the disease (Strick et al., 2006).

Recurrent herpes simplex virus infection needs two major treatments which are either episodic (treatment at the first sign or symptom of an outbreak) or suppressive treatment. Suppressive

treatment is a daily therapy given to an infected person to minimize asymptomatic viral shedding and to some extent reduce transmission (Brady et al., 2004). Oral antiviral drugs like valaciclovir are potent in managing recurrence of genital herpes infection by 1-2 days when administered at the onset of signs. When the regimen is administered as prescribed by the clinician, it is effective in for episodic treatment of HSV.

However, suppressive therapy is a treatment of choice for genital infection that has more than six (6) recurrent episodes a year. Most patients on suppressive antiviral therapy may show a greater tendency of lessened frequency of recurrence. Just like episodic treatment, acyclovir, famciclovir and valaciclovir can be used as suppressive antiviral regimen but at a different dosage and for a longer duration. Aciclovir can be administered either twice or four times depending on the drug dosage as prescribed by the clinician. Valaciclovir show the same efficacy with acyclovir when administered twice daily (250 mg), also, 500 mg of valaciclovir will be efficient in the treatment of recurrent disease. There are exceptional cases where the normal first episode treatment of HSV will not be effective like that of co-infection with HIV. In this case, the standard antiviral dosage will have to be doubled and in a situation where the lesions persist, a higher dose of antiviral would be prescribed (Patel et al., 2011).

Natural remedies are some of the ways by which herpes simplex virus infection can be treated. Certain dietary intakes most especially carbohydrate should be discouraged for an infected person because it has been implicated with HSV recurrences. Although the relationship between the two has not been fully substantiated but there are certain cases where sugar intake worsened herpes simplex infection. Also, increase in lysine intake or increase lysine to arginine ratio by an infected person will decrease the frequency of recurrence because lysine antagonizes the growth promoting tendencies of arginine which would have a great impact in the treatment of herpes simplex infection (Gaby, 2006).

CHAPTER 2

2.0 MATERIALS AND METHODS

2.1 Research Design

This is a retrospective laboratory study on patient samples donated by the hospitals selected in the North-West and KwaZulu-Natal provinces for the study. The study was objectively designed by the researcher to examine the molecular prevalence of herpes simplex virus and their interaction with Human immunodeficiency virus, and to statistically analyse the data outcome of the research.

2.2 Ethical Clearance

Ethical approval was obtained from the North-West University Research Ethics Committee (NWU-00068-15-A9) to conduct the study as seen in Appendix F and G.

2.3 Sample Collection

A total of forty-four stored sera were used in this study. Samples were randomly donated by laboratories from North-West Province (20) and twenty-four (24) from KwaZulu-Natal Province. The samples were anonymized as Gh1, Gh2 and Kz1, Kz2 etc., to conceal the identity of the patients in accordance to Good Laboratory Practice (GLP) and Biomedical Research Code of conduct. The samples were kept at -80 ^{0}C until further use.

2.4 Methods

2.4.1 Screening for HSV Using Enzyme Immunoassay Kits (ELISA)

Samples to be analysed were properly homogenized and diluted in a ratio 1:101 (10 µL of serum into 1 mL of diluent) and a concentration of 1 X washing buffer from 10 X wash buffer was prepared by adding 100 mL of the stock with 900 mL distilled water before the test. To every test conducted, a requisite of one negative control, 2 cut-off and one positive control were performed alongside with the test in this manner; 100 µL of diluted samples, 100 µL blank (cut-off) and 100 µL of positive and negative controls were transferred to each well of the test kits, covered with adhesive film, incubated at 37 °C for 45 min and washed with 300

µL of already prepared washing buffer four times. After which, 100 µL of conjugate was added into each well incubated at 37 °C for 45 min and washed four times. The test strips were left to be incubated at room temperature for 15 minutes after adding 100 µL of Substrate into each wells and the reaction was stopped with 100 µL of Stop Solution before it was read in a microplate reader at 450 nm within 30 minutes.

2.4.2 Screening for HIV Using ELISA

Elisa technique used to screen for HIV from the samples was performed as follows: 25 µL of the conjugate was applied into each of the wells and 75 µL of HIV Ag positive control, HIV Ab positive control, negative control in duplicates, and specimen were subsequently added as labelled on the microplates and mixed homogenously. The microplates were then covered with adhesive film, preventing reaction mixture from being contaminated and incubated at 37 °C for 60 min. After incubation, the film was removed and the solution was aspirated and washed using 370 µL of already prepared washing solution (40 ml of 20 X concentrate + 760 ml distilled water) three times. 100 µL of conjugate 2 working solution (Appendix C) were then dispensed into the wells, sealed with an adhesive film and incubated at room temperature for 30 min. The film was removed after incubation and the wells washed with 370 µL washing solution five times before 80 µL of freshly prepared substrate solution was added into each wells and left at room temperature in the dark for 30 min. 100 µL of stopping solution was immediately added to stop the reaction and read at an optical density of 450/620 nm with a microplate reader.

2.5 Analysis using Molecular Techniques

2.5.1 DNA extraction using QIAamp® MinElute® Virus Spin kit:

Deoxyribonucleic acid extraction from serum was done with QIAamp® MinElute® Virus Spin kit following the instructions of the manufacturer. The procedure can be summarized as follows: buffer AW1, AW2 and QIAGEN protease was prepared before the extraction began as illustrated in the Appendix D and E. 20 µl of already prepared QIAGEN protease was pipetted into a sterile 1.5 ml micro-centrifuge tube after which 200 µl of the sample was added as well as 200 µl of buffer AL (containing 28 µg/ml of carrier RNA). The reaction mixture was pulse-vortexed for fifteen seconds to ensure efficient lysis and immediately incubated at 56 ºC for 10 minutes. It was briefly spun in a centrifuge to remove the drops from the lids. 200 µl of

96 % ethanol was dispensed into the solution, mixed thoroughly by pulse-voltexing for few seconds and spun to remove drops from the lid. Transfer the mixture into QIAamp Mini spin column and centrifuge at 6000 g, 8000 rpm for one minute. When this was done, the filtrate was discarded and the QIAamp Mini spin column filled with 500 µl buffer AW1 was placed in a new and clean collection tube, centrifuged at 6000 g, 8000 rpm for one minute. The collection tube containing the filtrate was discarded as well and replaced with a new one after which the QIAamp Mini spin column was filled with 500 µl AW2 buffer centrifuged at 20000 g, 14000 rpm for three minutes. The filtrate was discarded just like the previous times and replaced with 1.5 micro-centrifuge tube, 200 µl of buffer AE was added to the QIAamp Mini spin column incubated at room temperature for five minute and spun at 6000 g, 8000 rpm for one minute.

2.5.2 RNA extraction using QIAamp® Viral RNA Mini kit (50):

Viral nucleic acid extraction of RNA from serum was done using QIAamp® Viral RNA Spin kit according to the manufacturer's instructions. 560 µl of buffer AVL containing carrier RNA was pipetted into 1.5 ml microcentrifuge tube mixed with 140 µl of serum by pulse-vortexing for fifteen seconds which was immediately incubated at room temperature for ten minutes. The tube was spun to remove fluids from the lid and 560 µl of 96% ethanol was added to the tube, mixed for few seconds by pulse-vortexing and spun homogenously to remove drops of fluid from the base of the tube lid. 630 µl of the solution was transferred into the QIAamp Mini spin column and centrifuged at 6000 g, 8000 rpm for one minute. The filtrate was discarded and the collection tube replaced. The step of transferring 630 µl of the solution was repeated until there was no more solution left. After, 500 µl of buffer AW1 was added to the column spun at 6000 g, 8000 rpm for one minute and the filtrate discarded. 500 µl of buffer AW2 was added into the column and centrifuged at a speed of 20 000 g, 14 000 rpm for three minutes. After this was done, the column was put in a clean 1.5 ml microcentrifuge tube and filled with 60 µl of buffer AVE equilibrated at room temperature for one minute and centrifuged at 6000 g, 8000 rpm for one minute. The extracted material was measured using nanodrop to determine the viral concentration and the purity ($A_{260/280}$) of the nucleic acid.

2.6 Control materials

The control materials used for this study were extracted Vircell Amplirun Herpes Simplex DNA control for both HSV-1 and HSV-2 bought from Davies diagnostic Ltd. The control

materials were reconstituted using the Vircell control reconstitution solution to a volume of 100 µl, shook to homogenously dissolve and stored at -20 ºC until further use.

2.7 HSV Amplification using Polymerase Chain Reaction Technique

2.7.1 HSV-1/2 Primers

The following primer pairs (Table 2.1) were used in the course of this research for HSV and HIV amplification. The target region for HIV is the integrase while the primers were used to target glycoprotein (gB) and glycoprotein (gG) for HSV-1 and HSV-2 respectively.

Table 2.1: The primer sequences used in conducting this research, the target regions and the fragment sizes.

PRIMER PAIR	NUCLEOTIDE SEQUENCE	FRAGMENT SIZE	REGION	REFERENCES
HSV-1 Outer pair forward ***(S4255)***	TGC TGG AGG ATC ACG AGT TTG	663bp	gB	Victória et al. (2005)
Outer pair reverse ***(S41AA)***	CAT CGT CTT TGT TGG GAA CTT			
HSV-1 Inner pair forward ***(S421AB)***	TGCAGAGCAACCCCA TGAAG	241bp	gB	Victória et al. (2005)
Inner pair reverse ***(S41AC)***	ATGACCATGTCGGTG ACCTTGG			

HSV-2 Outer pair forward ***(S30C1)***	TCAGCCCATCCTCCTTCGGCAGTA	184bp	gG	Schmutzhard et al. (2004)
Outer pair reverse ***(S30C2)***	GATCTGGTACTCGAATGTCTCCG			
HSV-2 Inner pair forward ***(S30C3)***	AGACGTGCGGGTCGTACACG	100bp	gG	Schmutzhard et al. (2004)
Inner pair reverse ***(S30C4)***	CGCGCGGTCCCAGATCGGCA			
HIV-1 Outer pair forward	ACAGTGCAGGGGAAAGAA	140bp	Integrase	Nie et al. (2011)
Outer pair reverse	CCCTTCACCTTTCCAGAG			

2.7.2 PCR amplification for the detection of HSV-1

Several researchers have carried out series of test on HSV using PCR technique but what differs from each reaction method is the temperature at which the primer anneals to the viral template (DNA) which is determined by (G+C) percentage composition present in the primer and the number of cycles. However, detection of HSV-1 glycoprotein B (gB) gene was carried out in a 25µl reaction mixture as seen in Table 2.2 below for both first and nested rounds.

Table 2.2: PCR reaction mixture and conditions for HSV-1 amplification run

Protocol		**1X Reaction (** µl)	
2 X Master mix with standard buffer		12.5	
10 µM Forward primer		0.5	
10 µM Reverse primer		0.5	
DNA Template		5	
Nuclease free water		6.5	
Total volume		25	
PCR CONDITIONS		FIRST ROUND	NESTED
Initial denaturation	94 ºC	30 seconds	30 seconds
	94 ºC	30 seconds	30 seconds
Annealing	55 ºC	60 seconds	61.4 ºC 60 seconds
Extension	68 ºC	60seconds	60 seconds
Final Extension	68 ºC	5 minutes	5 minutes
Hold	4 ºC	∞	∞

2.7.3 PCR amplification for the detection of HSV-2

PCR for HSV-2 glycoprotein G (gG) gene detection were carried out according to Schmutzhard et al. (2004) protocol with slight modification for optimisation. In a 25 µl reaction mixture, two successive PCR reactions (First and Nested) containing 5 µl of DNA template and 5 µl of PCR product respectively were used in amplifying HSV-2 as seen in Table 2.3. HSV-2 primers used are listed in table 2.1.

Table 2.3: Reaction mixture and conditions for First and Nested PCR amplification of HSV-2

Protocol	**1X Reaction (** µl)
2 X Master mix with standard buffer	12.5
10 µM Forward primer	0.5
10 µM Reverse primer	0.5
DNA Template	5
Nuclease free water	6.5
Total volume	25

PCR CONDITIONS		
Initial denaturation	94 ºC	30 seconds
	94 ºC	30 seconds
Annealing	55 ºC	60 seconds
Extension	68 ºC	60seconds
Final Extension	68 ºC	5 minutes
Hold	4 ºC	∞

2.7.4 PCR Amplification for HIV-1 detection

2.7.4.1 First strand cDNA synthesis

The extracted RNA materials were cooled down to room temperature and centrifuged to gather the samples to the base of the tubes before reverse transcribing the RNA template into cDNA with a superscript® Vilo™ cDNA synthesis kit. Preparation of the sample for cDNA was carried out using the manufacturer's procedure: a master mix of 4 µl 5X Vilo™ reaction mix, 2 µl 10X superscript® enzyme mix and 9 µl DEPC- treated water was prepared on ice in a 0.2 ml PCR tube for a single reaction before 5 µl of the RNA template was added. The tube was gently mixed, spun to collect the content into the bottom of the tube and incubated at 25 ºC for 10 minutes. The tube was transferred into the thermal cycler CFX96 (Bio-rad, Raymond Poincare,Marnes-la-Coquette, France) for further incubation at 42 ºC for 60 minutes and 85 ºC for 5 minutes. The cDNA was later amplified using the reaction mixture and conditions in table 2.4.

Table 2.4: Reaction mixture and conditions for one step PCR amplification of HIV-1

Protocol	**1X Reaction (** µl)
2 X Master mix with standard buffer	12.5
10 µM Forward primer	0.5
10 µM Reverse primer	0.5
MgCl2	1
cDNA Template	2
Nuclease free water	8.5
Total volume	25
PCR CONDITIONS	
Initial denaturation 94 ºC	30 seconds
94 ºC	30 seconds

Annealing	50 ºC	60 seconds
Extension	68 ºC	60seconds
Final Extension	68 ºC	5 minutes
Hold	4 ºC	∞

2.7.5 Precautions taken against contamination

Mistakes or errors caused by sample contamination are what a researcher should prevent while working in the laboratory as this may lead to either a false negative or a false positive result. No matter how trivial an error might be, it impacts a great havoc on the result making it irreproducible. However, some of the basic measures put in place for a better reproducible results are stated as follows:

- PCR reaction mixes was prepared in a clean room, a room free of DNA under a ultra-violet irradiation air hood.
- The prepared PCR reaction mixes were transferred to the DNA room where the templates were added under an air hood cabinet which has been properly sterilized.
- Transfer of materials from one room to another was avoided as each room was provided with its own laboratory materials like lab coats, micropipette, pipette tips of various sizes, sample holders etc.
- Reagents used were kept at optimal temperature as stipulated by the manufacturer in a DNA free room.
- Polymerase chain reaction (PCR) products were stored in the freezer at -20ºC. All reagents used for preparing PCR reaction mix were kept in the DNA free room
- All samples used were properly stored at -80ºC and was not thawed more than three times.
- The PCR tubes used were sterile DNA free tubes which was not contaminated in any form.

2.8 Analysis of PCR products:

2.8.1 Preparation of 2% agarose gel electrophoresis stained with ethidium bromide (EtBr)

To prepare a 100 ml 2% agarose, 2 g agarose powder was weighed on a weighing balance and poured into a 250 ml sterile graduated bottle before 100 ml of sterile distilled water was added to dissolve it. The solution was heated in a microwave for 30 seconds after which the bottle was swirled to allow for easy dissolution and reheated for another 60 seconds and allowed to cool. After it has cooled to a temperature of 50 ºC, 0.5 µl of ethidium bromide was added, mixed homogenously and allowed to settle to make sure there were no bubbles before pouring it into the electrophoresis casting tray. Comb was inserted and the tray left undisturbed for 30mins until the gel appears to have fully solidified.

To run the agarose gel, the gel was transferred into an electrophoresis tank filled with 0.5 X TBE used in preparing the gel and the comb was gently removed. 5 µl of the PCR product was added to 1 µl of 6 X loading buffer in a sterile 0.5 ml Eppendorf, mixed and loaded into the wells of 2% agarose stained with ethidium bromide (EtBr). The same protocol used for PCR product was used for the DNA ladder/marker and loaded into the first well on 2% agarose gel. The tank was then covered with the lid and connected to the power supply at a voltage of 50 V (6 V/cm), 412 Amp and run for 60 minutes. It was later disconnected from the power supply after DNA has migrated to about three-quarter of the gel and the gel placed on a sterile glass sheet. The expected DNA bands were visualised using ultra-violet light transilluminator at a wavelength of 320 nm and the image captured through AlphaImager™ 2200 system.

2.8.2 Sequencing

Four of the PCR positive co-infected products from nested PCR with high titre identified with ELISA were sent out to Inqaba Laboratory for Sanger and Next Generation Sequencing.

2.9 Data Analysis

The data obtained from this study were subjected to basic descriptive and statistical analyses. Statistical analyses were performed using the results obtained from the laboratory. The samples were analysed using Statistical Package for Social Science (SPSS) version 25. SPSS software

was used to perform Chi-Square and correlation test. Pearson Chi-Square was used to determine the significant association between HSV-1, HSV-2 and HIV-1. However, Pearson correlation test was used to determine the relationship between age, HSV-1, HSV-2 and HIV-1. KBase tool was used for the mapping of the sequenced data with the reference genome and Basic variant tool version 2.0 was used to call for variants.

CHAPTER 3

3.0 RESULTS

3.1 Overview of results

The summary of the results obtained from this study is schematically represented in Figure 3.1 below.

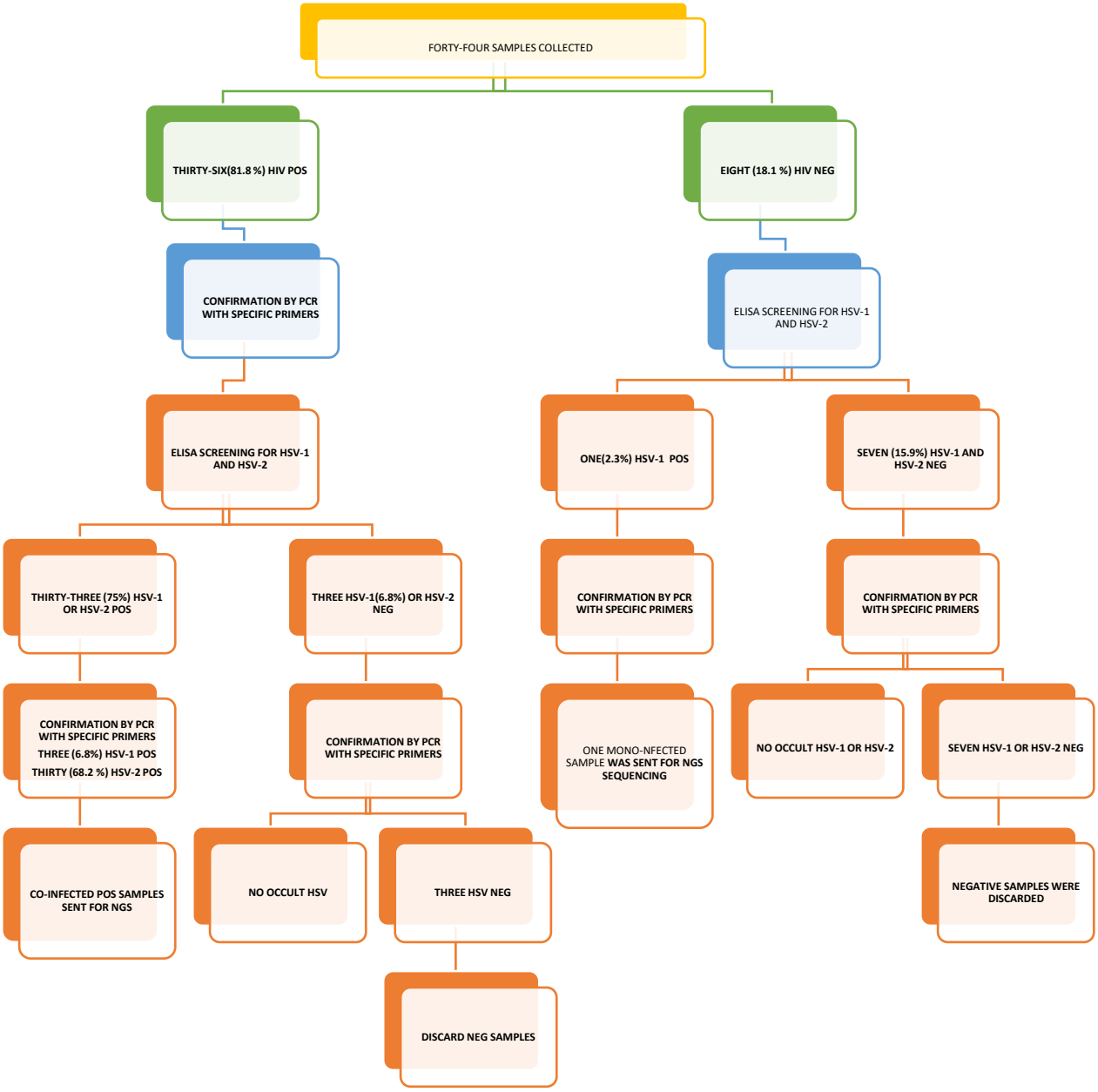

Figure 3.1: Schematic diagram of results obtained from each procedural methods.

3.2 Demographics

3.2.1 Gender

The study included serum samples from forty-four individuals with a standard. Majority of the samples were from female participants (79.5%) and the male population in this study was 20.5% as shown in figure 3.2.

3.2.2 Age

The participants in this study cohort falls within the age range of 11-60 years and they have a mean age of 33.09 years as depicted in figure 3.2.

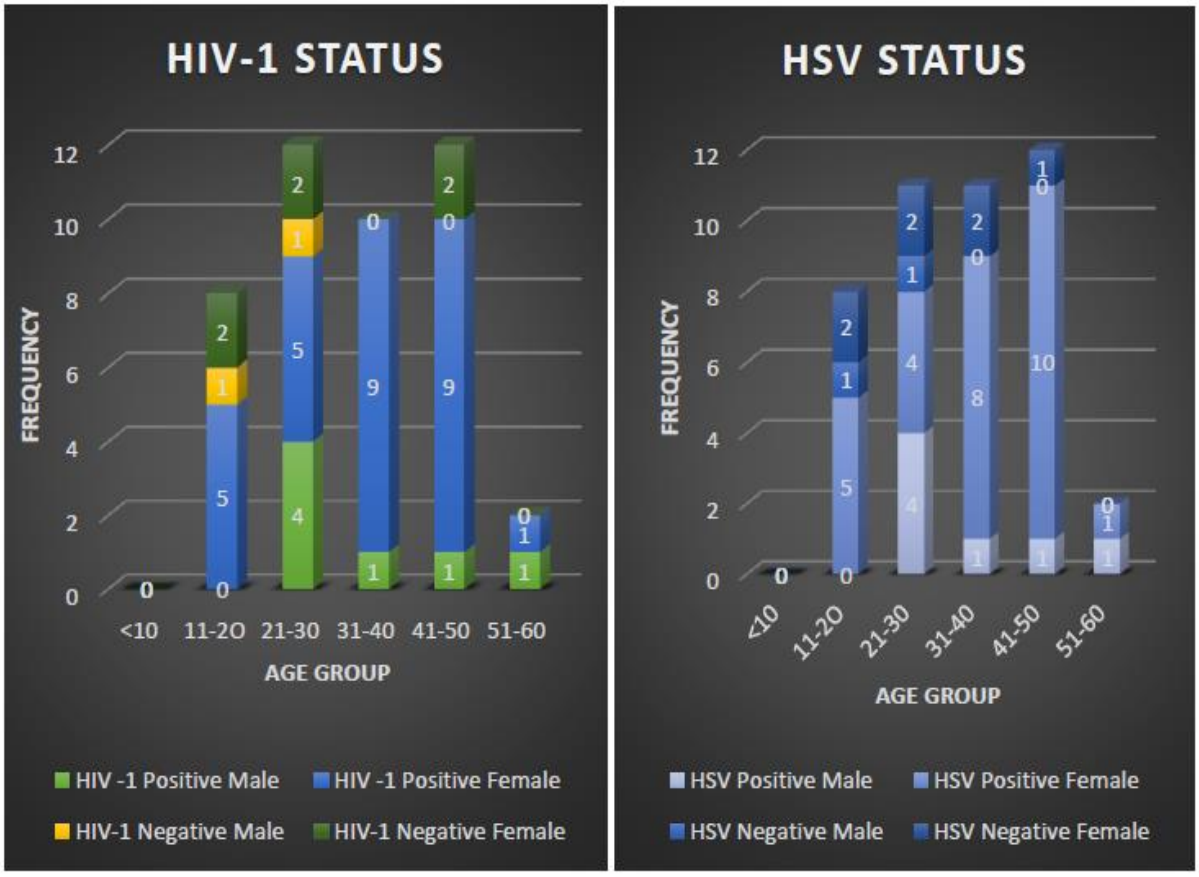

Figure 3.2: The frequency of distribution of male and female infected and negative for HIV-1 and HSV within certain age groups.

3.3 ELISA Screening Results

The sera samples collected for this study were screened for both HIV-1 and HSV. Greenscreen Ultra HIV Ag-Ab test kit was used in the ELISA screening of HIV-1 while HSV ELISA screening was carried out using Platelia HSV (1+2) IgG test kits. Although, the test kit was not type specific for Herpes Simplex Virus, however, it is highly sensitive. Firstly, the samples were screened for HIV-1 and eight (18.2%) out of the total of forty-four samples were seronegative. The samples were also tested for HSV and thirty-four (77.3%) from the forty-four sera were positive for either type 1 or type 2 Herpes Simplex Virus. The sera were categorised as either positive or negative following the manufacturer's instruction. It is considered positive for HSV specific IgG when the ratio of the optical density value of the serum and that of the cut-off is greater than 1.2 as shown in figure 3.3 and negative when the ratio is less than 0.8. Table 3.1 shows the viral distribution of HIV-1 and HSV between male and female in this study.

Table 3. 1: ELISA screening for HIV-1 and HSV in the sera samples collected from North-West and KwaZulu-Natal Provinces

	HIV -1 Positive		**HIV-1 Negative**			**HSV Positive**		**HSV Negative**	
Age Group	**Male**	**Female**	**Male**	**Female**		**Male**	**Female**	**Male**	**Female**
<10	0	0	0	0		0	0	0	0
11-20	0	5	1	2		0	5	1	2
21-30	4	5	1	2		4	4	1	2
31-40	1	9	0	0		1	8	0	2
41-50	1	9	0	2		1	10	0	1
51-60	1	1	0	0		1	1	0	0
TOTAL	7	29	2	6		7	28	2	7

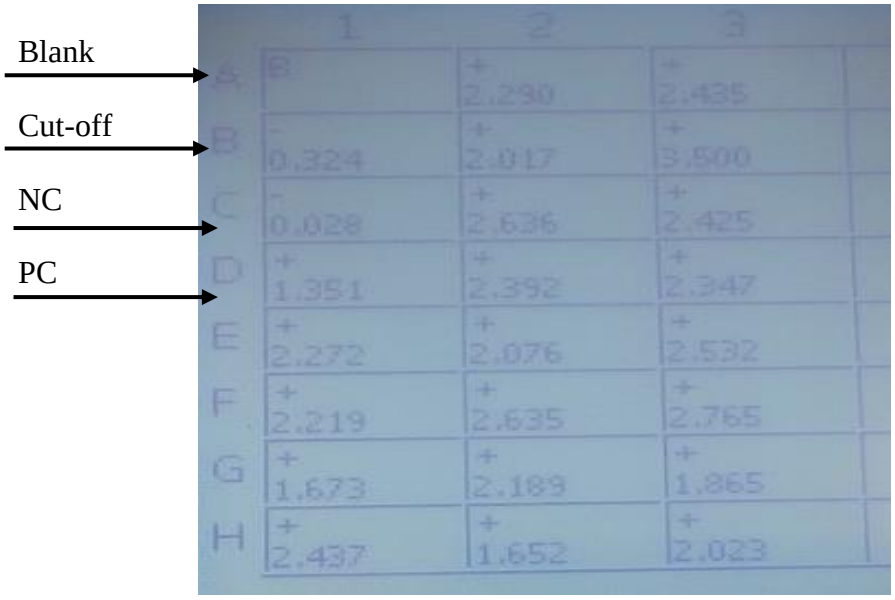

Figure 3.3: HSV ELISA result as read by LT-4000 Microplate reader. The arrows showed the value of blank, cut-off, negative control (NC) and positive control (PC) sera while the remaining values on the microplate reader were the test samples.

3.4 PCR amplification of integrase gene of HIV-1

One step viral amplification of the cDNA was carried out to target integrase gene using HIV-1 primer listed in Table 2.0. 81.8% (n=36) of the samples were positive with an expected band of 140bp for HIV-1 confirming the high level of specificity of the ELISA kit.

3.5 PCR amplification of glycoprotein B region of Herpes Simplex Virus Type 1

First and Nested PCR was carried out with gene specific primers to amplify glycoprotein B region (gB) giving a 241bp band. Out of the forty-four randomly collected sera, four (9%) samples tested positive for HSV-1 as shown in figure 3.4, 3.5.

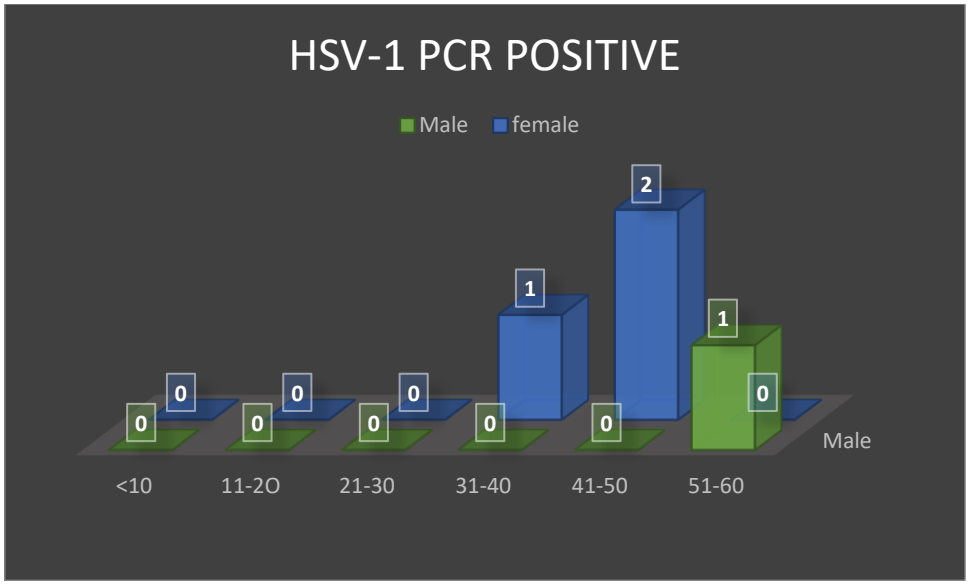

Figure 3.4: Age variation and the frequency distribution of HSV-1 in male and female participants**.**

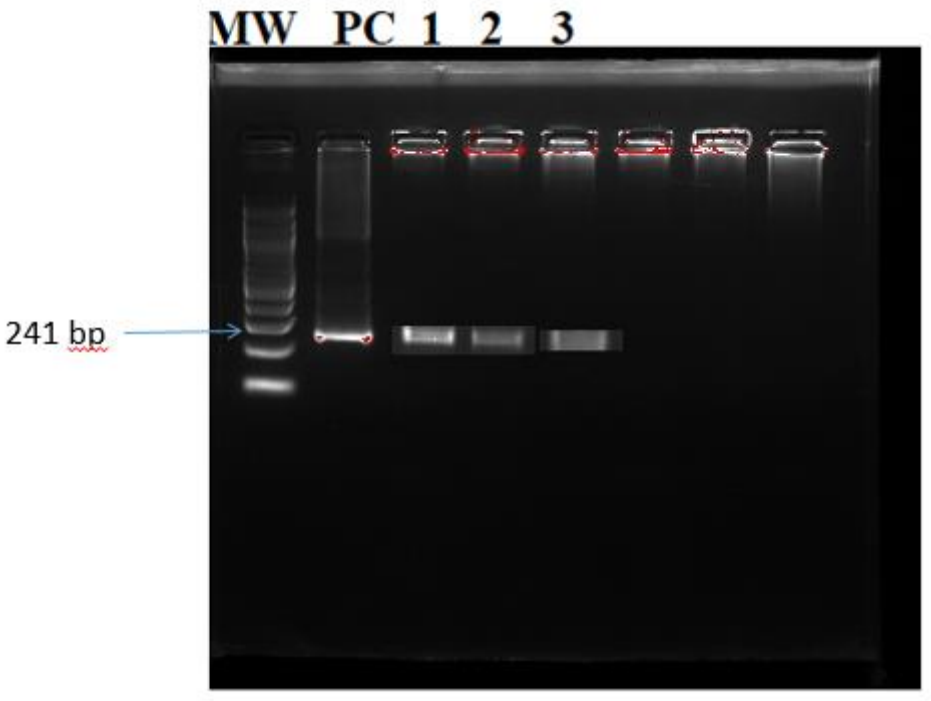

Figure 3.5: Composite gel electrophoresis of glycoprotein B (gB) region of an amplified HSV-1 PCR products (MW=Molecular weight marker, PC= Positive Control, samples= Lane 1 to 3).

3.6 PCR amplification of glycoprotein D region of Herpes Simplex Virus Type 2

Nested PCR using a specific primer to target glycoprotein D (gD) region was employed in the course of the study to amplify the 100bp region of the gene. Thirty (68.2%) of the total experimented samples tested showed that they were positive type 2 herpes simplex virus when the gel electrophoresis was viewed under UV trans-illuminator as represented in figure 3.6 and 3.7.

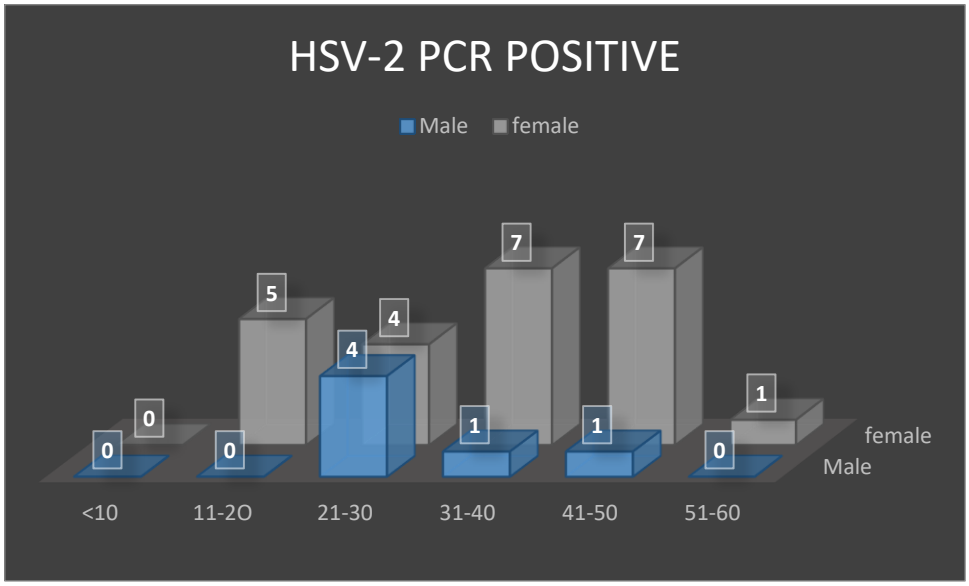

Figure 3.6: Age variation and the frequency distribution of HSV-2 in male and female participants

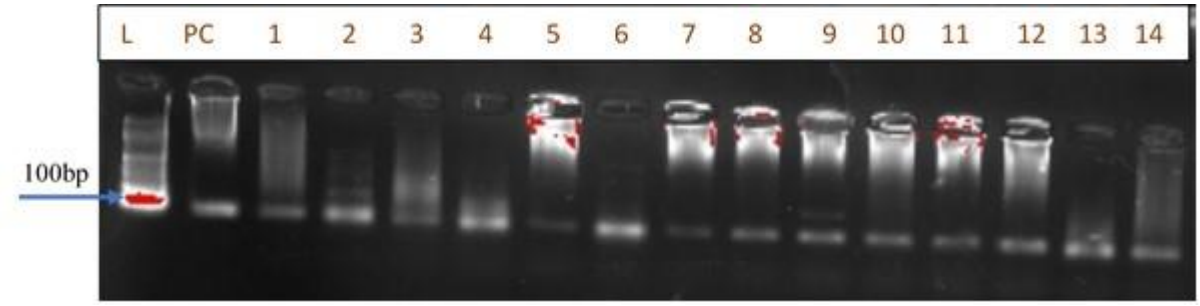

Figure 3.7: Gel electrophoresis of glycoprotein D (gD) region of an amplified HSV-2 PCR products (L=Molecular weight marker, PC= Positive Control, samples= Lane 1 to 14).

3.7 Relationship between Age, Herpes Simplex Virus and HIV

Statistical Package for the Social Sciences (SPSS) version 25 was engaged in the analysis of the demographic data. Forty-four samples were evaluated for significant relationship between Age, Herpes Simplex Virus and HIV. The data was analysed using a two-tailed correlation test.

The result of the study as represented in Table 3.2 showed that there is a statistical connection between age and sera samples infected with HSV-1(0.366**), HSV-2 with HIV-1 sera (0.690**) and an inverse relationship existed between herpes simplex virus type1 and herpes simplex virus type 2 samples (-0.463**) which mean that as there is an increase in the number of HSV-1 in the population, the rate of HSV-2 infection falls.

Table 3. 2: The relationship between Age, true HSV-1 PCR, HSV-2 PCR positive sera and HIV-1 ELISA positive samples.

	AGE	HSV-1 PCR	HSV-2 PCR	HIV-1 ELISA
AGE	1	0.366**	-0.061	0.208
HSV-1 PCR		1	-0.463**	-0.056
HSV-2 PCR			1	0.690**
HIV-1 ELISA				1

**Correlation is significant at the 0.01 level (2-tailed)

3.8 Association between HIV-1 samples and Herpes Simplex Virus positive samples

Chi-Square goodness of fit test was used to evaluate the claim that "ELISA HIV positive samples has an impact on the number of HSV-1 and HSV-2 PCR positive samples" with the null and alternate hypothesis stated as:

H_0: HSV has an impact on HIV-1 positive samples

H_1: HSV has no an impact on HIV-1 positive samples

From the data analysis result shown in table 3.3, the hypothesis was rejected while the alternate hypothesis was accepted for HSV-1, X^2 (1) = 0.138, P >0.05 because there is no significant association between HIV-1 and HSV-1. Also, the alternate hypothesis for HSV-2 was rejected and the null hypothesis retained because of the strong statistical association existing between HIV-1 and HSV-2, X^2 (1) = 20.952, P < 0.05. The same analysis was carried out to check for an association between age, gender, PCR HSV-1, PCR HSV-2 and HIV-1 ELISA as indicated in table 3.4 and 3.5, however, the result showed that there is no significant association between these variables.

Table 3. 3: Association between HIV-1 ELISA, PCR HSV-1 and PCR HSV-2

	HIV-1 ELISA			
	X^2 Value	**Degree of freedom (df)**	**Asymptotic Significance (2-sided)**	**Interpretation**
PCR HSV-1	.138	1	.711	No significant association
PCR HSV-2	20.952	1	.000*	Significant association

*Significant at the 0.05 level (2-tailed)

Table 3. 4: Association between AGE, PCR HSV-1, PCR HSV-2, HIV-1 ELISA

	AGE			
	X^2 Value	**Degree of freedom (df)**	**Asymptotic Significance (2-sided)**	**Interpretation**
PCR HSV-1	20.808	27	.795	No significant association
PCR HSV-2	22.489	27	.712	No significant association
HIV-1 ELISA	27.194	27	.453	No significant association

Table 3. 5: Association between Gender, PCR HSV-1, PCR HSV-2 and HIV-1 ELISA

	Gender			
	X^2 Value	**Degree of freedom (df)**	**Asymptotic Significance (2-sided)**	**Interpretation**
PCR HSV-1	.056	1	.813	No significant association
PCR HSV-2	.012	1	.913	No significant association
HIV-1 ELISA	.124	1	.725	No significant association

KEY: X^2= Chi-Square, df = degree of freedom

3.9 HSV and HIV-1 Co-infection

Out of the forty-four samples used in conducting this study, 11.1% (n=1) male and 22.2% (n=2) female were co-infected with HSV-1 and HIV-1 while a significant proportion of the population, 17.1% male and 68.6% female were HSV-2 and HIV-1 co-infected. This is illustrated in table 3.6 for HSV-2/HIV-1 co-infection.

Table 3. 6: HSV-1 and HIV-1 Co-infected sera samples

Age group	Male	female	HSV-1/HIV-1 Co-infection
<10	0	0	0
11-20	0	0	0
21-30	0	0	0
31-40	0	1	1
41-50	0	1	2
51-60	1	0	1
TOTAL	1	2	3

3.10 Gene Sequencing

3.10.1 Direct sequencing of the PCR products

Four (Three HSV-2 positive and one HIV-1 positive) PCR products were sent to Inqaba Biotech for next generation sequencing (NGS). The samples were sequenced on the illumina MiSeq NGS platform using the following reference genomes; NC_001802, used for HIV-1 samples (G20) while KF781518 was used as the reference genome for HSV-2 samples (G13, G15, G34). The raw sequences were processed to obtain high quality reads using KBase platform (Arkin et al., 2016). To check for the quality of the sequenced data, FastQC v 1.0.1 was used. Trimmomatic v 0.36 was used to trim, remove adapter or low quality sequences and ambiguous reads. The trimmed sequences were aligned with the reference genomes using BowTie 2 v 2.3.2 (Langmead & Salzberg, 2012) as seen in table 3.7 which was later assembled using Spades v. 3.12 (Nurk et al., 2013). The assembled sequences were annotated using Prokka v 1.12 to determine the viral genome for each of the samples (Seemann, 2014). The output results of the mapping showing the coverage of the samples across the reference is seen in figure 3.8, 3.10 and 3.12 respectively for the HSV-2 samples (G13, G15, G34). Basic variant

tool version 2.0 was then used to call for variants. The called variants represent the single nucleotide polymorphism (SNP) differences that exist between the samples and the reference genomes which satisfy the criteria of the detection tool. The table of variants for each of the sequenced samples is represented in the Tables 3.8, 3.9, 3.10 and 3.11.

Table 3. 7: Mapping quality of the glycoprotein D sequenced samples against the reference HSV-2 genome from NCBI data base in KBase mapping tool.

Parameters	G13	G15	G34
Reference size	113,796	113,796	113,796
Number of reads	92,056	237,742	149,530
Mapped reads (%)	68.48	52.59	92.8
Unmapped reads (%)	31.52	47.41	7.2
GC Percentage	67.3	67.3	67.29
Mean coverage	55.384	110.9148	122.3325
Standard Deviation coverage	1,855.6953	3,679.5997	4,096.5959
Mean Mapping Quality (%)	61	61	61

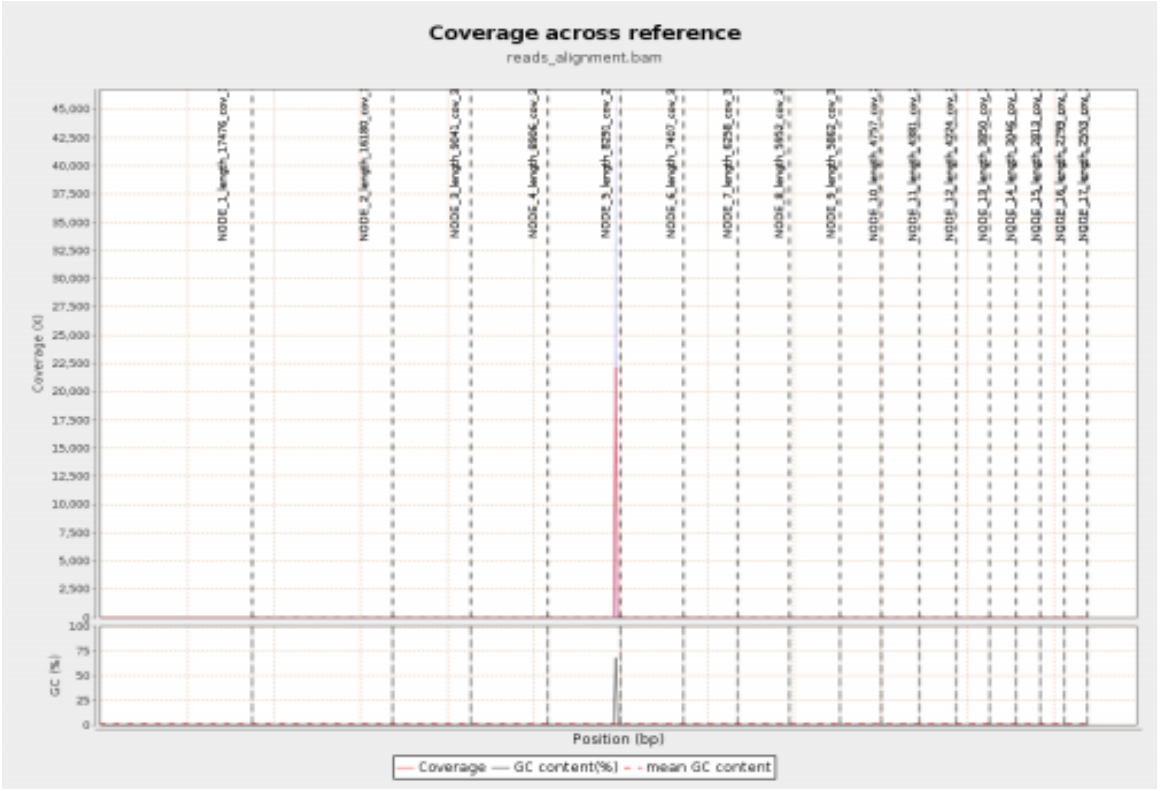

Figure 3.8: coverage of G13 sample paired reads with HSV-2 reference genome

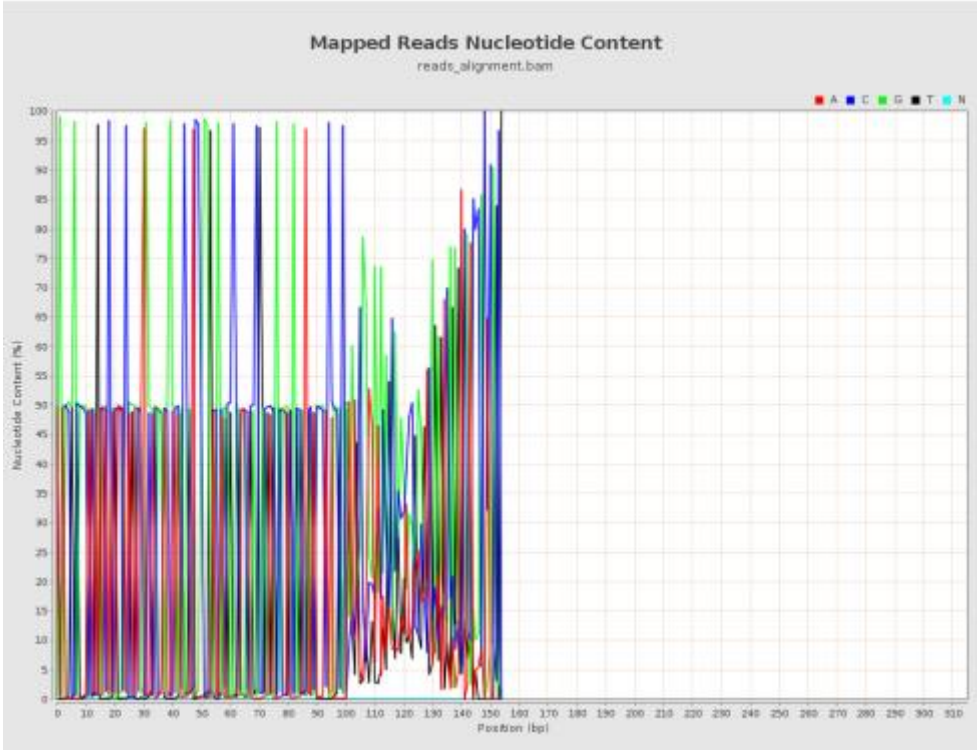

Figure 3.9: The percentage of mapped nucleotide contents of G13 sample that fully align with the reference genome

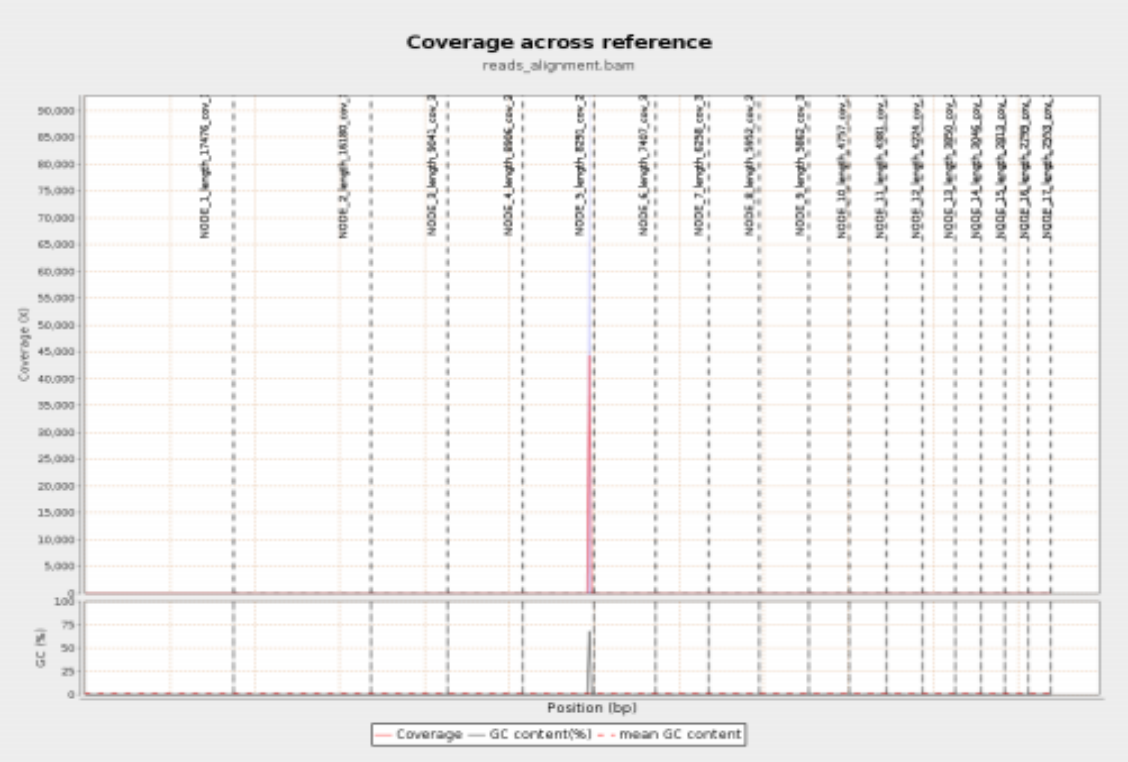

Figure 3.10: Coverage of G15 sample paired reads with HSV-2 reference genome

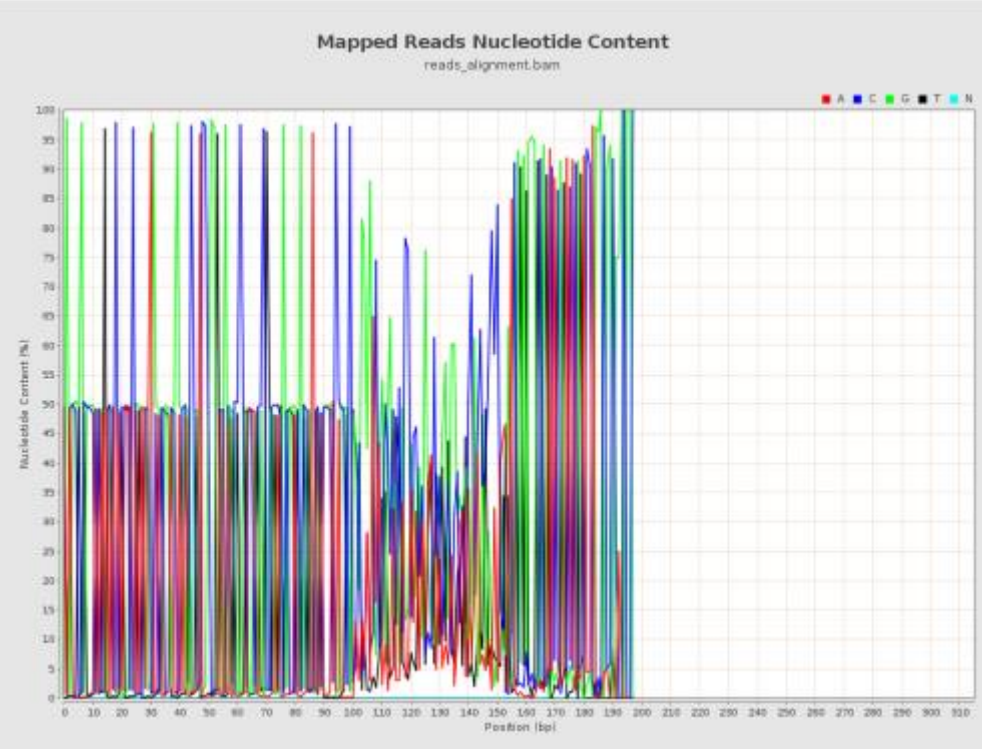

Figure 3.11: The percentage of mapped nucleotide contents of G15 sample that fully align with the reference genome

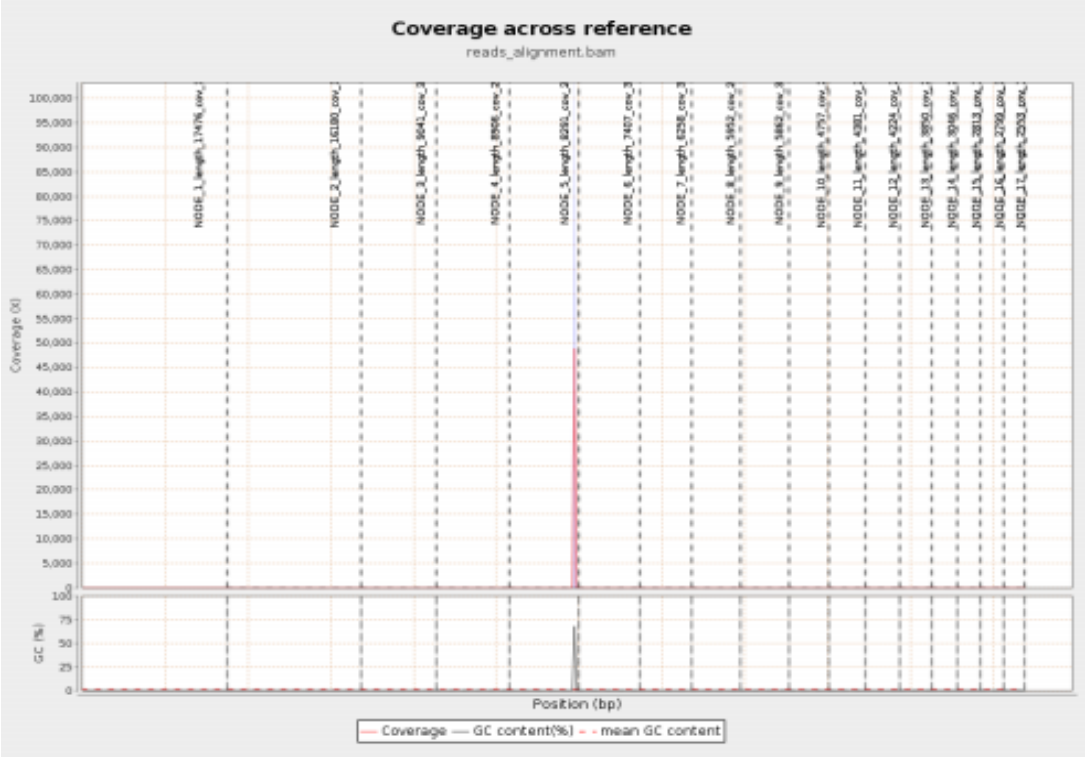

Figure 3.12: Coverage of G34 sample paired reads with HSV-2 reference genome

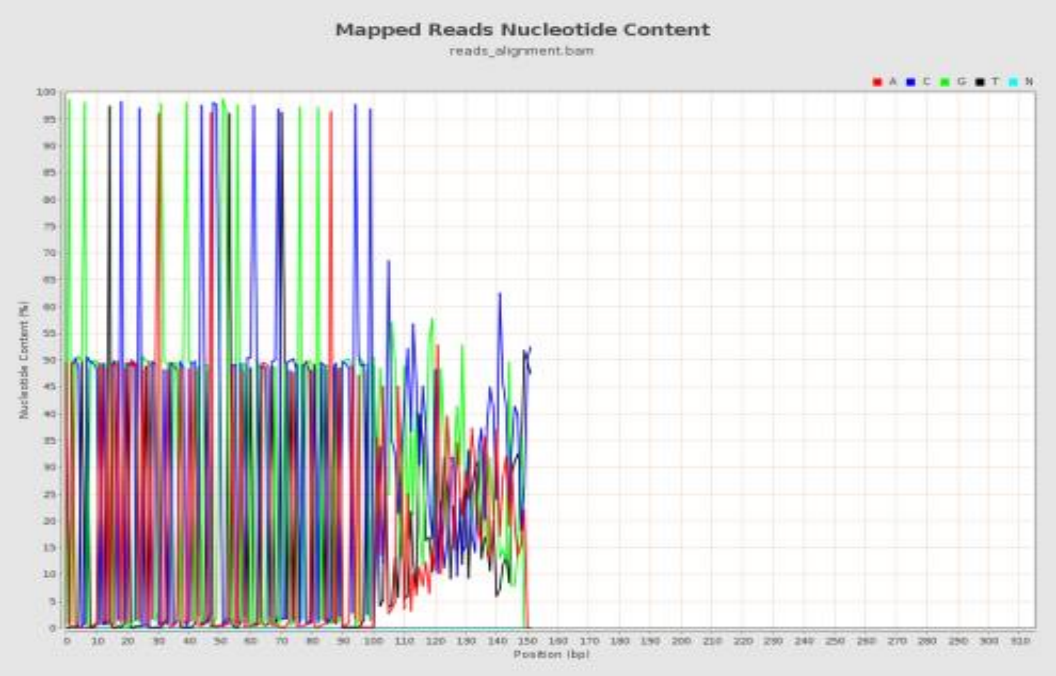

Figure 3.13: The percentage of mapped nucleotide contents of G34 sample that fully align with the reference genome

3.10.2 Evolutionary analysis

From the study samples, four samples with high quality PCR products selected for sequencing were utilised in conducting the phylogenetic analysis of HSV-2 and HIV-1. The FASTA data of the reference genomes from National Center for Biotechnology Information (NCBI) were imputed in Molecular Evolutionary Genetics Analysis (MEGA) with the samples to draw the phylogenetic tree.

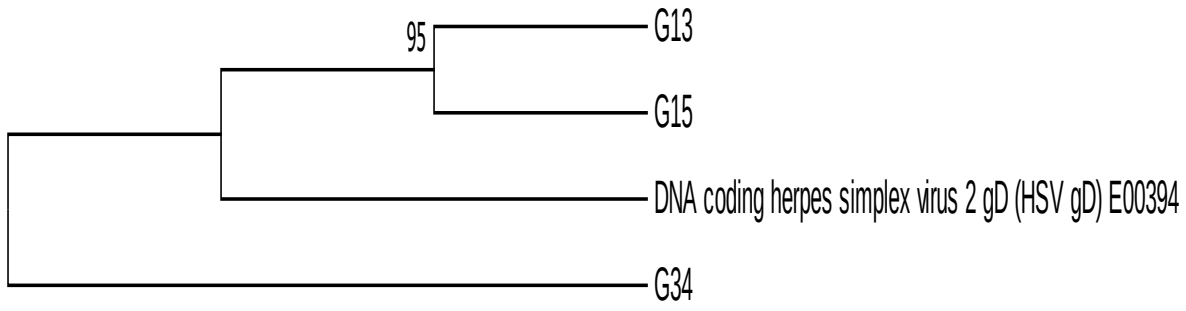

Figure 3.14: Evolutionary relationships of the PCR products of gD samples (G13, G15, G34) with reference genome of HSV-2 from NCBI data base.

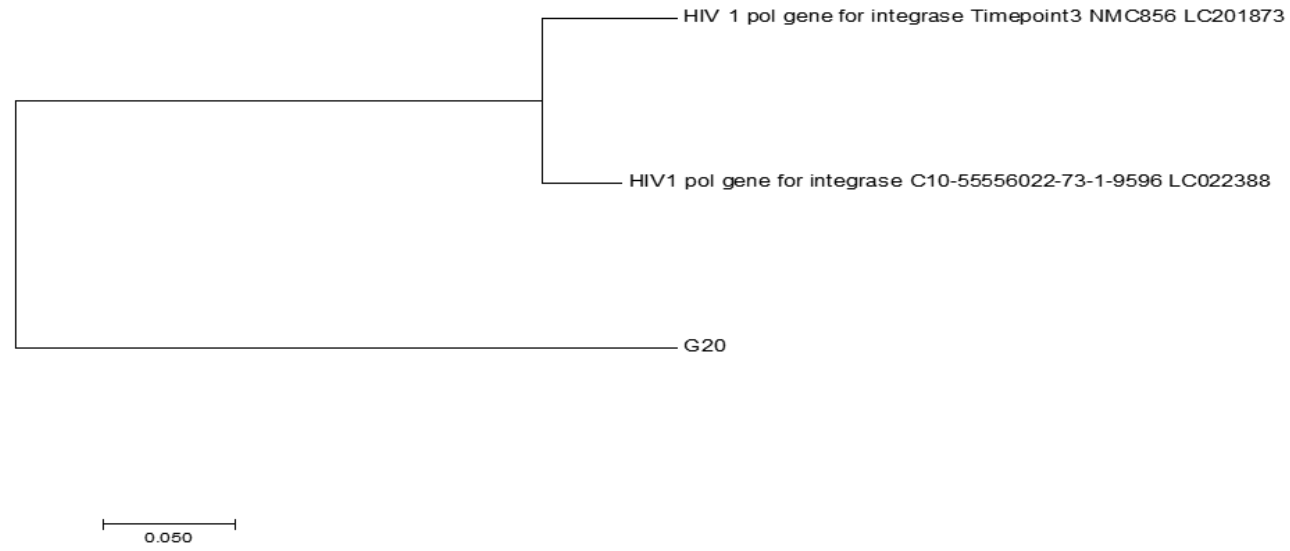

Figure 3.15: Evolutionary relationships of the sequenced PCR products of sample (G20) with reference genomes of HIV-1 from national center for biotechnology information (NCBI) database.

Table 3. 8: Variant table of sample G13

Chromosome	**KF781518**	**KF781518**	**KF781518**	**KF781518**
Region	138078^138079	138078^138079	138120	138123
Type	Insertion	Insertion	SNV	Deletion
Reference	-	-	T	A
Allele	G	-	C	-
Reference allele	No	Yes	No	No
Length	1	0	1	1
Linkage				
Zygosity	Heterozygous	Heterozygous	Homozygous	Homozygous
Count	40	78	5	5
Coverage	118	118	5	5
Frequency	33.89830508	66.10169492	100	100
Probability				
Forward read count	29	63	3	3
Reverse read count	15	24	2	2
Forward/reverse balance	0.340909091	0.275862069	0.4	0.4
Average quality	35.775	19.03846154	33.6	36.2
Read count	44	87	5	5
Read coverage	131	131	5	5
# Unique start positions	9	14	3	3
# Unique end positions	16	12	4	4
BaseQRankSum	6.08	0	0	0
Read position test probability	0.31954891	0.843638712	1	1
Read direction test probability	0.979821518	1	1	1
Hyper-allelic	Yes	yes	No	no
Homopolymer	No	No	No	No
Homopolymer length	1	1	1	1

Table 3. 9: Variant table of sample G15

Reference position	**137944**	**137949**	**137949**	**137951**	**137955**	**138074**	**138120**	**138123**
Type	SNV	SNV	SNV	Deletion	SNV	Insertion	SNV	Deletion
Length	1	1	1	1	1	2	1	1
Reference	T	G	G	T	G	-	T	A
Allele	C	C	T	-	T	CG	C	-
Linkage								
Zygosity	Homozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Homozygous	Homozygous	Homozygous
Count	439	3	4	6	115	64856	7499	7090
Coverage	449	12	12	14	304	71795	7628	7189
Frequency	97.77283	25	33.33333	42.85714	37.82895	90.33498	98.30886	98.6229
Forward/reverse balance	0.496583	0	0.5	0.5	0	0.499106	0.329111	0.342454
Average quality	37.24601	9	38	38	16.10435	35.8625	32.99533	30.01693
Overlapping annotations	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Coding region change	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Amino acid change	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table 3. 10: Variant table of sample G34

Reference position	**137944**	**138079**
Type	SNV	Insertion
Length	1	1
Reference	T	-
Allele	C	G
Linkage		
Zygosity	Homozygous	Heterozygous
Count	770	223
Coverage	775	383
Frequency	99.35484	58.22454
Forward/reverse balance	0.492208	0.145299
Average quality	37.30519	31.95516
Overlapping annotations	N/A	N/A
Coding region change	N/A	N/A
Amino acid change	N/A	N/A

N/A means not applicable

Table 3. 11: Variant table of sample G20

Chromosome	**NC_001802-1**	**NC_001802-1**
Region	4497..4498	4497..4498
Type	MNV	MNV
Reference	TC	TC
Allele	CG	TC
Reference allele	No	Yes
Length	2	2
Linkage		
Zygosity	Heterozygous	Heterozygous
Count	3	6
Coverage	10	10
Frequency	30	60
Probability		
Forward read count	1	6
Reverse read count	3	6
Forward/reverse balance	0.25	0.5
Average quality	36	36.71429
Read count	4	12
Read coverage	18	18
# unique start positions	2	4
# unique end positions	3	4
BaseQRankSum	1.33	
Read position probability	0.937039	0.81253
Read direction test probability	0.85542	0.998216
Hyper-allelic	Yes	Yes
Homopolymer	No	No
Homopolymer length	1	1

CHAPTER 4

4.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

4.1 Discussion

4.1.1 Introduction

The principal purpose of this research was to examine the molecular prevalence of herpes simplex virus types in HIV-1 positive and HIV-1 negative sera samples collected from two provinces, North-West and KwaZulu-Natal Provinces. A total of forty-seven samples were originally collected from the two provinces. Two of the samples however were haemolysed and were discarded and one was insufficient. Therefore, only forty-four sera samples were analysed. The samples were subjected to series of laboratory procedural tests which include ELISA screening, PCR and Sequencing. Some researchers believe that HSV has a major contribution in the transmission of HIV-1. Therefore, understanding the impact of the relationship between these two viruses could help reduce, if not totally eliminate the incidence of the disease and act as a prognosticator to inform HIV-1 epidemic. It is then imperative to study the molecular prevalence of HSV types in HIV-1 positive and negative antibody sera.

4.1.2 ELISA screening

Serology screening is a very important tool in identifying persons with high possibility of acquiring HSV infection. A comprehensive analysis of the data in Table 3.1 showed that 77.3% of the population were positive for both types of herpes (HSV1/2) virus and 81.8% were seropositive for HIV-1 with a predominant female prevalence. Approximately 18.2% and 22.7% of the study population have antibodies that were negative for HIV-1 and HSV respectively. The population with highest number of HSV and HIV-1 prevalence falls between the age group 41-50 years. The ELISA kit used for HSV is not type specific but it is highly sensitive. Kasubi (2006) stated that most sera samples from African nations have a high tendency of producing false positive results regardless of the procedure used in the analysis. The large percentage of true positivity experienced in this study could be as a result of the high sensitivity of the Platelia HSV (1+2) IgG test kit used in conducting the test. Three (6.8%) sera samples were HSV-1/HIV-1 co-infected and thirty (68.2%) samples were HSV-2/HIV-1 co-infected when screened with ELISA.

4.1.3 PCR Results

In order to determine the true positivity of each sample for either HIV-1, HSV-1 or HSV-2, the samples were further analysed using Polymerase Chain Reaction (PCR). The procedure was used because of its high sensitivity and the specificity increased with second (Nested) PCR. The inner primers used for nested amplification differ from those used for the first round amplification which was able to reduce unnecessary amplification of non-specific products. Each pair of primers used for nested amplification targeted glycoprotein B, glycoprotein D (gD) and integrase region for HSV-1, HSV-2 and HIV-1 respectively at 241bp, 100bp and 140bp when viewed on gel electrophoresis. Four (9.1%) of the samples were positive for HSV-1, thirty (68.2%) were HSV-2 seropositive and thirty-six (81.8%) of the total samples tested were positive HIV-1 as shown in figure 3.5 and 3.7 respectively.

The PCR results showed that 6.8% females and 2.3% males of the study participants are true positive for herpes simplex type 1 while 54.5% and 13.6% of females and males respectively are true positive for HSV-2. This is in tandem with the prevalence rate of HSV-2 in sub-Saharan Africa as stated by (Looker et al., 2015; Paz-Bailey et al., 2006). It also agrees with Debra et al., (2018) where high prevalence rate of HSV-2 of 58% in Uganda, 68% in Zimbabwe, 55% in Zambia and 28% in Gambia. The seroprevalence of HSV-2 increased to a peak of 26.6% (n=8) in 31-40 age group and became steady until it falls by 3.3% (n=1) between the ages of 51-60 years. A study conducted by Cunningham et al., 2006 revealed a higher HSV-1 prevalence in gender (80% in women and 71% in men) and a relatively lower prevalence of HSV-2 of 16% and 8% in women and men respectively. There was a reduction in HSV-1 prevalence rate as shown in this study which could be as a result of reduced sample size or a shift in trend of the disease spread in this population as a lot of people lack HSV-1 antibodies in the early age which could be a predisposing factor towards HSV-2 acquisition when exposed to the virus which is well illustrated in figure 3.4.

However, the sera that were co-infected (HIV-1/HSV) when screened with ELISA were also confirmed using PCR. The true co-infection rate of HSV-1/HIV-1 and HSV-2/HIV-1 set at (11.1%, 22.2%) and (17.1%, 68.6%) for male and female respectively as shown in table 3.6. The co-infection is common in both provinces because most of the samples that were also with

HIV-1. The result of the co-infection is similar to the high prevalence rate of HSV-2/HIV-1 co-infection of 65% in men and 91% in women as identified by Celum et al. (2004).

4.1.4 Sequencing

Viruses have tendency of developing resistance against antiviral drugs. These tendencies have become a major concern over the past decades to the treatment and management of infections (Kimberlin et al., 1996). More often than not, the infections or disease caused by resistant viruses are common in immunocompromised hosts. From the variant tables, the sequenced HSV-2 genomes differ from the reference genome with one or more allele at different regions. Insertion of G loci occur at the 138078 region in G13 as seen in table 3.8 and also in G34 (table 3.10) which was not present in the reference genome. Also, single nucleotide variant (SNV) occur in both G34 and G15 at 137944 region replacing T with C allele. However, SNVs at different region are present in G15 with a few deletion and insertion of alleles. The deletion of T and A from the reference genome at 137951 and 138123 respectively is responsible for the genetic variant in G15. Although, the exome of genomes contains approximately 13,000 SNVs (Sayitoğlu, 2016), its role in the infection spread and treatment is what we do not know. The frequency of SNVs is high in each variant calls when compared with the frequencies of variant calls caused by deletions and insertions. This high frequency in variant calls can be used to confirm the prediction of a difference in the nucleotides of the sample as compared with the reference genomes and could be a factor responsible for the increase in antiviral resistance. Certain antiviral drugs were used for the treatment of HSV infection and acyclovir is the most commonly used among the choice drugs Singh et al. (2005), however, resistant strains have been discovered to these choice of drugs which could be due to the possible variation in the loci of different Herpes Simplex Virus strain. This difference could be the reason why there is no current cure for Herpes Simplex Virus infection.

HSV resistance originates from the mutations of the viral genome. Mutations within the viral DNA polymerase gene and activating/phosphorylating genes also result in acyclovir resistance because most antiviral drugs for HSV, target the DNA pol gene for effectiveness. Alterations in the DNA pol enzyme will hinder the performance of the drug which on the other hand will cause resistance (Jiang et al., 2016). Multiple nucleotide variants (MNV) occur in sample G20 which is different from the reference genome at 4497 region. This variant occurs when CG

replaces the reference allele (TC) at a very low coverage. This low coverage of the alignment does not give us the confidence required to confirm that there is a variant call at this position (4497).

4.1.5 Phylogenetic analysis

This was done to check for the phylogenetic relationship between the molecular sequences of the samples and the reference genome. Evolutionary history was inferred using neighbour-joining method which was run with 1000 replicates to construct the phylogenetic tree for G13, G15, G34 and HSV 2 gD E00394 alignment, and G20, LC022388, LC201873 HIV-1 alignment. HSV-2 sequenced samples aligned with each other and the reference genome at 113,796 region as shown in table 3.7. They have a high GC content that is similar to that of HSV-2 of 68% (Brown, 2007). From the evolutionary tree, G34 appeared to be closely related with the reference genome with a high mapping reads of 92.8% than G13 and G15 with a percentage alignment reads of 68.48% and 52.59% respectively. Although, the two samples (G13 and G15) are closely related but they are also distantly related to the rooted evolutionary tree.

The phylogenetic relatedness of G20 with the two reference genomes of HIV-1 showed that a distant relation between the sequence and the two genomes existed.

4.2 Descriptive analyses

4.2.1 Relationship between Age, HSV and HIV-1 transmission

"Among young adults, HSV-2 prevalence consistently increases with age in different countries, populations, and within sexes, with increases depending in particular on geographic area, population group, and sex". These were the words of Smith et al. (2002). These words instigate the need to examine for a relationship between age, HSV-1, HSV-2 and HIV-1. This was evaluated on SPSS using a two-tailed Pearson correlation test and the result was tabulated in Table 3.2. The cut-off significance value was set at 0.01 (99%) and 0.05 (95%). From table 3.2, age appears to strongly correlate with HSV-1 at P value of (0.366^{*}) but there was no correlation between age, HSV-2 and HIV-1. This significant relationship between age and HSV-1 is consistent with the findings of Smith et al. (2002) that HSV-1 increases with age in

the general population and it is also in conflict with it because there is no relationship between age and HSV-2 as shown in this study.

The relationship between HSV-1, HSV-2 and HIV-1 was also analysed. Table 3.2 showed that there is no correlation between HSV-1 and HIV-1. However, there is significant inverse correlation between HSV-1 and HSV-2 at 99% cut off with a P value (-0.463^{**}). This finding shows that as the number of HSV-1 increases in the population, there is a decline in HSV-2 prevalence rate. This finding agrees with WHO (2017) outcomes that individuals infected with oral herpes are less likely to be affected with genital infections.

The correlation between HIV-1 and HSV-2 in this study is statistically significant with a strong P value (0.690^{**}) at 99% cut off level which is not so with age and HSV-1 as shown in table 3.2. This revealed that increase in HSV-2 infection will lead to the steady rise in HIV-1 acquisition. One possible explanation for this is that both share a similar route of transmission and the impact of one is significant on the other as seen in the micro-ulceration of the genitalia in a HSV-2 patient which provides a port of entry for HIV-1 (Sheth et al., 2008).

4.2.2 HIV-1 and HSV association

We examined the association between HIV-1 and HSV for all data. This was done using Pearson Chi-Square and P values with significance set at $P < 0.05$ as seen in Table 3.3. From the study population, the number of HIV-1 positive sera that has the type 1 Herpes Simplex Virus gene were three and the total number of samples with negative antibodies to HIV-1 and positive HSV-1 gene was one. This implies that the prevalence of HSV-1 in this study population is low predisposing the region to further acquisition of HSV-2 when in contact with a carrier of type 2 HSV infection. The P value of HSV-1 (0.711), $X^2(1) = 0.138$, $P > 0.05$ showed that there is no significant association between HSV-1 and HIV-1, meaning that HSV-1 is not the sole cause of HSV/HIV-1 co-infection in the study population which could be as a result of the difference in the route of transmission. However, the same analysis was conducted for HSV-2 to check for an association between the virus and HIV-1. The result showed a strong association with a P value (0.000^{*}), $X^2(1) = 20.952$, $P < 0.05$. In the work published by Kasubi (2006), he recorded that no substantial association existed between HSV-2 and HIV which is in conflict with our research findings and that of other researchers. Kouyoumjian et al. (2018)

found that there is a solid association between HSV-2 and HIV-1 prevalence with HSV-2 being consistently higher than HIV-1 in all population. Here, HIV-1 prevalence was consistently higher than HSV prevalence in the study population and are significantly associated. The result is dependent on existing facts that these two viruses are circulating in the population that share similar route of disease transmission.

Some researchers believed that there is a significant association between Herpes simplex virus and demographic factors like gender and age (Smith et al., 2002). Ghebremichael et al. (2009) discovered that the age at coitarche is strongly associated with the incidence rate of sexually transmitted infection. This is why we decided to check in this study for any possible association. Contrasting results were found after the data was analysed on SPSS using Pearson Chi-Square. The result recorded that there is no association between age and herpes simplex virus types with the P value of HSV-2 (0.712), X^2 (27) = 22.489, P >0.05, HSV-1 (0.795), X^2 (27) = 20.808, P >0.05 and similar result was found between age and HIV-1, P value (0.453), X^2 (27) = 27.194, P >0.05 suggesting that age is not a key factor in contracting these viruses as anyone regardless of their age when exposed can be infected with the virus. This is well illustrated in Table 3.4.

Furthermore, gender was categorised as either male or female. This was then cross-tabulated to check for an association with HIV-1 and HSV. Table 3.5 shows the summary of the association between these variables. The association between gender and HSV-2 informed that HSV-2 (0.913), X^2 (1) =0 .012, P >0.05, HSV-1 (0.813), X^2 (1) = 0.056, P >0.05 and that of HIV-1 was HIV-1 (0.725), X^2 (1) = 0.124, P >0.05 respectively. Hence, no significant association was recorded between gender, HSV-2, HSV-1 and HIV-1.

4.3 Summary of study

The results generated from this study showed us that the majority of the study participants that were infected with HSV and HIV were predominantly female and the infection increases relatively with age for HSV-1 which was not the case with HSV-2 and HIV-1. Also, people who are positive for HSV-2 have a higher chance of contracting HIV-1 infection, although, we were unable to infer if the infection was due to the route of transmission sexually or non-sexually acquired because we do not have the information about the route of infection. Co-

infection with these viruses could cause a variation in the genomes of an infected individual, altering the genes by deletion, insertion or lead to the transposition of an allele with single nucleotide variation (SNVs).

4.4 Conclusions

The main essence of this study was to determine the molecular prevalence of HSV1/2 from HIV-1 positive and HIV-1 negative sera samples collected from North-West and KwaZulu-Natal Provinces and to evaluate for a possible association between the herpetic viruses and HIV. From this study, a few drawn inferences were listed below:

- There is increase prevalence rate of Herpes Simplex Virus in North-West and KwaZulu-Natal Provinces and the prevalence rate differs in gender with women having the highest infection rate. This means that if the infection is not properly treated, its spread to other geographical region is imminent which will pose a threat to global eradication of HIV as HSV fuels the transmission of HIV.
- The spread of HSV-2 increases with HIV-1 in all population within the age group which is not so in a population that is infected with type 1 Herpes as expected. Also, age do not appear to be a factor in contracting HSV-2 and HIV-1 but it is a strong factor in HSV-1 transmission. This means that anyone can be infected with HSV-2 and HIV-1 as long as they are predisposed to the infection source. A decline in HSV-1 acquisition in childhood means teenager are vulnerable to genital herpes infection caused by HSV-1 and a clinical HSV-2 disease is expected among persons with no HSV-1 antibodies.
- There are possible variations in the genome allele of HSV-2/HIV-1 co-infected samples showing a distant relationship with the evolutionary outgroup.

4.5 Limitations and Strength of the study

The objectives of the study were achieved in spite of the following encountered challenges:

- Few samples were available to conduct the study because of stringent procedures for ethics approval concerning handling of human samples

- Inaccessibility to the patients was also considered a limiting factor as there was no communication between the researcher and the patients to ask valuable demographic questions like the site of infection, which would have been useful in the data analysis.
- ELISA is a sensitive and good technique in diagnosing Herpes Simplex Virus and HIV but Polymerase Chain Reaction is a better choice for ruling out false positive results that usually comes with ELISA technique.

4.6 Recommendations

The information garnered from this study agrees with the high prevalence of Herpes Simplex Virus in sub-Sahara Africa. In order to alleviate the spread of these diseases, the following recommendations were suggested from this study:

- Enlightenment programme should be fully encouraged in all provinces with a focus on the rural areas because most of the infection carriers are not even aware of their possible infection and what they need to do to limit the spread of the disease.
- Government should encourage routine testing for Herpes Simplex Virus especially for pregnant women before delivery. If this is done, neonatal herpes caused by the transmission of HSV from an infected mother would be reduced if not totally averted.
- A similar study should be done in the future with a larger sample size that will generalize the entire population, covering all the Provinces of South Africa. Samples to be used for the study must contain proper demographics and information about the patients like the site of infection, number of sexual partners, the time of coitarche and race should be put into consideration while conducting the research.

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APPENDIX

Reagent preparation

A. Preparation of 0.5X Tris-borate-EDTA (TBE) buffer:

For 1 litre of 0.5 X TBE buffer, 950 ml of sterile distilled water was added to 50 ml 10 X TBE stock buffer autoclaved and stored at room temperature for further use.

B. Preparation of carrier RNA

To prepare a solution of 1 µg/µl of carrier RNA, 310 µl of buffer AVE was added to 310 µg of lyophilized carrier RNA. However, the carrier RNA was prevented from losing its yields of viral isolated RNA by storing them in aliquots to minimize denaturizing effect by freeze-thawing. The volume of carrier RNA added to buffer AVL before the commencement of RNA extraction was calculated using this formula:

$N \times 0.56$ ml = y ml

Y ml × 10 µl /ml = z µl where N = number of samples

Y = calculated volume of buffer AVL

Z = volume of carrier RNA-Buffer AVE to add to buffer AVL

C. Preparation of Conjugate 2 working solution (Reagent 7a (R7a) + Reagent 7b (R7b)):

This was prepared by adding the content of conjugate diluent vial (R7b) into lyophilised conjugate 2 (R7a) vial and allowed to stand on the bench for 10 minutes while it is been properly mixed for adequate dissolution.

D. Preparation of protease or proteinase k

Proteinase k or QIAGEN Protease was prepared according to the manufacturer's instruction by adding 1.2 ml protease resuspension buffer into the vial containing lyophilized QIAGEN Protease and allowed to dissolve. This was later stored at 2–8°C.

E. Preparation of Buffer AW1

Buffer AW1 supplied as a concentrate was prepared by adding 25 ml of 96 % ethanol to the vial containing AW1 buffer and mixed homogenously.

F. Ethical Clearance

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Ethics Committee
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ETHICS APPROVAL OF PROJECT

The North-West University Research Ethics Regulatory Committee (NWU-RERC) hereby approves your project as indicated below. This implies that the NWU-RERC grants its permission that provided the special conditions specified below are met and pending any other authorisation that may be necessary, the project may be initiated, using the ethics number below.

Project title: Characterization of HIV-HBV and HIV-HCV co-infections in a HIV-infected pregnant cohort in Southern Africa.

Project Leader: T Sithebe and L Modise

Ethics number:

N	W	U	-	0	0	0	6	8	-	1	5	-	A	9
Institution				Project Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Approval date: 2015-05-22 **Expiry date**: 2020-05-21

Special conditions of the approval (if any): None.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- *The project leader (principle investigator) must report in the prescribed format to the NWU-RERC:*
  - *annually (or as otherwise requested) on the progress of the project,*
  - *without any delay in case of any adverse event (or any matter that interrupts sound ethical principles) during the course of the project.*
- *The approval applies strictly to the protocol as stipulated in the application form. Would any changes to the protocol be deemed necessary during the course of the project, the project leader must apply for approval of these changes at the NWU-RERC. Would there be deviated from the project protocol without the necessary approval of such changes, the ethics approval is immediately and automatically forfeited.*
- *The date of approval indicates the first date that the project may be started. Would the project have to continue after the expiry date, a new application must be made to the NWU-RERC and new approval received before or on the expiry date.*
- *In the interest of ethical responsibility the NWU-RERC retains the right to:*
  - *request access to any information or data at any time during the course or after completion of the project;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the project are revealed or suspected,*
    - *it becomes apparent that any relevant information was withheld from the NWU-RERC or that information has been false or misrepresented,*
    - *the required annual report and reporting of adverse events was not done timely and accurately,*
    - *new institutional rules, national legislation or international conventions deem it necessary.*

The Ethics Committee would like to remain at your service as scientist and researcher, and wishes you well with your project. Please do not hesitate to contact the Ethics Committee for any further enquiries or requests for assistance.

Yours sincerely

Linda du Plessis

Digitally signed by Linda du Plessis
DN: cn=Linda du Plessis, o=North-West University, ou=Vice-Rector: VTC, email=Linda.duPlessis@nwu.ac.za, c=ZA
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Prof Linda du Plessis
Chair NWU Research Ethics Regulatory Committee (RERC)

G. Supporting letter for Ethical Clearance

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06/12/2018

Attention:

Examinations" Administrator

Dear Sir/Ma'am

Re: Ethics Clearance Certificate (NWU-00068-15-A9)

This serve to confirm that the following students operated under the umbrella of the above mentioned ethics clearance certificate with me and Ms Modise as project leaders. They are:

1. Mr N Mokgautsi (22999035)– M degree
2. Mr OS Obisesan (27521680)– M degree
3. Ms T Jantjie (23975874)– Honor's degree
4. Mr B. Bodumele (25330152)– Honor's degree

Should you require any further information, please do not hesitate to contact me.

Sincerely yours

Nomathamsanqa Patricia Sithebe(21905088)

PhD Medical Virology

Ass Prof Microbiology