Molecular characterization of Major Viral Hepatotrophs Co-infected with HIV from sera collected in the North West and Kwa-Zulu Natal Provinces of South Africa

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DECLARATION

I Lorato Mosetsanagape Modise hereby declare herewith that the dissertation entitled "Molecular Characterization of Major Viral Hepatotrophs in HIV-infected individuals from Kwa-Zulu Natal and North West Provinces of South Africa", which I herewith submit to the North-West University upon completion of the requirements set for for the degree Doctor of Philosophy in Biology (Medical Virology), is my own work and has not been submitted elsewhere before and all the sources used or quoted have been indicated and acknowledged.

Signed at…….this…….day of……2018

DEDICATION

This work is dedicated to my precious children Kganya and Kgothatso, who are my strength and motivation in life. Behind a good Mom there are great children and you have been those children. When I think of my success, sacrifices, joy and peace I think of you two. Mommy Love you whole heartedly and more than anything else.

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PREFACE

PUBLICATIONS IN PROCESS

Human Immunodeficiency Virus type 1 (HIV-1) genotyping in Mahikeng, South Africa: assessing antiretroviral drug-resistance associated mutations in an HIV type-1 infected pregnant women cohort.

Molecular characterization of HBV in HIV infected cohort from KwaZulu-Natal Province

Prevalence of HCV in HIV/ HBV co-infected individuals from North West and KwaZulu-Natal Province.

PRESENTATIONS

2017- YOUNG SOUTH AFRICAN SCIENTIST CONFERENCE: Molecular Characterization of HIV and HBV among the HIV-infected Pregnant Women in Mahikeng, South Africa: Advocating the need for Policy Implementation on Routine Genotyping of HIV and HBV. Birchwood, Johannesburg, South Africa.

2016- THE 21ST INTERNATIONAL AIDS CONFERENCE: Assessment of Human Immunodeficiency Virus Type-1 Subtype C Drug Resistance Mutations in North West Province, South Africa. Durban, KwaZulu-Natal, South Africa.

2016 SOUTH AFRICAN SOCIETY OF MICROBIOLOGY- Molecular Prevalence of Major Viral Hepatotrophs in a Cohort of HIV-infected Pregnant Women in the North West Province.

2015- VIROLGY AFRICA CONFERENCE: Molecular Prevalence of Major Viral Hepatotrophs in a Cohort of HIV-infected Pregnant Women in the North West Province, Cape Town, South Africa.

2015- PATHOLOGY RESEARCH AND DEVELOPMENT CONGRESS (Pathred/NHLS): Assessment of Human Immunodeficiency Virus Type-1 Subtype C Drug Resistance Mutations in North West Province, South Africa. Johannesburg, South Africa.

ABSTRACT

Blood borne infections by HBV, HCV and HIV represent a major public health problem globally. The co-infection of HIV with HBV/ HCV is globally common owing to shared route of bloodborne transmission. From 36 million people infected with HIV, close to 4 million people were reported to be infected with HBV and 5 million were infected with HCV (WHO, 2017). These two major hepatotrophs increase the HIV pathogenesis and have emerged as a major cause of death in HIV-infected patients. In South Africa HIV/HBV co-infections substantially out-number HIV/HCV co-infections; close to 63% South Africans living with HIV have been exposed to HBV with chronic HBV ranging from 5-20% in various studies of HIV-infected individuals (Scheibe, 2017). But in South Africa the HCV prevalence is reported to range from 0.16 to 1.8% (Hecht *et al.*, 2018). HCV co-infection is less common, reflecting the magnitude of injecting drug use. Currently, the prevalence of HIV-infected individuals co-infected with both HBV/ HCV is uncertain. There is still limited data on the HBV/ HCV genotypes circulating in HIV-infected individuals in the North West Province and KwaZulu-Natal, these two provinces have the highest number of people infected with HIV suggesting that co-infection with HBV and HCV might be present. Co-infection of HBV and HCV in HIV infected people may hinder treatment and management of these viruses in the context of co-infections and result in progressive HIV replication and death in HIV-infected patients.

The aim of this study was to determine the molecular prevalence of HBV and HCV in HIV-infected individuals from Durban, KwaZulu-Natal and pregnant women from Mahikeng, North West Province. Using serology and genotyping methods we identified the prevalence of HBV/ HCV in HIV infected individuals and we characterized HIV, HBV and HCV genetic types in co-infected individuals. Co-infected individuals were further characterized for mutations associated with drug resistance.

HBV/ HIV co-infection from Mahikeng cohort was 9% (2/23) based on Polymerase chain reaction amplification of the HBV partial surface gene. From the Durban cohort, HBV/HIV co-infection was 78% (41/50) based on PCR amplification of the HBV partial surface and 10% (5/50) for amplification of the BCP/PC gene. There was no HBV/HCV and HCV/HIV co-infection identified from both cohorts. Genotyping of HBV and HIV was done through PCR amplification, Sanger sequencing and phylogenetic analysis. Sequence and phylogenetic analysis indicated that all HIV sequences from both cohorts from this study clustered with HIV genotype 1 and HBV sequences belonged to HBV genotype A. Both HBV and HIV genotypes identified in this study are predominant among heterosexual populations in South Africa. The prevalence of mutations on the HIV *pol* region from Mahikeng cohort was 17% (4/23). HIV *pol* region contained mutations associated with resistance to NRTIs and NNRTIs drugs such as K103N, M184I, M230L and K65R which according to the Stanford Genotypic Resistance Interpretation Algorithm are considered major mutations, were detected indicating possible transmission of anti-retroviral drug resistance mutations since the cohort was antiretroviral therapy naïve. The prevalence of mutations on the HIV *pol* region from Durban was 0% (0/50). The prevalence of mutations on the BCP/PC region of HBV from Mahikeng was 0% (0/23) whilst it was 16% (6/38) from Durban cohort. The BCP/PC sequences had mutations; A1762G, T1753C, A1762T, G1764A, C1766T, G1862A and also GCAC Kozak sequence was identified as a variant and may cause immune escape HBV. From the Mahikeng cohort prevalence of mutants on the overlapping HBsAg region of HBV was 25% (1/4) and the prevalence on RT region of the HBV polymerase region was 75% (3/4). Mutant identified on the overlapping HBsAg region of HBV was S207N and on the RT region of the HBV polymerase we identified M129L, V163I and I253Y. S207N mutant is associated with poor HBsAg antigenicity whilst V163I and I253Y mutants on the RT region are associated with resistance to LMV, LdT and ADV.

From the Durban cohort, mutations prevalence in the HBsAg region was 47% (18/38). The most common mutation was S207N at 71%, followed by L216V and A194V at 23%, P70H at 21%, L209V at 18%, P217L at 8%, F134L, E164D and T189I at 5%, S204R, S117N, T143S, G145R, Y206H, P127T, Y200T, F129T and K122R all at 3%. From the Durban cohort, the prevalence of mutations associated with drug resistance was 50% (7/14) within the RT region. Drug resistance mutations included LMV resistance at 57% (4/7), LdT at 57% (4/7), 14% (1/7) for ETF and 43% (3/7) for ADV resistance. Mutations causing resistance to LMV and LdT were M204V, L180M, V163I, and S202K; with S202K also being resistance to ETF and ADV resistance mutation were I253Y, I223V and M250I. The drug susceptibility prevalence was 68% (26/38). Multiple drug resistance mutations within a single sample were identified from sample QQ46 containing L180M, M204V, S202K and M250I mutations. All the mutations associated with drug resistance identified in the polymerase (RT) were identified from genotype A sequences. We have identified HBV genotypes in HIV-infected patients and the HBV mutations present in HBV/HIV co-infected individuals.

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LIST OF ABBREVIATIONS

C: Degree Celsius

µl: Microlitre

3TC: Lamivudine

Aa: Amino acid

ADV- Adefovir

AIDS- Acquired Immunodeficiency Syndrome

ALT- Alanine aminostransferase

Anti-HBc- Antibody to Hepatitis B core antigen

Anti-HBe- Antibody to Hepatitis B e antigen

Anti-HBs- Antibody to Hepatitis B surface antigen

BCP- Basal core promoter

bp- Base pair

cccDNA- Covalently closed circular DNA

CTL- Cytotoxic T lympocyctes

DNA- Deoxyribonucleic acid

DR- Direct repeats

dsDNA- Double-stranded DNA

Enh- Enhancer

EPI- Expanded Programme on Immunization

ER- Endoplasmic reticulum

ETV- Entecavir

FTC- Emtricitabine

HAV- Hepatitis A Virus

HBcAg- Hepatitis B core antigen

HBeAg- Hepatitis B e antigen

HBsAg- Hepatitis B surface antigen

HBV- Hepatitis B Virus

HBx- Hepatitis B x protein

HCC- Hepatocellular carcinoma

HCV- Hepatitis C Virus

HEV- Hepatitis E Virus

HIV- Human Immunodeficiency Virus

HLA- Human leukocyte antigen

IG- Immunoglobulin

IgG- Immunoglobulin G

IgM- Immunoglobulin M

kb- Kilobase

kDa- Kilodalton

Ldt- Telbivudine

LHBs- Large Hepatitis B surface protein

MHBs- Medium Hepatitis B surface protein

MHC- Major histocompatibility complex

MHR- Major hydrophilic region

ml- Millilitre

mRNA- Messenger RNA

MTCT- Mother to child transmission

NRTI- Nucleos(t)ide reverse transcriptase inhibitors

nm- Nanometre

ORF- Open reading frame

PCR- Polymerase chain reaction

pgRNA- Pregenomic RNA

Pre-C- Pre-core

Pre-S- Pre-surface

Pol- Polymerase

qPCR- Quantitative PCR

rcDNA- Relaxed circular DNA

RNA- Ribonucleic acid

RT- Reverse transcriptase

ssDNA- Single stranded DNA

TDF- Tenofovir disoproxil fulmarate

ULN- Upper limit of normal

WHO- World Health Organization

LIST OF NUCLEOTIDES

A- Adenine

C- Cytosine

G- Guanine

T- Thymine

LIST OF AMINO ACIDS

A- Alanine

C- Cysteine

D- Aspartic acid

E- Glutamic acid

F- Phenylalanine

H- Histidine

I- Isoleucine

K- Lysine

L- Leucine

M- Methionine

N- Asparagine

P- Proline

R- Arginine

S- Serine

T- Threonine

V- Valine

W- Tryptophan

Y- Tyrosine

CHAPTER 1

1. BACKGROUND AND RESEARCH PROPOSAL

1.1 Background

The bloodborne infections caused by hepatitis B virus (HBV), hepatitis C virus (HCV) and Human Immunodeficiency Virus currently, represent a major public health problem that requires resolving (Giordano *et al.*, 2018). Indicated by the staggering increase in the number of people infected with HIV (33 million), HBV (350 million) and HCV (170 million) globally (WHO, 2017). From 36 million people infected with HIV in 2015, close to 4 million people were reported to be co-infected with HBV and 5 million people were co-infected with HCV (Hebo *et al.*, 2019).

The HBV/HIV and HCV/HIV co-infections are common owing to shared route of transmission among these viruses through blood. The continent of Africa is having the highest prevalence of HIV infections, with South Africa having the largest number of people administering antiretroviral (ARVs) drugs introduced in South Africa on April 2004 and is also an HBV endemic area (Bland, 2011; Venter *et al.*, 2017). More than 2.4 million people in South Africa have successfully received ARV treatment which has reduced HIV related deaths, enhanced the quality of life and prevented mother-to-child HIV transmission by suppressing the HIV viral progression (Venter *et al.*, 2017).

Even though antiretroviral therapy (ART) has substantially reduced the progression of HIV infection to AIDS, there is still a challenge of the HIV/AIDS related deaths caused by opportunistic pathogens such as *Cytomegalovirus*, *Pneumocystis jirovecii*, *Mycobacterium avium* complex, *Toxoplasma gondii*, *Cryptococcus neoformans* and high virulence opportunistic pathogens including hepatotrophs to thrive (Ayoade and Chandranesan, 2018).

However, there are limited reports on the association on co-infections of the major hepatotrophs namely; hepatitis B virus (HBV) and hepatitis C virus (HCV) among HIV infected people in South Africa.

The sub-Saharan region was reported to have the second largest prevalence of hepatitis B and hepatitis C infections after Asia (Sonderup *et al.*, 2017). Over the years the co-infection of HBV and HCV in HIV-infected persons has been previously studied extensively and has been reported that HIV influences the rapid progression of hepatitis infections into liver fibrosis and malignancy (Lacombe *et al.*, 2017).

Whilst the impact of HBV on HIV pathogenesis is unclear, it is suggested to be worse and there is contradictory evidence about the impact of HCV on the HIV pathogenesis (Matthews *et al.*, 2014). In most Sub-Saharan regions, including here in South Africa HIV/HBV co-infections substantially out-number HIV/HCV co-infections (Andersson and Van Rensburg, 2011; Matthews *et al.*, 2014; Musyoki *et al.*, 2015; Tathiah, 2015).

Sub-Saharan Africa accounts for 70% of HIV infections globally and has a high seroprevalence of chronic HBV infection because of perinatal and early childhood transmission patterns (Croome *et al.*, 2017) . Chronic HBV co-infection was reported in up to 36% of all HIV-positive persons, with West Africa and Southern African cohorts having the highest prevalence (Spearman *et al.*, 2017) . HCV co-infection is less common in Sub-Saharan regions and the explanation is still unclear but it is reported to be reflecting the magnitude of injecting drug use (Sonderup *et al.*, 2017).

Serological assays such as immunoblotting or enzyme-linked immunosorbent assay were reported to be relevant to use on detecting HIV, HBV, and HCV markers because of their inexpensive and easy to perform (Nguyen *et al.*, 2011). Nucleic acid testing (NAT) by detecting the viral loads using real-time PCR (qPRC) and genotyping of HCV 5’ UTR, core

and NS5B; HBV BCP and pre core regions by direct sequencing are considered the most successful and reliable methods to determine the prevalence of HBV and HCV in HIV-infected persons (Shin *et al.*, 2018).

Mixed infection with HCV and HBV in HIV-infected persons in South Africa has been reported uncommon. The HBV vaccine is given to the infants at 6 weeks, 10 weeks and 14 weeks after birth and fails to protect the infant who had HBV vertically transmitted to them during birth (Bittaye *et al.*, 2019)

In South Africa, there is no routine screening of HCV in HIV-infected people in most rural areas. More-over, among pregnant women due to financial constraints and also may be due to low prevalence of HCV in this region. However, it is important to continue screening for HCV in HIV-infected pregnant women; they pose a risk for a mother to transmit HCV to her baby.

Currently, the prevalence of HIV-infected pregnant women co-infected with both HBV and HCV is uncertain. There is a need for such reports because liver diseases by these two hepatotrophs have emerged as a major cause of death in HIV-infected patients. In addition, studying the prevalence and association of these three viruses is important for epidemiology and treatment of these mixed infections.

1.2 Problem Statement

Blood borne infections caused by HBV, HCV and HIV represent a major public health problem globally due to limited management and treatment care of these viral infections. However, the efficiency of transmission, risks of transmission and genotypes of these viruses differ geographically. There is extensive data on the HBV, HCV prevalence and genotypes in HIV-infected people globally; however, this data cannot be extrapolated to South Africa context.

There is limited data on the prevalence of HBV and HCV in HIV-infected people and the insufficient reports are making it difficult to quantify the true burden of HCV and HBV prevalence in HIV-infected people moreover, among pregnant women in South Africa and identify the clear risks associated with these mixed infections. Identification of mono HBV and HCV genotypes in patients infected with hepatitis have been reported in South Africa.

There is still limited data on the HBV and HCV genotypes circulating in HIV-infected individuals in the North West Province and KwaZulu-Natal, these two provinces have the highest number of people infected with HIV suggesting that co-infection with HBV and HCV might be present. Co-infection of HBV and HCV in HIV infected people may hinder the efficiency on treatment and management of these viruses in context of co-infections and result in progressive HIV replication and death in HIV-infected patients.

1.3 Aim of the Study

The aim of this study was to determine the molecular epidemiology of HBV and HCV from HIV-infected individuals from Durban, KwaZulu-Natal and Mahikeng, North West Province.

1.4 Objectives of the Study

1.4.1 Determine the serological markers of HBV and HCV in HIV-infected individuals from the Durban and Mahikeng cohorts.

1.4.2 To determine molecular epidemiology of HBV and HCV among HIV infected individuals from Durban and Mahikeng cohorts.

1.4.3 To molecular characterize of HBV, HCV and HIV mutations in coinfected individuals

1.4.4 Identify and characterize HBV mutations associated with vaccine escape and drug resistance

1.4.5 Determine and characterize HIV mutations associated with drug resistance

1.5 Significance of the Study

Based on the literature review there is limited data on the recent epidemiology of HBV and HCV confections in HIV infected pregnant women as compared to HIV non-infected women in the North West Province and the general population from KwaZulu-Natal regardless of the two provinces having the highest HIV infections and HBV being endemic to South Africa. Knowing the prevalence of HBV, HCV in HIV infected patients is important to understand the epidemiology of the disease.

Based on these conditions this study sought to determine the magnitude of HBV/HIV and HCV/HIV co-infections in HIV-infected pregnant women from the North West Province (Mahikeng) and the general population from KwaZulu-Natal Province (Durban) to come up with information that will contribute to the current literature. Moreover, studying the prevalence and association of these three viruses is important for epidemiology and treatment of these mixed infections.

Therefore it is of great importance to identify HBV, HCV genotypes in HIV-infected people and variants associated with drug resistance to generate epidemiology knowledge relevant to South Africa, so that understanding on the effect of the two or three viruses can be achieved which will aid in developing better and improved treatment and management of these viruses in context of co-infections.

CHAPTER 2

2. LITERATURE REVIEW

2.1 Virology of the Human Immunodeficiency Virus

2.1.1 HIV Classification

HIV is classified into two types, HIV type-1 (HIV-1) and HIV type-2 (HIV-2); both viruses belong to the *Lentivirus* genus and are members of the non-oncogenic Retroviruses affiliated with the *Retroviridae* family (Zajac, 2018). Retrovirus members of the *Lentivirus* genus cause chronic diseases associated with long incubation periods and not directly implicated in any malignancies.

Lentiviruses share common structural, biological and genomic properties. The Retrovirus unique genome organisation consist of the two identical copies of single-stranded, positive-sense RNA (+ssRNA) which upon entry into host cell is converted into double-stranded DNA (dsDNA) by a viral encoded reverse transcriptase (RT), integrated into the host DNA and the virus DNA might become latent. *Lentiviruses* infect a wide range of vertebrate hosts from apes, cow, monkeys, e.t.c. with HIV being common to human primate and SIVin non-human primate hosts (Sharp and Hahn, 2011)

Both HIV-1 and HIV-2 originated from West and Central Africa from non-human primates simian immunodeficiency virus (SIV) (Filippone *et al.*, 2017). Transmission was obtained by humans during the early 20th century through numerous zoonotic transmissions (Zajac, 2018).

The genome sequence analysis showed that HIV-2 is related to SIV from Sooty mangabeys (SIVsmm) also known as (*Cercocebus atys*) (Palesch *et al.*, 2018).

The HIV-2 consists of eight identified genetic groups A to H, circulating recombinant forms (CRFs) such as HIV-2_CRF01_AB and the A and B strains are predominantly circulating in

the HIV-2 endemic areas (Ibe and Sugiura, 2013). The HIV-1 originated from the evolution of SIV from chimpanzees (SIVcpz), a virus that infects wild chimpanzees in Southern Cameroon (Palesch *et al.*, 2018).

2.1.2 Mature HIV-1 Virion Morphology

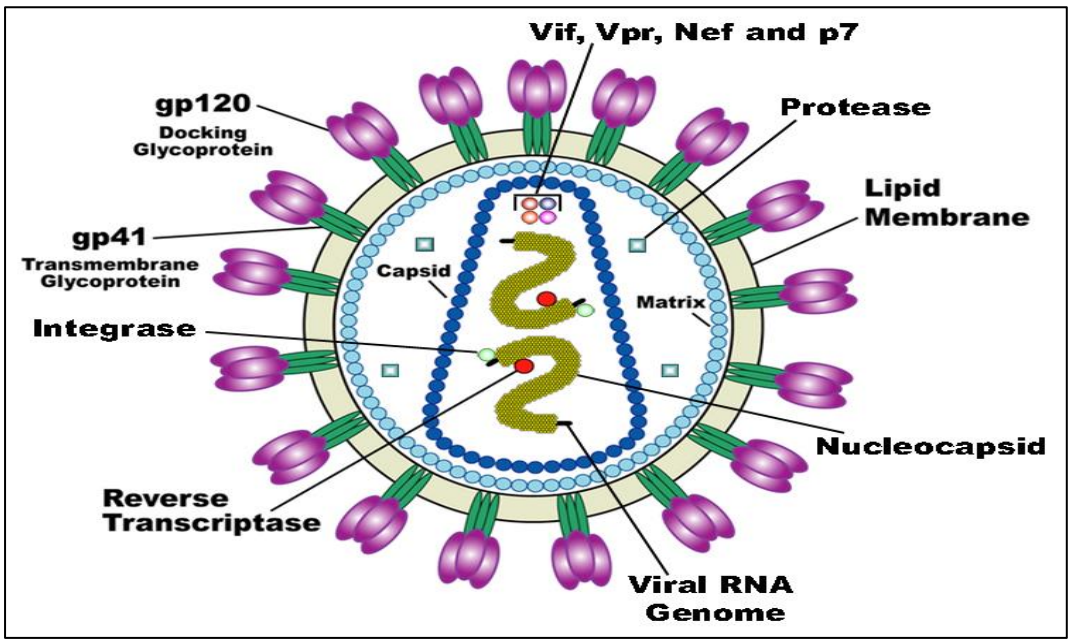

Figure 2.1: Schematic diagram of mature HIV-1 viron. This figure was obtained from: http://www.infohow.org/science/biology-ecology/hiv-viron/. Accessed 2019-06-22

HIV virion is around 129 nanometer (nm) in diameter has a roughly spherical shape and contains two +ssRNA enclosed by a conical capsid made up of the viral protein p24 (Figure 2.1) (Lu *et al*., 2011). Inside the capsid contains +ssRNA molecule bound to the nucleocapsid protein P7 and P9 as well as reverse transcriptase, protease, ribonucleases, integrase and some copies of accessory vif, vpr, nef proteins and $tRNA^{lys3}$ (Lu *et al.*, 2011). The capsid is surrounded by a nucleocapsid with p17 protein matrix, which in turn lines the inner surface of the viral membrane (Kirchhoff, 2013) . The capsid is enclosed in a phospholipid bilayer envelope with spikes of glycoproteins derived during budding from the host cellular

membrane (Arthur *et al.*, 1992). The bilayer contains external gp120 which mediates viral attachment and gp41 transmembrane that allows viral infusion (Chan *et al.*, 1997).

2.1.3 HIV Genome

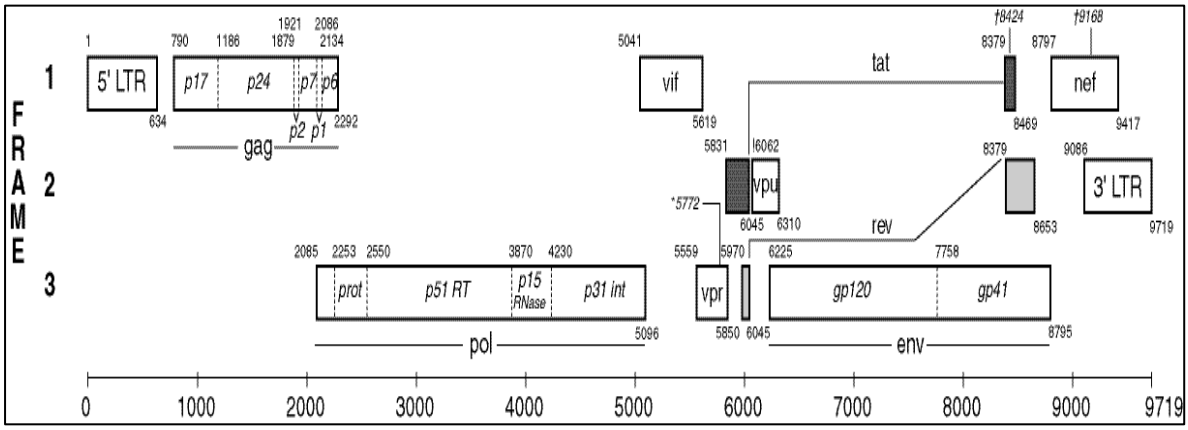

Figure 2.2: The complete HIV-1 genome organisation HXB2 (K03455). The open rectangles represent the open reading frames, the gene start, indicated by the small number in the upper left corner of each rectangle, adapted from (Freed, 1998).

The HIV genome is composed of two identical copies of non-covalently linked, unspliced +ssRNA. The RNA component is approximately 9.7 kilo base (Kb) nucleotides long containing 9 genes (Wain-Hobson *et al.*, 1985). The 9 genes can be classified into three structural genes group-specific antigen, polymerase and envelope (*gag*, *pol* and *env*), two regulatory genes Trans-Activator of Transcription and Regulation of HIV gene expression (*tat* and *rev*) and accessory genes viral protein U, Viral Protein R, Viral infectivity factor and Negative Regulatory Factor (*vpu*, *vpr*, *vif* and *nef*) (Figure 2.2) (Cohen *et al.*, 2008). The genome is flanked at both ends by the 634 base pair (bp) long terminal repeats (LTR) sequences containing U3, R and U5 regions and numerous open reading frames (ORFs). The 5´-end of the LTR sequence acts as a promoter coding for the transcription of the viral genes after the provirus is integrated into the human genome, whilst the LTR sequence at the 3´-end is responsible for the addition of a Poly (A) tail to the messenger RNA (mRNA) (Castelli *et al.*, 2002).

Table 2.1: HIV-1 structural and regulatory encoded proteins and their functions

Gene	Protein	Function
Gag	p24 caspid protein (CA)	Involved in the making of a conical capsid
	p17 matrix protein (MA)	Making the inner membrane
	p7 nucleocaspid protein (NC)	formation of the nucleoprotein
	p6	Involved in budding of viral particle
Pol	p66, p51 (reverse transcriptase)	Forms ds DNA copy from a ssRNA strand
	p15 (RNase H)	Degrade RNA template in the DNA-RNA hybrids
	p32 (Integrase)	Insert proviral DNA into host genomic DNA
Env	gp120(surface glycoprotein) (SU)	Allows the attachment of virus to the host cell
	gp41transmembrane	Fusion of viral and host cell membrane
Rev	p19	Facilitate the export of spliced and unsliced mRNAs
Tat	p17 transcription activator	Activate the transcription from LTR
Nef	p27 regulating factor	Down regulate surface expression of CD4 receptors
Vif	p23 infectious protein	Increase the infectivity of HIV particle
Vpr	p15 virus protein r	Tethering the viral genome to the nuclear pore
Vpu	p16 virus protein unique	Modulate intracellular trafficking
Vpx	p15 virus protein x	Involved in early step of HIV-2 replication
Tev	p26	Regulate the activation of Tat and Rev in a nucleus

Adapted from (Cohen *et al.*, 2008).

The *Gag*, and *Env* genes code for viral structural proteins whilst the *Pol* gene encode for functional proteins (Mushahwar, 2007). The *Gag* gene code for precursor (Pr55) which is cleaved by viral protease into four proteins, outer membrane (MA, p17), capsid protein (CA, p24), the nucleocapsid (NC, p7) and p6 (Lel, 2018). The second ORF, polymersase (pol) gene sequence code for the enzymes protease (PR, p12), reverse transcriptase (RT, p51), ribonuclease H (RNase H, p15) and integrase (IN, p32). The pol reading frame is followed by the adjacent envelope gene reading frame coding for the envelope precursor (Pr160) which is cleaved into two glycoproteins gp120 (surface protein, SU) and gp41 (transmembrane) (Rajarapu, 2013).

The small ORFs code for two regulatory proteins (tat and rev) and four accessory proteins (nef, vif, vpr and vpu) and HIV-2 codes for vpx (virus protein x) instead of vpu (Costin, 2007; Cohen *et al.*, 2008; Votteler and Schubert, 2008). Transactivator protein (tat) and RNA splicing-regulator (rev) are responsible for initiation and enhancing HIV replication. The functions of structural, regulatory and accessory proteins encoded by the 9 genes are detailed in (Table 2.1).

2.1.4 Heterogeneity of HIV

During the reproductive cycle of HIV, several errors introduce genetic variation due to the lack of proofreading of nucleotide bases by the reverse transcriptase. HIV-1 has shown extensive diversity. Phylogenetic analysis of HIV samples has led to the classification of HIV-1 into four groups: main group (M), outlier group (O), non-M-non-O group (N) and P group (P) (Vallari *et al.*, 2011).

Each group arose from an independent transmission of SIVcpz from non-human primates into humans. HIV-1 group M viruses are responsible for the majority of HIV infections globally, with HIV group O and N causing minor epidemics in Central Africa (Buonaguro *et al.*,

2007). HIV-1 group P was isolated in 2009 from a Cameroonian woman residing in France (Plantier *et al.*, 2009).

HIV-1 group M represents the predominant group responsible for the pandemic, and diversified into 9 subtypes designated by the letters (A, B, C, D, F, G, H, J to K), sub-subtypes (A1, A2, F1 and F2); 72 CRFs, unique recombinant forms (URFs) and second generation recombinants (SGRs) (Wolf *et al.*, 2003). Within Group M, the average inter-subtype genetic amino acid variations variability is 15% for group-specific antigen (Henquell *et al.*) gene and intra-subtype of 25% for the envelope gene (Rambaut *et al.*, 2004). This variation has led to the split of virus strains into sub-subtypes. For an example, subtype A has been subdivided into sub-subtypes A1, A2, A3, A4, and A5 and subtype F into F1 and F2 (Lihana *et al.*, 2012).

HIV subtypes A, B and C are predominant HIV-1 genetic variants globally, with subtype C responsible for 50% of all HIV-1 infections. There is an uneven distribution of HIV subtypes and CRFs throughout the world, with some genetic forms becoming dominant in certain geographic regions. Subtype A was originally common in West Africa but has since had temporal trends on HIV-1 epidemics identified in Russia (Tebit and Arts, 2011). CRF02_AG is dominating in Western Africa (Tongo *et al.*, 2016).

Subtype B predominates in Western and Central Europe, in North America, in Australia and it was also common in South East Asia. It accounts for 66% of HIV infections in America, 12% in Europe and 10% in East Asia (McCutchan, 2006). HIV-1 subtype B has been identified among the South Africans and Russian homosexual men (Buonaguro *et al.*, 2007). There has been an increase in the prevalence of HIV-1 non-B strains in Western countries due to infected immigrants' invasion with subtype C from Asia and Africa (Bredell *et al.*, 2002; Tebit *et al.*, 2007).

HIV-1 Subtype C is dominant in the Southern African region, in India, in Brazil, and the Southern province of China (Hemelaar *et al*., 2006). The epicentres of HIV-1 subtype C have been verified in Southern Africa namely, in Botswana, Zimbabwe, Malawi, Zambia, Namibia, South Africa, Lesotho and in Swaziland (Selabe *et al*., 2007; Velayati *et al*., 2007). HIV subtype C strains have been identified in South Africa (van Harmelen *et al.*, 2001; Hemelaar *et al.*, 2006). Subtypes, sub -subtypes and recombinants which have been reported in South Africa include A1, B, D, G, BC recombinants, URF_AD, URF_AC and complex URFs (Iweriebor *et al.*, 2011; Jacobs *et al.*, 2014; Wilkinson *et al.*, 2015). Diversification of HIV-1 has caused changes in HIV geographic distribution and epidemiology with CRFs evolving in Africa regions such as CRF02_AG which is dominating in Cameroon, Ghana, Cote d'Ivoire and in Senegal. CRF06_cpx is common in Burkina Faso (Wilkinson *et al.*, 2015). CRF are defined as the identical recombinants identified at least in 3 epidemiological unlinked people and is characterized by full length genome sequencing (Thomson *et al.*, 2002).

Africa is an area of very high HIV endemic, the majority of the HIV-1 subtypes have been identified in sub-Saharan Africa and in Congo Basins with limited data on subtype G, H, F and J (Tongo *et al*., 2016). However, subtype F has been identified in Central Africa, subtype G being prevalent in West Africa, subtype J reported in Central, in North and West Africa and subtype K is limited to the Democratic Republic of Congo (Montavon *et al.*, 2000; Vidal *et al.*, 2000).

Subtype D accounted for approximately 40% infections in East and Central Africa. There is insufficient data on the prevalence of subtype E which has been renamed as CRF01_AE which was reported in Central Africa, Thailand, China, the Philippines and in Vietnam among infected intravenous drug users (IDUs) (Woodman and Williamson, 2009). The CRFs plays a significant role in the global pandemic accounting for 18% of infections (Lau and

Wong, 2013). Similarly, CRFs influenced the regional epidemics, with CRF02_AG common in West and in Central Africa being responsible for close to 75% of the circulating strains in Cameroon and CRF01_AE dominant in South East Asia (Fischetti *et al.*, 2004).

HIV-1 genome mosaic forms have been reported in Africa such as CRF_06_cpx in Burkina Faso, in Mali and CRF09_cpx in Senegal (Montavon *et al.*, 2002). High level of genetic variability of HIV-1 may have an important implication for pathogenesis, transmission, treatment and vaccine development. Genetic heterogeneity of HIV may challenge diagnosis and treatment by affecting the sensitivity and specificity of serological and molecular diagnosis assays which may increase spreading of unidentified infections.

HIV-1 genetic variants pose different pathogenesis and immunological properties demonstrated by subtype A which uses CCR5 for attachment into host cell whilst subtype D use CXCR4 co-receptor (Clapham and McKnight, 2001). In terms of transmission, subtype A has been reported to be associated with higher risk of vertical transmission as compared to subtype D (Renjifo *et al.*, 2001).

It was reported that HIV-1 subtypes A, C and D from Sub-Saharan Africa are better adapted for heterosexual transmission than subtype B which is effectively transmitted by IDU and homosexual route (Kliks *et al.*, 2000). It is necessary to continue monitoring the evolution and spread of HIV-1 in South Africa and world-wide. Understanding HIV-1 diversity in South Africa will play an important role in HIV-1 prevention strategies.

2.1.5 Epidemiology/ Distribution

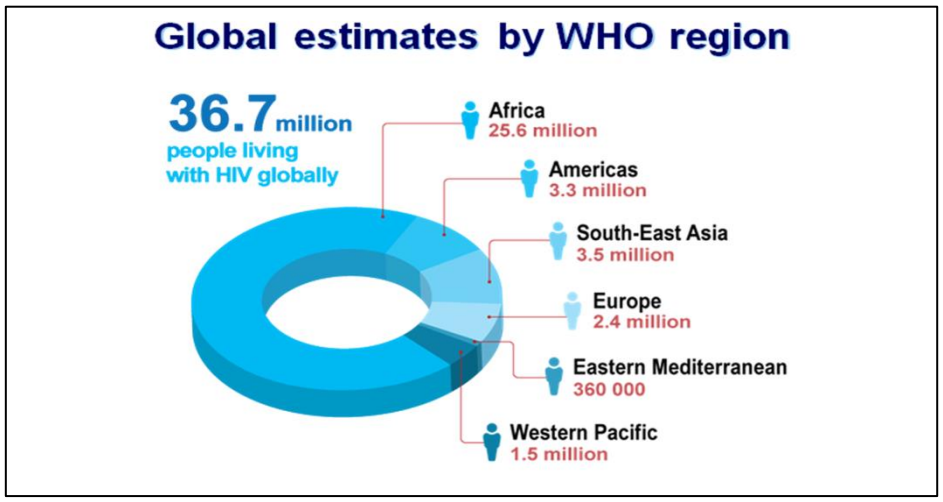

Figure 2.3: The HIV global prevalence and distribution (UNAIDS, 2017).

In 2016 an estimated 36.7 million people were infected with HIV worldwide, with 25.6 million people in Sub-Saharan Africa, 3.5 millions in South East Asia, 3.3 in America, 2.4 million in Europe, 1.5 million in Western Pacific and 360 000 people in Eastern Mediterranean (Figure 2.3). Although Africa is hit the hardest by the HIV epidemic, the prevalence rates vary geographically which can be divided into regions of low, intermediate and high endemicity. The prevalence of HIV/AIDS in Africa was determined as the total percentage of the population aged 15-49 years in 2011 (Figure 2.4), with the North Africa region having a low prevalence of HIV with 0.2% reported in Morocco (UNAIDS, 2017).

Eastern and Central African countries had moderate to high HIV incidence, ranging from 2.1% in Angola, 5.0% in Gabon to 7.2% in Uganda (Yamaguchi *et al.*, 2004). Although Western Africa is dominated by HIV-2, HIV-1 is endemic in some regions, with an estimated 7% prevalence reported in Nigeria and in Cote d Ivore (Velayati *et al.*, 2007). The Southern Africa countries have the highest HIV epidemics with four countries out of nine having a prevalence rate greater than 15% including in Swaziland (26%), Botswana (23%), Lesotho (23%) and in South Africa (17%) (Selabe *et al.*, 2007).

South Africa had the highest epidemic in the world with 7.1 million people infected in 2016, associated with 270, 000 new infections and 110,000 AIDS-related deaths (WHO, 2016b) . HIV prevalence in South Africa is variable among the 9 provinces, with the highest rate reported at 40% in Kwa-Zulu-Natal (KZN), 35% in Mpumalanga, 30% in North West and Gauteng, and the lowest being 17% in Western Cape (Figure 2.5) (Shisana *et al.*, 2014; Msimanga *et al.*, 2015) (Msimanga *et al.*, 2015; Shisana *et al.*, 2014). Sexual route is the main method of HIV transmission in South Africa and includes male-to-female, male-to-male, with the male-to-male HIV prevalence reported to be approximately 26.8%. The risk of HIV infection by sex workers is estimated at 57.7% and the percutaneous transmission is limited. Mother-to-child HIV transmission (MTCHT) is another contributing factor of HIV transmission in South Africa due to inefficiency of antenatal therapy and feeding contaminated breast milk with 12,000 infections due to MTCHT (UNAIDS, 2017).

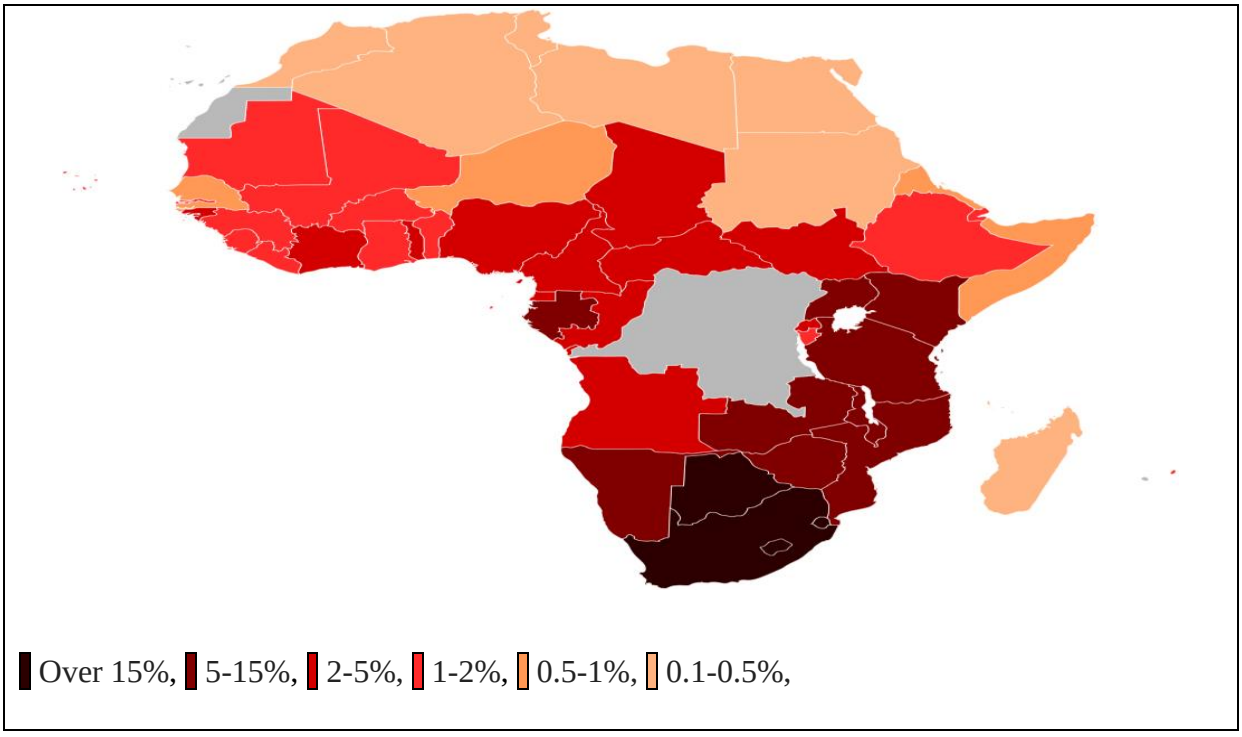

Figure 2.4: HIV prevalence in Africa (total % of population ages 15- 49), adapted from (Tambo *et al.*, 2016).

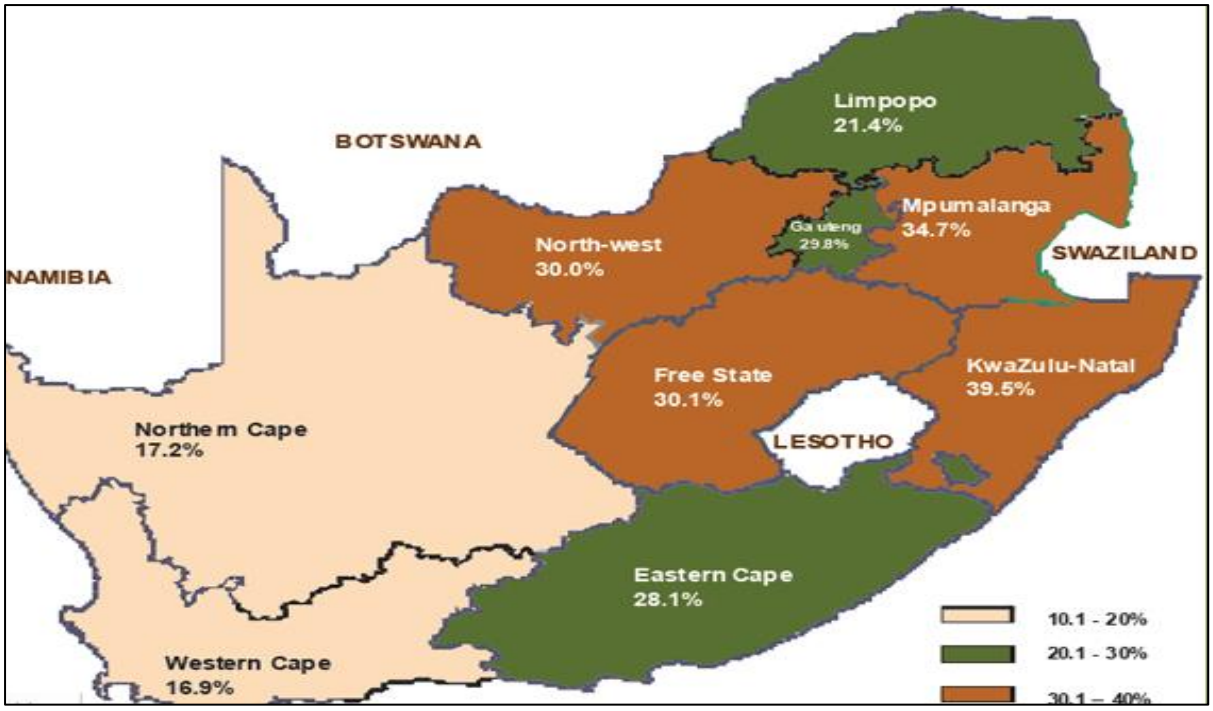

Figure 2.5: HIV prevalence in South Africa within the nine different provinces. Adapted from (Shisana *et al.*, 2014).

2.1.6 Transmission

HIV transmission is through percutaneous and mucous membrane exposure to infectious body fluids such as blood; other body fluids such as semen, pre-seminal, rectal, anal, vaginal fluids and breast milk (Alter, 2006). These fluids must come in contact with a mucous membrane or damaged tissue or be directly injected into the bloodstream. The HIV routes of transmission include sexual, percutaneous and perinatal and while each route is associated with a distinctive risk of infection (Royce *et al.*, 1997).

The sexual routes are the main method of HIV transmission and include male-to-female, female-to-male, male-to-male and fellatio. HIV is sexually transmitted through exposure of epithelium membranes (vaginal, endocervical, rectal, glans, urethra and penis foreskin) (Figure 2.6). Parenteral transmission includes transfusion of HIV-infected blood and needlestick injuries, tattooing and circumcision.

Vertical HIV transmission is from mother-to-child; includes the risk of infection during antenatal therapy with azidothymidin (AZT) (Cohen *et al.*, 2008). Transmission of HIV-1 from mother to infant may occur in utero, intra-partum, or postpartum through breast-feeding. In KZN, South Africa, mother-to-child transmission (MTCT) accounts for 34% of HIV infections (Rollins *et al.*, 2002).

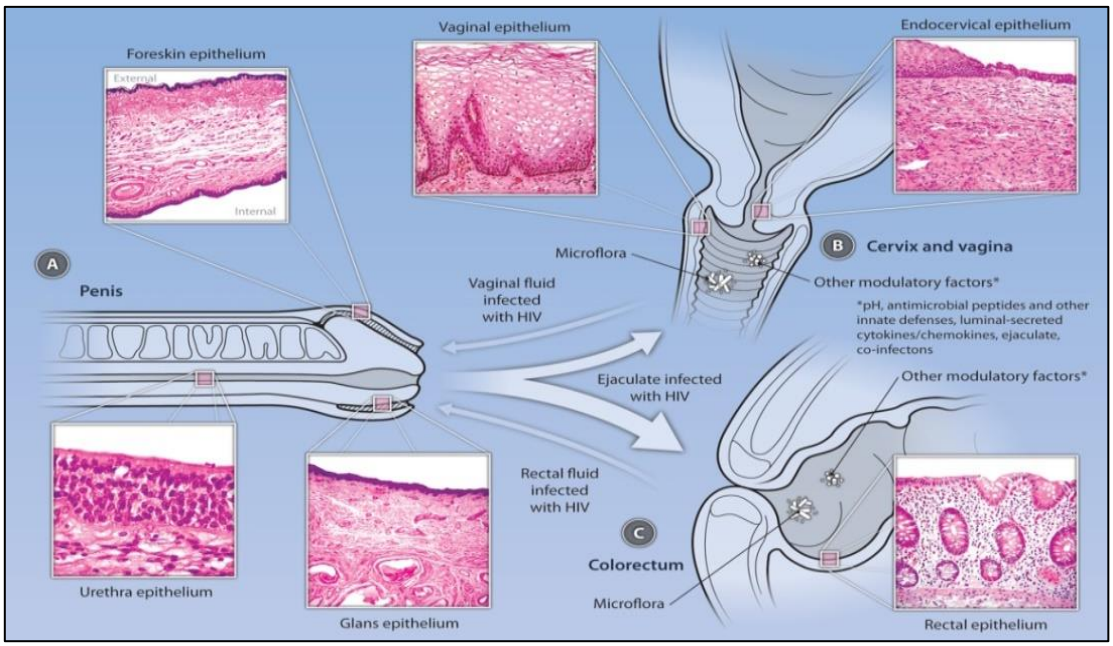

Figure 2.6: Different methods of heterosexual HIV transmission , adapted from (Anton and Herold, 2011).

2.1.7 Pathogenicity

There are 3 stages of HIV infection (i) acute HIV infection, (ii) chronic HIV infection and (iii) the AIDS syndrome (Figure 2.7). Acute HIV is the first stage of asymptomatic HIV infection and it normally develops within 4 weeks after exposure to HIV. Acute HIV infection is characterized by the high level of the viral RNA due to rapid viral replication. Two days after exposure to HIV, it can be detected in the regional lymphatic tissue and in the regional lymph nodes (Maher *et al.*, 2005). At 14 days post infection HIV can be detected in the whole blood. Between weeks 5 to 6, the humoral immune system responds against HIV

infection and produces antibodies to HIV and HIV p24 antigen is detectable, but the viral load in the whole body remains relatively high.

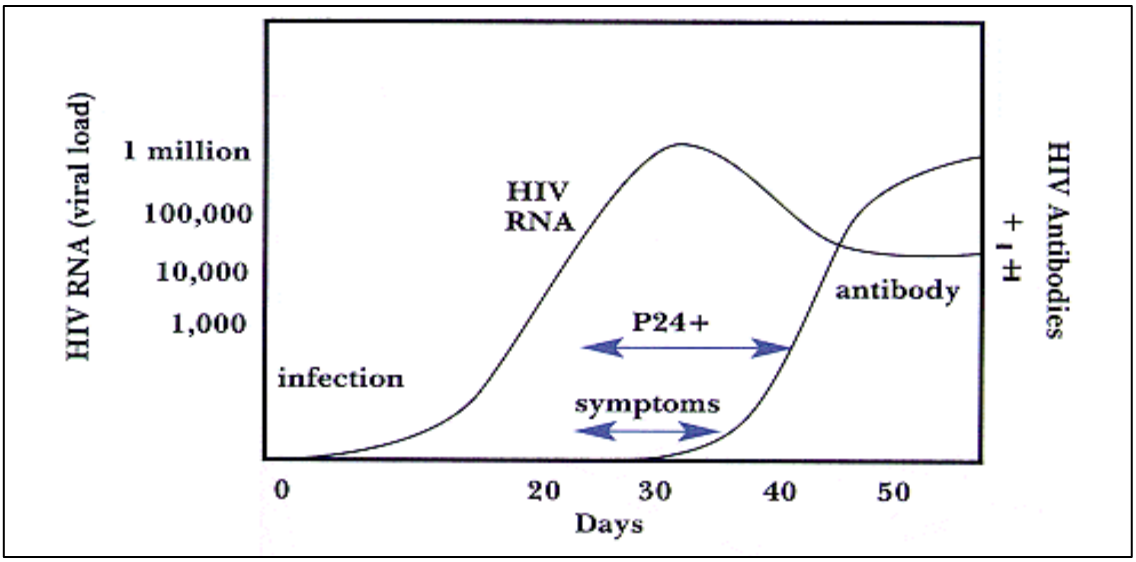

Figure 2.7: Phases of primary acute HIV-1 infection, adapted from (Rosenberg, 2002).

Within 5 weeks of HIV infection, specific antibodies in the plasma can be detected by commercially available antibody screening kits (Brochot *et al.*, 2013). At 4 weeks after exposure to HIV infection, previously uninfected individual exposed to HIV usually present nonspecific clinical symptoms such as fever, rash, pharyngitis, loss of appetite, weight loss, malaise, fatigue, nausea, headaches, infected gums, diarrhoea, genital and anal sores which can last close to 2 months (Figure 2.8).

The symptomatic acute HIV stage may be followed by asymptomatic latency stage also called chronic HIV infection, which is the second stage of HIV infection and this can last for many years characterized by the low level of the virus in the peripheral blood and high in plasma due to low viral replication. Chronic HIV- infected individuals may be asymptomatic but they can still infect other people. Chronic HIV infections may advance to AIDS in approximately 10 years.

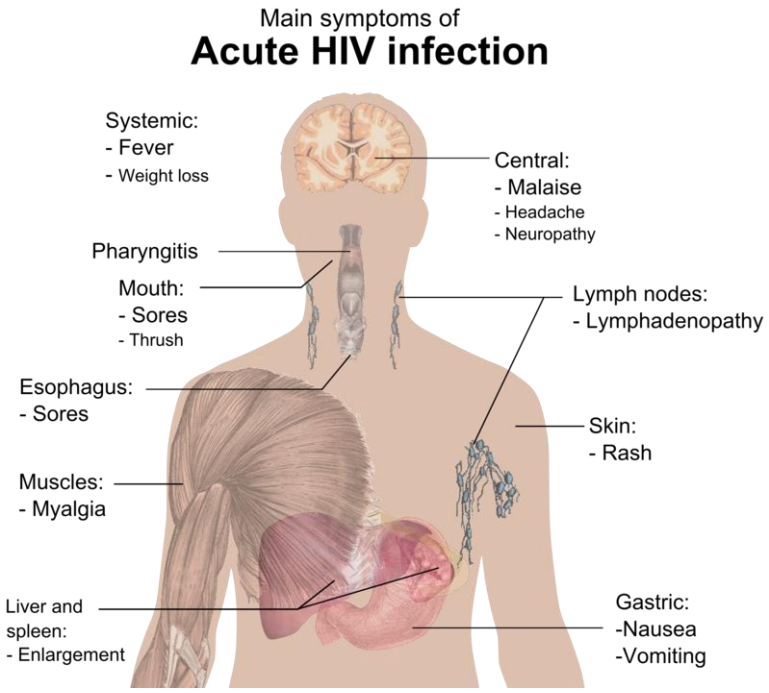

Figure 2.8: Clinical signs and symptoms displayed during acute HIV infection, adapted from (Kelley *et al.*, 2007).

The final stage of infection is known as AIDS during this stage HIV has weakened the immune system severely. HIV- infected people may be classified into categories A1-C3 based on clinical symptoms and CD4 cells counts (Brettle *et al.*, 1993). HIV- infected people are diagnosed with AIDS when they have CD4 cells count below 200 cells/ mm^3 and during this stage individuals are at a greater risk of developing susceptibility to opportunistic infections (Chibatamoto *et al.*, 1996). Symptoms may include persistent thrush, weight loss, fever, diarrhoea, may increase to Kaposi's sarcoma, tuberculosis neurologica dysfunction, *Cryptosporidium parvum, Pneumocystis jirovecci* (Hays and Shapiro, 1992). Concurrent infection with opportunistic microorganisms such as hepatotrophs (HBV, HCV) results in fast progression of HIV infection into AIDS (Ockenga *et al.*, 1997).

2.1.8 Diagnosis

Combination of immunological and molecular assays is used to detect HIV and diagnose HIV infections. Acute HIV-1 infection is characterized by HIV-1 RNA or p24 antigen in serum or plasma using commercial immunoassays kits. Samples that are reactive on an initial antigen/antibody (Ag/Ab) assay should be tested with an immunoassay that differentiates HIV-1 from HIV-2 antibodies and HIV-p24 antigens. However, the immunoassays have limited specificity and molecular techniques may be used to confirm HIV infections. Samples reactive to HIV-2 antibodies and HIV-p24 antigens should be tested for quantitative or qualitative HIV-1 RNA; a negative HIV-1 RNA test result indicates that the original Ag/Ab test result was a false positive because HIV-1 RNA levels in acute infection are generally very high (e.g., >100,000 copies/mL). Quantitative or qualitative HIV-1 RNA detection should be followed by sequencing of the HIV nucleotides.

2.1.9 Treatment

HIV infection or AIDS is treated and managed using ARV drugs and treatment of HIV infection by ART was begun by the use of zidovudine in 1987. The ART evolved with the development and approval of the administration of a combination of multiple ARVs drugs known as highly active antiretroviral therapy (HAART) in 1996 and in 2010, more than 20 ARVs were being used to treat and manage HIV infection (Ayuk, 2013).

South Africa has the largest ART programme worldwide with approximately 2.4 million people, reported to be on ART in 2013 (UNAIDS, 2017). With 85% of the ART administered through public health sector, 11% through private health sectors and 4% through non-government community treatment programmes. HAART provides maximal suppression of viral load, by suppressing viral replication to a level that can no longer be detected at <50 RNA copies/ ml with current blood tests. Other functions of HAART are to restore immune function, preventing opportunistic infections that can cause death and prolong life expectancy (Moore and Chaisson, 1999).

There are five classes of ART drugs available in South Africa which are nucleoside/nucleotide reverse transcriptase (NRTIs/NtRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase inhibitors (INSTIs), fusion inhibitors (FIs) and chemokine receptor antagonists (CCR5 antagonists). ARVs drugs are grouped according to drug inhibition mechanisms at different stages of the HIV-1 life cycle.

It was previously recommended that ART be initiated for all HIV- infected individuals with CD4+ cell < 350 cells/ ml (Donnell *et al.*, 2010). In Southern Africa, Initiation of ART is recommended for all HIV+ individuals regardless of their CD4+ count. Individuals with CD4+ count of 200 cells/ µl are at a risk of opportunistic infections and should be initiated on ART within a week of diagnosis (Ford *et al.*, 2017). The ARV drugs include a fixed dose combination (FDC), which reduces the burden of multiple pills. All HIV-infected adolescence and adults in South Africa are started on a first-line regimen including a daily uptake of 300 mg tenofovir (TDF), 300 mg lamivudine (3TC), 200 mg emtricitabine (FTC), and 600 mg efavirenz (EFV) at night. Nevirapine (NVP) is used on women who have contraindication to EFV or are pregnant; the NVP dosage is 200 mg daily for 2 weeks, followed by 200 mg intake every 12 hours (Chersich *et al.*, 2006).

TDF is being replaced by the use of stavudine (d4T) due to accumulation of toxicity however, TDF is still recommended in susceptible patients. Patients failing first line regimen are switched to 2nd-line regimen which consists of ritonavir-boosted PIs (atazanavir or lopanavir) and 2 NRTIs (zidovudine or TDF). The 2nd line with FDC in South Africa include TDF, 3TC/FTC and lopanavir/ritonavir (LPV/r) or AZT, 3TC and LPV/r, the dosage for LPV/r is 400/100 mg every 12 hours (Donnell *et al.*, 2010).

Similarly 3rd line regimen indicated for patients with PIs resistance should be recommended and it includes; PIs darunavir (DRV), INSTIs, dolutegravir (DTG), raltegravir (RAL), NNRTIs (etravirine (ETR), riipivirine (RVP), CCR5 blocker Maraviroc (MVC) (Hansoti *et al.*, 2017).

Selection of a new regimen for a patient failing the previous regimen should be determined by an experienced clinician and genotyping test should be determined to prevent ART inefficiency. ART drug resistance is a vital challenge to selecting and maintaining ART effectiveness in addition to presence microorganisms such as TB, HBV and HCV. ART efficiency must be monitored by measuring the viral load at baseline, successively at 3 month, 6 month and 12 month intervals. The efficiency of ART is defined by the decline in viral load to <50 copies/ ml within 6 months of administration whilst ART ineffectively is increased in viral load to >1600 copies/ ml with between 2-3 months of measure.

2.1.10 HIV-1 Life Cycle

Viral Entry: HIV targets active susceptible CD4+ T–helper cells which are key regulators of humoral and cellular immune response cells. Other cells targeted by HIV are macrophages, monocytes, glial and chromaffin cells (Kirchhoff *et al.*, 1995). HIV attachment and penetration are facilitated by cell tropism, binding and fusion of HIV-1 into CD4+ cells. HIV infection is initiated by the interaction of gp120 with the cellular CD4 receptor, binding of gp120 to CD4 receptor induce change in the envelope trimer allowing the interaction of gp120 with either CXCR4 (X4) or CCR5 (R5) co-receptors (Figure 2.9).

Interaction between gp120 and co-receptor causes conformation changes on the viral envelope, allowing gp41 transmembrane to insert hydrophobic fusion peptides into cell membrane. After the trimeric gp41 complex form helical bundle structure which pulls the

viral and cellular membranes together, thus allowing fusion which introduces the contents of the virion into the cytoplasm (Figure 2.7) (Wilen *et al.*, 2012).

Viral entry into the host cell is followed by uncoating of the viral capsid. The process of uncoating is the disassembly of the viral capsid before the import of the viral genome into the nucleus. The exact timing and mechanisms of uncoating is poorly understood and further studies are needed to fill this gap.

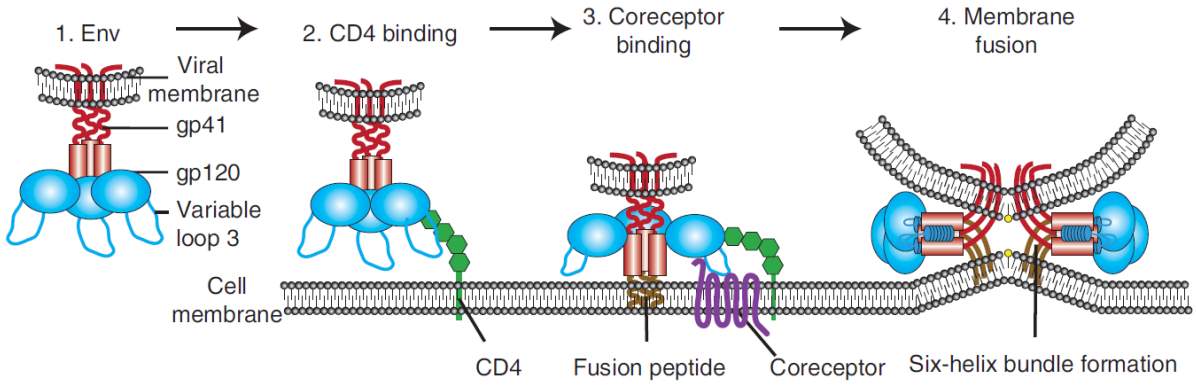

Figure 2.9: Overview of HIV entry, through the interaction of gp120 with the cellular CD4 receptor induce and CXCR4 (X4) or CCR5 (R5) co-receptors. Adapted from (Wilen *et al.*, 2012)

2.1.11 Transcription, Replication and Translation of HIV

The two +ssRNAs are converted into linear dsDNA by a process of reverse transcription (Figure 2.8, stage 4). The synthesis of the first DNA strand is initiated by the primer tRNAlys3 whose 3´ end is base paired to a complementary sequence near 5´ end of the viral RNA called the primer binding site (pbs) which is 180 nucleotides in size (Khorchid *et al.*, 2000). Once the primers binds to the RNA strand synthesis of the first DNA strand is initiated which results into a RNA-DNA duplex which is processed by a RNase H, degrading the 5´ end of the viral RNA, exposing the newly synthesised minus single -strand complementary DNA (-sscDNA) (Telesnitsky and Goff, 1993).

The -sscDNA is transferred to the 3´ end of viral RNA by use of direct repeat sequence called R. After the first strand-transfer, extension of –sscDNA occurs to copy the whole viral RNA resulting into a duplex of minus cDNA strands and viral RNA. RNase digestion occurs until the RNase resistance purine rich sequence (PPT). The PPT acts as primer for synthesis of plus strand strong stop cDNA (+sscDNA); +sscDNA forms a duplex with PBS region of tRNA primer, after digestion of PBS by RNAse H, +sscDNA is transferred to the 3´ end (2nd strand transfer).

The +sscDNA act as a primer to synthesis plus-strand cDNA resulting into a linear dsDNA molecule (Khorchid *et al.*, 2000). The newly synthesised dsDNA is transported across the nuclear membrane; the viral DNA is incorporated into cellular DNA by an integrase enzyme. The integrated DNA (proviral DNA) replicates along with the host DNA replication, the provirus can become infectious to other cells or remain latent for years in other cells such as the old memory T-cells (Figure 2.10, stage 5) (Sauter and Kirchhoff, 2016).

HIV-1 genome transcription is regulated by the 5´ LTR promoter located in the U3 region of 5´ UTR, regulatory proteins tat and rev, transcriptional factors (containing NF-kB and NFAt binding motif) (Nabel and Baltimore, 1987). The tat, transcriptional-responsive region (TAR) induce transcription by expression of the receptor; tat bind specifically to the specific sequence in the R region of 5´ LTR TAR and increase transcription under pTEFb control, allowing synthesis of full length HIV transcript, which includes unspliced 9 Kb mRNA, spliced 4 Kb mRNA and spliced 1.8 Kb mRNA. The 9 Kb encodes for gag and pol precursors, 4 Kb encodes for env, vif, vpr, and vpu and a multiply spliced 1.8 Kb encode for tat, rev and rev (Feinberg and Moore, 2002).

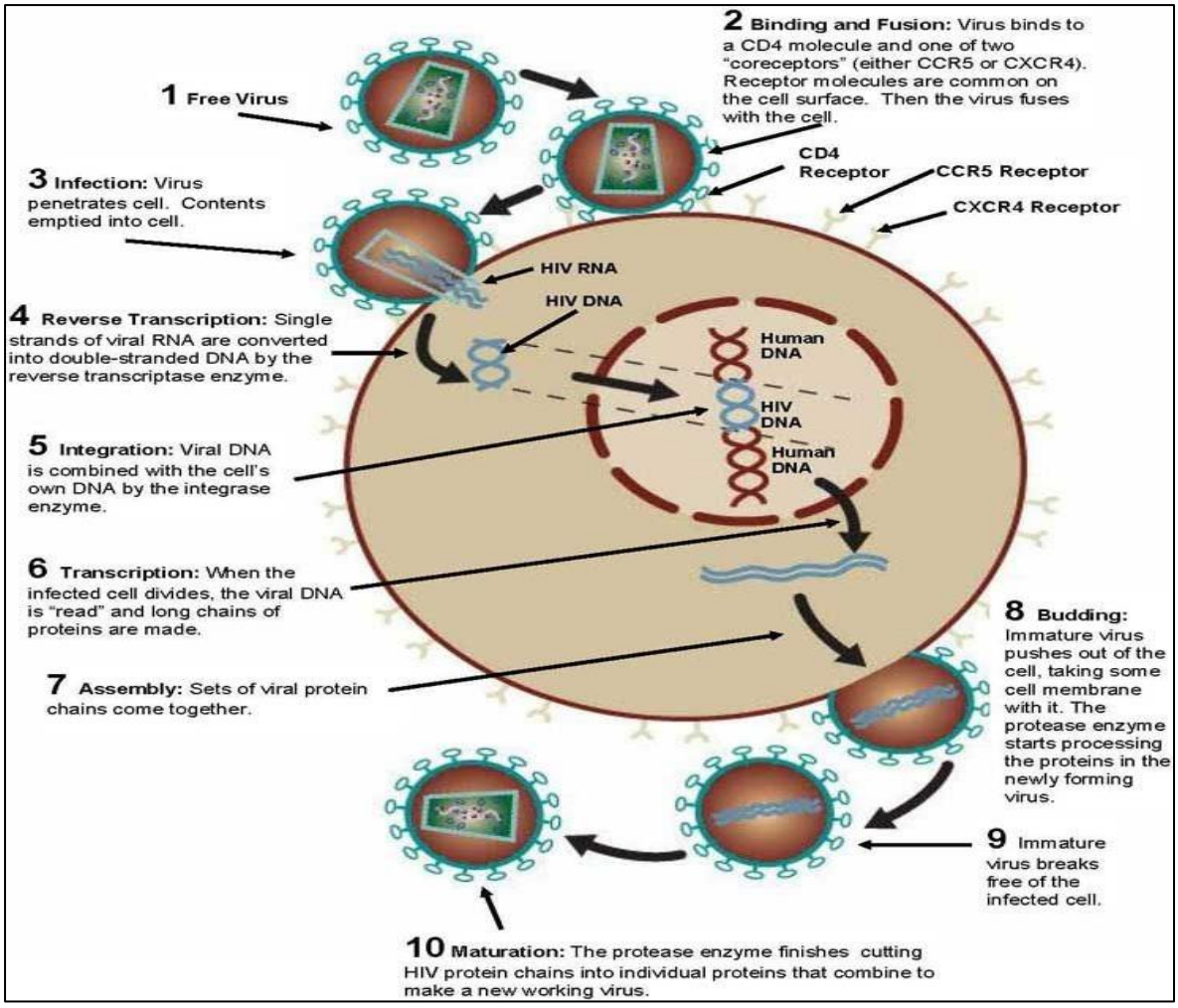

Figure 2.10: Schematic diagram of the HIV-1 life cycle, adapted from (Laskey and Siliciano, 2014).

The HIV-1 RNA spliced and unspliced transcripts are transported out of the nucleus via nuclear pore complex (Köhler and Hurt, 2007).The 9 Kb and 4 Kb mRNAs are transported by the rev-dependent export route whilst the completely spliced 1.8 Kb mRNA is exported to the cytoplasm by the normal mRNA export route in the absence of rev (Karn and Stoltzfus, 2012).

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The viral mRNAs are translated into viral proteins; gag-pol precursors are translated into major structural and enzymatic proteins which along with the genome RNA are assembled into new virus particles (Marr *et al.*, 1998).

2.1.12 Assembly and Release of HIV

HIV-1 virion assembly occurs at the plasma membrane mediated by the gag-pro-pol polyprotein. The virion assembly packages all of the components including gag-pro-pol, 2 copies of +ss RNA genome, cellular tRNALys3, envelope, gag, protease, reverse transcriptase and integrase.

The release of progeny virion from the infected cells is mediated by the association between the HIV-1 p6Gag and host endosomal sorting complex (ESCRT) machinery (Peel *et al.*, 2011). The HIV-1 p6Gag (domain of the p6 of gag) interact with the Tsg101 protein (a subunit of ESCRT) resulting in the recruitment of ESCRT factor which allows the HIV-1 to pinch off the host cell, process is illustrated in stage 8 (Figure 2.10) (Demirov *et al.*, 2002; Martin-Serrano and Neil, 2011) (Martin-Serrano and Neil, 2011; Demirov *et al*., 2002).

The HIV particles are released as immature non-infectious form characterized as arranged gag and gag-pol-precursor, after budding protease is activated as cleaves , gag and gag-pol-polyprotein at 10 different sites into mature molecules (MA, CA, NC, p6, PR, RT, and IN) (Hill *et al.*, 2005). These molecules initiate the configuration of proteins and formation of the dense conical core making particle infectious, shown in stage 9 (Figure 2.8) (Briggs and Kräusslich, 2011).

2.1.13 HIV Mutations caused by Reverse Transcription

The lack of proof reading activity by the viral reverse transcriptase during the HIV replication causes drug resistance mutations and immunological escape mutants. The risk of

development of HIV mutants is influenced by large heterogeneity of the HIV-1 population, rapid HIV replication in untreated patients during therapy (Ockenga *et al*., 1997; Coffin, 1995). There are polymerases that are crucial during HIV replication and can contribute to mutations found in HIV, such as DNA polymerase, host RNA poly II and viral RT. The mutation errors in genome replication are of great importance in the viral evolution, they allow virus mutants to escape host immune response. Therefore this results in changes which bear on the progression of the disease, virus replication and expression of viral proteins.

NNRTIs are group of compounds that are potent and specific inhibitors of HIV-1 replication. The NNRTIs consists of structurally dissimilar hydrophobic compounds that selectively bind to a hydrophobic binding pocket in the RT but distinct from the active sites to which accommodates deoxynucleoside triphosphates (dNTPs) and NRTIs (Domaoal and Demeter, 2004). NNRTIs compounds inhibit HIV-1 replication by displacing the catalytic aspartate residue and that leads to non-productive binding of the incoming nucleotide during DNA polymerization. HIV-1 resistance to NNRTIs is caused by mutations in the NNRTIs binding pocket which interfere with drug binding (Domaoal and Demeter, 2004). A single mutation might result into high-level resistance to 10 or more NNRTIs and may emerge rapidly when NNRTIs are administered separately as monotherapy.

Reverse transcriptase of HIV-1 is a heterodimer of p66 and p51 subunits of which p66 consists of polymerase and RNase H domains whilst p51 facilitates template primer binding. The p66 has a polymerase and RNase H active sites, p51 is derived from p66, by proteolytic cleavage and contains the first 440 amino acid residues of p66 it assumes a very different structure and does not have a catalytic active site (Kohlstaedt *et al.*, 1992). The NNRTIs binding pockets include residues from amino acids 100 to 108, 180-190, 220-240 in the RT (von Wyl *et al.*, 2010). Efavirenz is the most commonly used NNRTIs, because of its high tolerability, combination of efavirenz and nucleoside analogue has shown vital clinical

efficacy (Albrecht *et al*., 2001). K103 is the most frequently observed NNRTIs resistance mutation and causes between 20 to 50-fold resistances to each of the available NNRTIs (Albrecht *et al.*, 2001).

K103N is a common NNRTIs resistance mutation identified from patients failing efavirenz , other efavirenz-resistant mutants reported are G190A, G190S, V106A and Y181C; K103 variant also confer resistance to NVP (Corbett *et al.*, 2000). The M230L mutation was reported to be uncommonly causing resistance to NNRTIs at approximately 20-fold resistance to efavirenz and 40-fold to NVP (Soriano and Mendoza, 2002).

NRTIs are prodrugs that are triphosphorylated by host cellular enzymes; they are discriminated by the RT from competing with natural dNTPs for incorporation into newly synthesised viral DNA chain where they cause chain termination. This mechanism of resistance to NRTIs involves HIV with point mutation K65R, Q151M and M184V which cause diminished affinity of RT for specific NRTIs with little or no change in affinity for the corresponding dNTP substrate. The consequence is a diminished incorporation of drugs into the DNA chain (Marcelin *et al.*, 2004).

The common mutation occurring in HIV+ in people receiving NRTIs are the zidovudine resistance mutations which occurs at codon 41, 67, 70, 210, 215, 219 (K70R, T215Y, T215S/C/D). Amino acid variants at positions 65, 74, 113, M184I/V and K65R causes resistance to abacavir and >100 fold resistance to lamivudine (Marcelin *et al.*, 2004). K65R confers intermediate levels of resistance to didanosine, abacavir, zalcitabine and lamivudine (Angus *et al.*, 2003). The other biochemical mechanisms are mediated by mutations that increase the rate of hydrolytic removal of chain terminating NRTIs and enable continued DNA synthesis.

HIV-1 protease has 2 non-covalently associated monomers 99 amino acids in length. Mutations at 20 positions have been associated with PI resistance such as cleft, the flap which occurs during treatment with indinavir, nelfinavir, saquinavir, ritonavir, prenavir and lopanavir (Shafer *et al.*, 2007). However, there is limited information on PIs mutants evolving in HIV-infected people.

It is vital that drug resistance testing is performed before initiation and during ART. The data of drug resistance before starting a new drug regimen is an independent predictor of virological response to that regimen. HIV resistance testing includes phenotypic assays, both methods are required to identify the mechanisms of resistance to new drugs and drug combination. However, genotypic tests are used commonly in clinical settings and provide better insight into the population of drug-resistance variants.

2.2 The Virology of the Hepatitis B Virus (HBV)

2.2.1 Classification

Hepatitis B virus is an enveloped virus a prototype of the Hepadnaviridae family and the *Orthohepadnavirus* genus is important human pathogens, posing a significant global health problem. According to the Baltimore virus classification HBV belongs to the Class VII viruses, it contains a double-stranded DNA genome, they replicate via a ssRNA intermediate (Gust *et al.,* 1986). HBV is estimated to infect more than 300 million people worldwide and is a common cause of liver disease and liver cancer with approximately 1 million deaths from chronic liver disease and hepatocellular carcinoma (HCC) per year.

2.2.2 HBV Virion Organisation

The HBV virion is of 42 nm in diameter and consists of inimical relaxed and circular (RC-dsDNA) double-stranded DNA of 3.2 Kb genome and the virion is also known as the Dane particle (Figure 2.11a). Attached to the 5´-end of the (-) strand, is the viral DNA polymerase. The genome is bound by a capsid which encompasses of HBV core protein (HBcAg) (Summers *et al.,* 1975). The capsid is surrounded by an outer lipid envelope containing three varied size surface glycoproteins designated as small (S), middle (M) and large (L) HBsAgs (Hoofnagle *et al.*, 1973).

The surface proteins can appear in other forms known as HBV particles (Figure 2.11b), these occur when HBsAg get discharged from the virion envelope to produce sub-viral particles namely spherical and tubular particles (Figure 2.11b). As compared to the Dane particle, these HBV particles are smaller in diameter size (22 nm) but they vary in their length and they are not infectious because they are made up of envelope glycoproteins without nucleic acids. HBV particles are produced in high quantity and over the Dane particle (Lee and Locarnini, 2004) (Figure 2.11b). The virion also consists of the X protein and HBV e antigen (HBeAg) referred to as the non-particulate proteins (Figure 2.11a) (Kramvis, 2016).

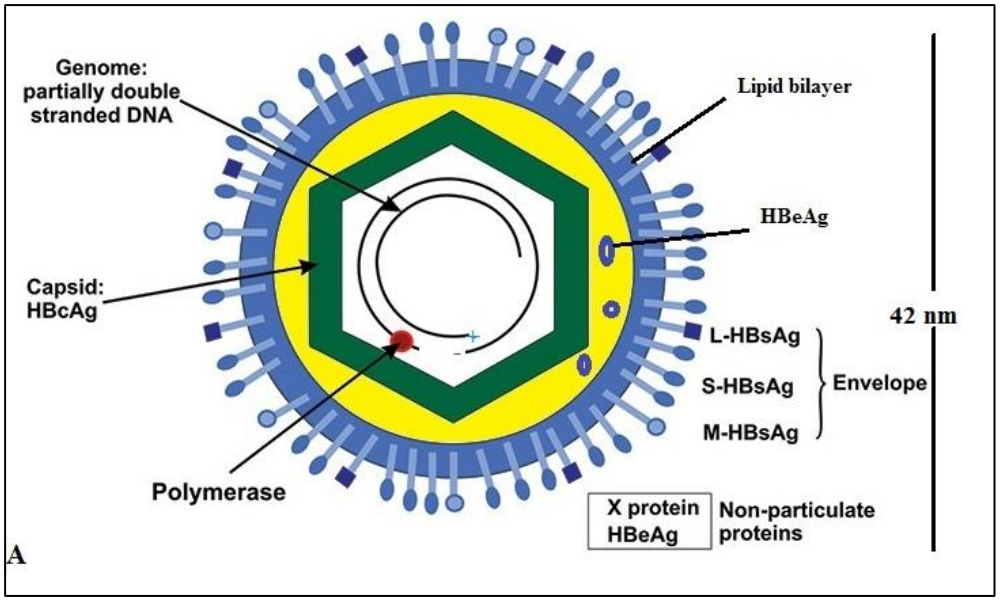


Figure 2.11a: The schematic representation of the HBV infectious Dane partcicle, encompassing the dsDNA, bound by a capsid containing HBcAg, surrounded by a lipid bilayer with HBsAgs. Virion also contains of non-particulate HBeAg and X protein. Adapted from (Bukhari and Tsiquaye, 1990).

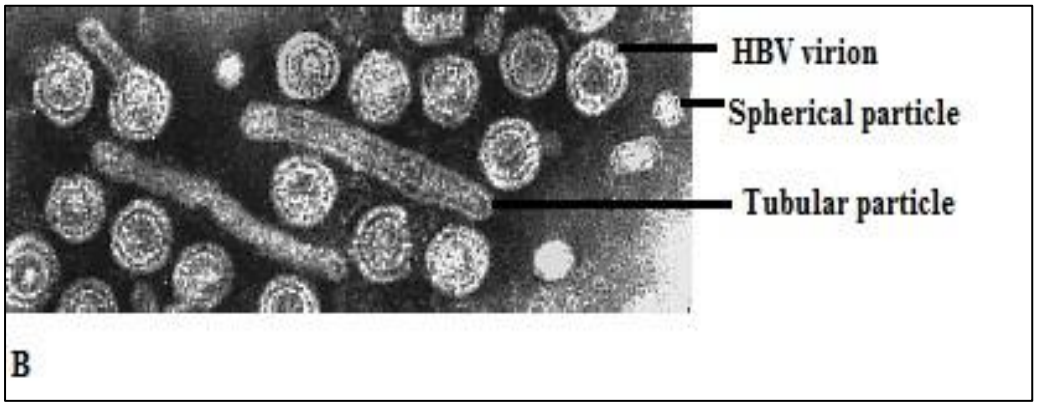


Figure 2.11b: Non-infectious HBV spherical particles and tubular particles, adapted from (Bukhari and Tsiquaye, 1990).

2.2.3 HBV Genomic Organisation

The 3.2 Kb, RC-dsDNA genome has a long complete (+) strand with a viral DNA polymerase attached to its 5´ end and the end of an incomplete (-) strand, has a 5´ capped short oligo ribonucleotides sequence (Figure 2.12) (Panigrahi *et al.*, 2012). Interaction between the 5´ ends of these two strands retains the relaxed circular structure of the HBV genome. The (-) strand encodes for the viral proteins while the (+) strand does not code for the viral proteins (Galibert *et al.*, 1979).

The genome of all HBV genotypes has four ORFs namely, S (surface or envelope gene); preCore/core, (core gene); X (X gene) and P (polymerase gene). The upstream region of the HBV genome consists of the promoters, that initiate transcription of the genes into encoded proteins and the promoters facilitate this process together with enhancer elements, polyadenylation and encapsidation signals (Shepard *et al.*, 2006). The HBV genome encompasses four promoters: core promoter (CP), large surface (S1 promoters), middle surface (S2 promoter) and the X promoter. The CP controls the transcription of the 3.5 Kb transcripts. The S1 and S2 promoters control the transcription of the 2.4 Kb and 2.1 Kb mRNA transcripts (Moolla *et al.*, 2002).

Promoters regulate the transcription process with enhancer elements; enhancer I and enhancer II. Enhancer I (EN I) controls the upregulation of pregenomic RNA (pgRNA), preCore (preC) mRNA and HBX mRNA (Moolla *et al.*, 2002). EN II stimulates the S1, S2 and X promoters; the two-signal induced mechanism regulate gene expression; polyadenylation and encapsidation signals function in regulating transcription and initiating reverse transcription (Yuh *et al.*, 1992).

The core promoter directs transcription initiation and the production of 3.5 Kb preC mRNA and pgRNA. The core promoter consists of the subunits basal core promoter (BCP) and a regulatory sequence located on the promoter upstream BCP is a site on the core promoter

which directs the exact initiation of preC mRNA and pgRNA (Yuh *et al.*, 1992). However, the BCP, PreC mRNA and pgRNA transcripts are produced from a different promoter with a TATA box-like promoter initiator because the BCP does not have the orthodox TATA box sequence to initiate production (Chen *et al.*, 1995).

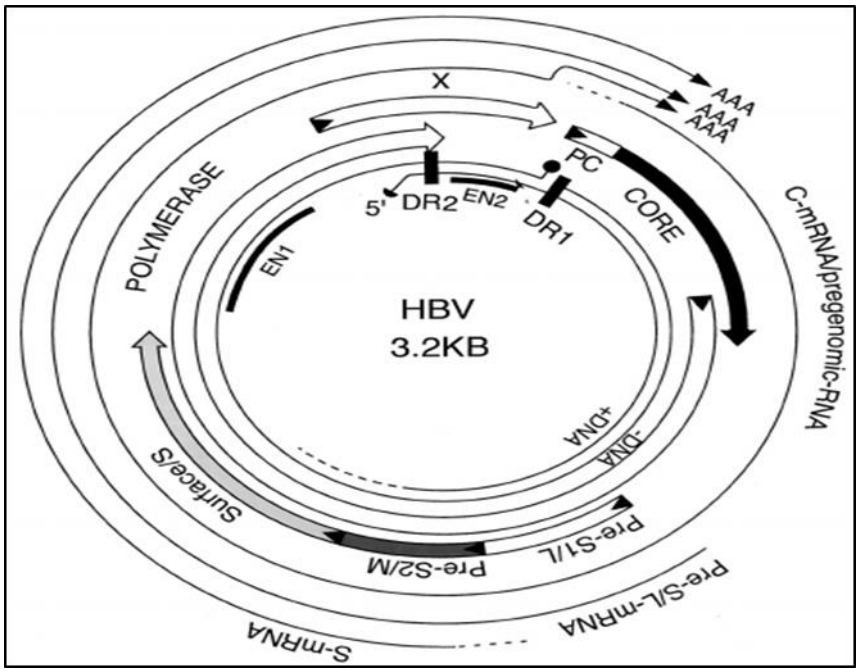


Figure 2.12: The HBV genome, containing the partially ds DNA with a complete (-) strand and (+) incomplete. Four open reading, preCore/Core, P (polymerase), S (surface) and X. Adapted from (Seeger and Mason, 2000).

Upstream of the BCP there is regulatory sequence known as the core upstream regulatory sequence (CURS) which functions in activating the BCP and also the BCP contains the negative regulatory element (NRE) which is responsible for down-regulation of the core promoter activity (Moolla *et al.*, 2002).

The S1 and S2 promoters initiate the transcription of the 2.4 Kb and the 2.1 Kb transcripts. Large hepatitis surface antigen (LHBsAg) is translated from the 2.4 Kb mRNA. Middle

(HBsAg or MHBs) and small (HBsAg or SHBs) hepatitis surface antigens are translated from the 2.1 Kb mRNA (Feitelson, 1992). The S1 promoter controls the synthesis of the LHBsAg, situated 5´-to the preS1 region, and is the only HBV promoter with a classical TATA-box sequence (Siddiqui *et al.*, 1986).

The S1 promoter contains sequence elements that positively regulate transcription of the 2.4 Kb transcripts. However, the S1 promoter is negatively regulated by the downstream sequences in the S2 promoter. The S2 promoter, a distinctive TATA-less promoter, located between the polymerase and surface (preS1) ORF controls transcription of the 2.1 Kb mRNA. This promoter induces the synthesis of a 2.1 Kb transcript from the S2 promoter but also represses production but it also limit expression of a 2.4 Kb transcript. The fourth HBV promoter, the X promoter, regulates transcription of the smallest 0.7 Kb mRNA (Siddiqui *et al*., 1986).

Gene products: polymerase, core, X and surface proteins

The four synthesised mRNA transcripts are translated into the seven coded proteins (polymerase, HBe; core; LHBsAg, MHBsAg, SHBsAg, and HBx). Polymerase is a 95 kDa protein encoded by the viral (P) ORF, it is very essential during HBV replication, because it is made up of four domains having distinct catalytic activities. The domains include: conserved DNA polymerase/ RT, RNase H, the terminal protein (TP) domain and a non-conserved spacer domain (Summers and Mason, 1982).

Initiation of DNA synthesis is activated by the TP domain of the polymerase by initiating protein-priming (Miyake-Stoner *et al.*, 2009). The TP tyrosine residue uses a bulged region within the pgRNA ε stem-loop structure as template for a short DNA oligonucleotide and initiate (-) strand DNA synthesis from the pgRNA (Weber, 2005). Upon completion of the pgRNA/DNA complex, RNase H cleaves the RNA from the pgRNA/DNA and polymerase

synthesise the plus-strand DNA, leading to the formation of RC-dsDNA of the HBV genome (Nassal, 2008).

The core protein: The structural protein of the hepatitis B core antigen (HBcAg) and the hepatitis B e antigen (HBeAg) are generated from alternative two translation start codons, HBcAg is synthesised from the internal translation start codon (AUG) (Ganem and Prince, 2004). HBcAg protein self-assembles to form particles that function in the encapsidation of the viral DNA and polymerase. Initiation at the upstream AUG gives rise to a HBeAg precursor, the precursor, gets processed in the endorecticular (ER) including C terminal cleavage in the golgi and produce a 18-kDa mature HBeAg protein (Milich *et al.*, 1987).

The HBeAg is significantly used as a serological marker in HBV diagnosis, however, the function of HBeAg in HBV infection and replication is not properly known, but it has been speculated for a long period of time since its discovery 30 years ago (Bile *et al.*, 1987). But it is assumed to be regulating the host immune and promoting viral persistence (Hwang *et al.*, 1983).

The HBx (X protein): It is a 17 kDa protein translated from the X ORF; the X protein functions are not exactly known but it is assumed to have multifunctional properties such as increasing the viral replication and spread (Zoulim and Seeger, 1994).

The Surface proteins: They are synthesized from a single preS/S ORF with triple multivariable in-frame translation initiation codons. The largest of these, the preS1 protein (or large HBsAg or LHBs) is transcribed from the 2.4 Kb pre-S1 mRNA; it is the largest envelope protein with 389-400 amino acids (aa) residues. The pre-S1 has been reported to be associated with viral entry into the host hepatocytes and binding site for the core protein during HBV particle assembly. The middle HBsAg or MHBs is transcribed from the 2.1 Kb mRNA initiated by second codon preS2. The MHBs consists of both pre-S2 domain and S

region (55 aa and 226 aa) and function of MHBs is still not being elucidated into details (Fernholz *et al.*, 1993). HBsAg initiation at the third start codon produces small hepatitis B surface antigens (or small HBsAg or SHBs)

2.2.4 Heterogeneity of HBV

HBV genomes have been classified into ten major genotypes designating A to J based on the whole genome genetic heterogeneity of about >8% (Tatematsu *et al.*, 2009). The genetic heterogeneity results from the lack of proofreading mechanism of the viral polymerase during viral DNA replication (Rahman *et al.*, 2016). However, the different HBV genotype distributions differ geographically genotype A dominant in Europe, North America, Africa and in India (Figure 2.9). Genotype B and C are the dominant genotypes in East and in Southeast Asia, genotype D prevails in India, genotype E is commonly found in sub-Saharan Africa. Genotypes F and H are scarce and genotype G is dominant in France and Germany (Norder *et al.*, 1993; Kramvis *et al.*, 2005; Tanwar and Dusheiko, 2012) (Tanwar and Dusheiko, 2012; Kramvis *et al*., 2005; Norder *et al*., 1993). The HBV is further divided into ten different subtypes *adw2, adw4, adrq+, adrq−, ayw1, ayw2, ayw3, ayw4, ay*r and *adw3* which were based on the "a" determinant region and its reaction to amino acid patterns (Rahman *et al.*, 2016).

2.2.5 Epidemiology/ Distribution

It is estimated that approximately two billion people worldwide have evidence of past or present infection with hepatitis B virus (HBV); there are 248 million chronic HBV carriers in the world with 600,000 deaths each year from HBV-related liver disease. Chronic HBV carriers are characterised by present of hepatitis B surface antigen (HBsAg). The wide range in the prevalence of patients with chronic HBV in different parts of the world is largely

related to differences in the age at infection, which is inversely related to the risk of chronicity**.**

The overall prevalence of Chronic HBV (HBsAg positive) is reported to be 3.6 percent globally; however, it varies depending upon the geographic area (Shi *et al.*, 2013). The prevalence of chronic HBV ranges from <2 percent in low-prevalence areas (eg, United States, Canada, Western Europe) to 2 to 7 percent in intermediate-prevalence areas (eg, Mediterranean countries, Japan, Central Asia, Middle East, and parts of South America) to ≥8 percent in high-prevalence areas (eg, Western Africa, South Sudan) (Figure 2.13) (Sunbul, 2014).

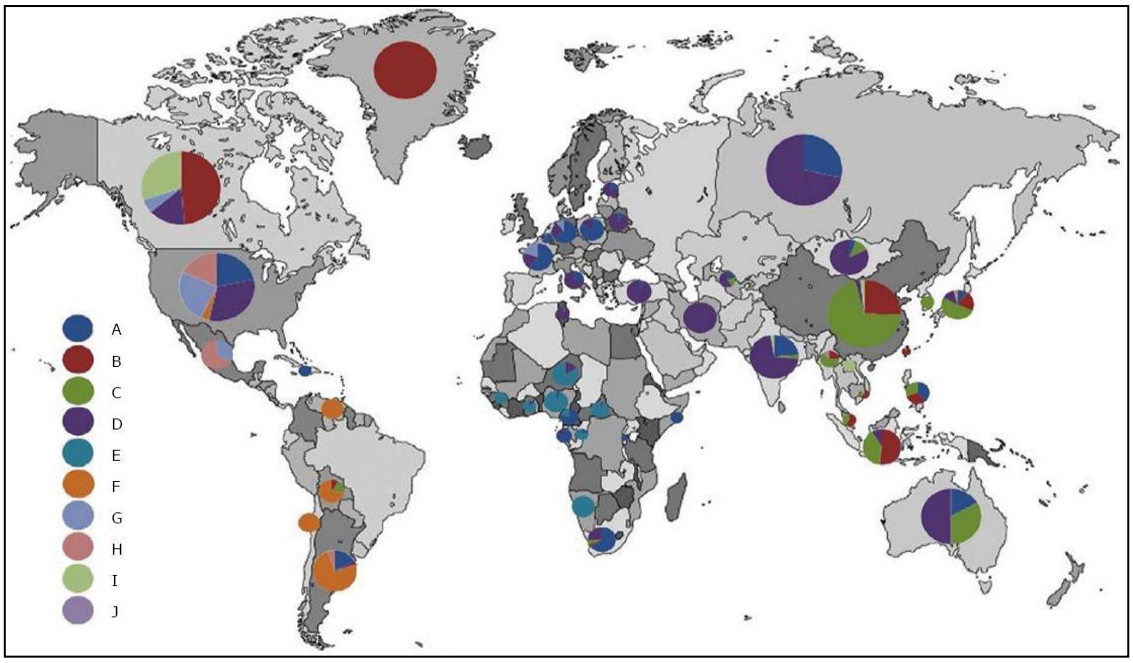


Figure 2.13: HBV prevalence and genotypes distribution globally, adapted from (Shi *et al.*, 2013; Sunbul, 2014).

2.2.6 Transmission

The HBV can be transmitted vertically (perinatally) or horizontally through contact with infectious blood or other infectious body fluids containing blood with numerous forms. Mode of HBV transmission include: sexual contact, blood transfusion and using infectious sharp

needles for injecting drugs, tattooing and body piercing, MTCT during birth or breast feeding. HBV MTCT during breastfeeding can occur if the infected mother did not take immune-prophylaxis during and before giving birth.

2.2.7 Pathogenicity

Hepatitis B virus infects cells in the liver the hepatocytes causing liver infection, the liver infection caused by HBV may be divided into acute and chronic. Acute hepatitis is characterised by inflammation of the liver (hepatitis) and individuals with acute HBV hepatitis can either present symptoms of infections or be asymptomatic. The acute HBV incubation time ranges from three to six months depending on the HBV variant and host immunity. Symptomatic individual can present neither non-specific, clinical symptoms such as fatigue, nausea nor specific clinical symptoms such as liver inflammation. This can be followed by progressive symptoms over a period of time such as fatigue, nausea, anorexia, dark urine, jaundice, abdominal pains, increase in alanine aminotransferase (ATL), HBsAg, HBeAg and HBV DNA levels in serum (Liang, 2009).

In the sixth month, the HBV DNA level reduces the HBsAg reduces and is converted to detectable levels of anti-HBs (sero-convention). However, less than 1% individuals might develop acute HBV liver failure, fulminant hepatitis, and this may lead to death. Individuals who have high levels of HBsAg, HBeAg and HBV DNA after six month and beyond are diagnosed as having chronic HBV hepatitis. Acute HBV hepatitis if not treated may proceed to chronic HBV hepatitis.

Chronic HBV hepatitis may either be asymptomatic or may present clinical symptoms depending on the severity of the liver damage, most individuals with chronic hepatitis remain asymptomatic for a long period of time. Symptoms of hepatitis by chronic hepatitis B (CHB) range from relatively mild symptoms to severe symptoms of liver failure (cirrhosis). Mild

symptoms include fatigue, rash on the palms, osteoporosis these can progress to symptoms associated with cirrhosis such as encephalopathy, portal hypertension, ascites, anaemia and kidney failure (Figure 2.14).

Associated with liver inflammation such as acute necrotizing vasculitis, membranous glomerulonephritis and papularacro dermatitis this liver inflammation may lead to cirrhosis over a period of years. This dramatically increases the incidence of HCC. Chronic HBV hepatitis is characterised into four stages, namely, immune tolerance, immune active, inactive HBsAg and reactivation stages. All stages are dependent of the HBV variant, host cell, host immunity and external factors (Liang, 2009).

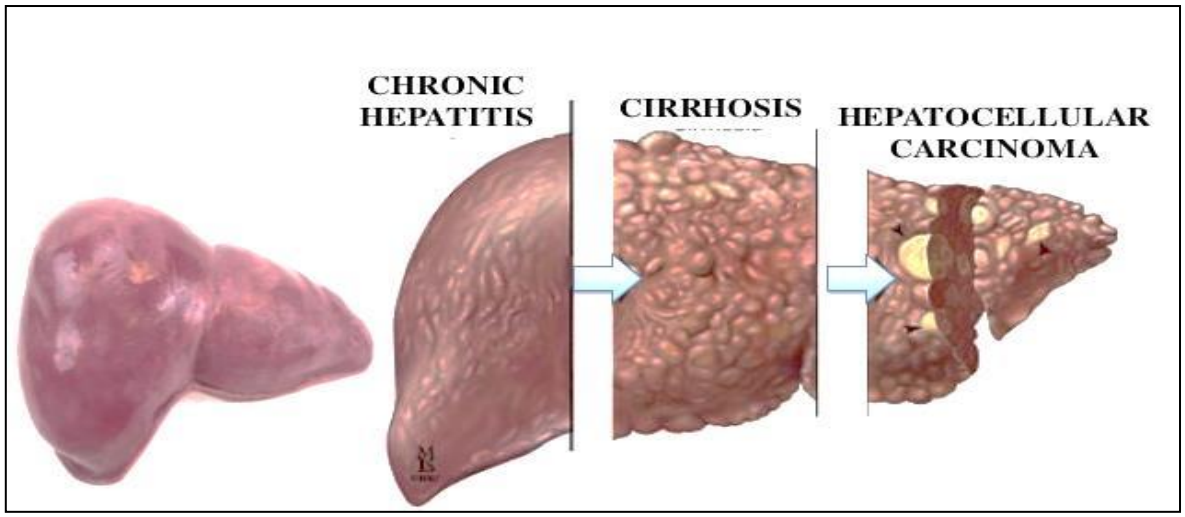


Figure 2.14: Stages of liver damage by Hepatitis B Virus, adapted from (Nawaz *et al.*, 2015).

2.2.8 Diagnosis

To diagnose and differentiate HBV as a source of hepatitis from other viral agents laboratory testing for HBV must be done. Numerous tests such as serology and molecular are available to diagnose hepatitis and can be used to distinguish acute and chronic infections. The serological testing by ELISA kits diagnose HBV by determining the HBV antibodies and antigens such as antigen (HBsAg); antibody to hepatitis surface antigen (anti-HBs); antibody

to hepatitis B core antigen (anti-HBc); and IgM antibody subclass of anti-HBc (IgM anti-HBc). HBsAg is the main clinical marker indicating acute or chronic infection and prevalence as well as endemicity of HBV infection is defined by the presence of HBsAg. Molecular techniques diagnose HBV by determining the quantitative HBV-DNA.

2.2.9 Treatment

HBV can be prevented by subjecting infants to hepatitis B vaccine as soon as possible after birth. There is no specific treatment for acute hepatitis B. However, there are different treatment methods for patients with chronic hepatitis infection available and include immunomodulators such as standard or pegylated interferon-α, as well as long-term treatment with the nucleos(t)ide analogues lamivudine, adefovir dipivoxil, entecavir, telbivudine, or tenofovir. IFNs are cytokines which interfere with viral replication in host cells by inhibiting viral DNA synthesis, and enhancing the cellular immune response against HBV-infected hepatocytes. However, it was shown that IFNs efficacy was improved through pegylation which indicated and showed high HBV DNA suppression, and ALT normalization as compared to use of IFN-α-2a alone. PEG-IFN is also recommended over nucleos(t)ide analogues especially, lamivudine because of effective HBV DNA suppression, and delay in HBsAg seroconversion.

WHO recommends the use of oral treatments - tenofovir or entecavir, because these are the most potent drugs to suppress hepatitis B virus. They rarely lead to drug resistance as compared to other drugs, are simple to take (one pill a day), and have few side effects, so require only limited monitoring (WHO, 2016a). Similarly entecavir and adefovir are recommended over lamivudine. Tenofovir disoproxil fumarate and adefovir are used preferably over lamivudine. Lamivudine resistance poses a major problem in the management of patients with chronic HBV infection since close to 80% of patients develop lamivudine

resistance after 5 years of therapy. Treatment guideline for HBV differs geographically based on genotypes and stage of infections.

2.2.10 HBV Life Cycle

Viral Entry

HBV mechanisms of attachment and entry into the host hepatocytes are still not understood in detail due to the lack of HBV *in vitro* infection models. Mechanisms of how HBV attaches and enters into the hepatocytes remains controversial, with some studies describing LHBs as the main site involved in cell attachment, on the contrary, SHBs has also been reported to be a main HBV site for HBV attachment (Paran *et al.*, 2001). HBV interact with specific receptors on the host cell membrane to initiate attachment of the HBV. However, just like the HBV surface proteins, the host membrane specific receptors and co-receptors involved in HBV attachment and entry are still not fully understood.

Some binding receptors recognised by the HBV binding proteins have been identified such as carboxypeptidase. HBV attach to the host membrane by utilising the carbohydrate side of hepatocyte-associated heparin sulphate proteoglycan receptor (Leistner *et al.*, 2008). The sodium taurocholate co-transporting poplypeptide (NTCP) has recently been identified as a receptor for HBV attachment (Yan *et al.*, 2012).

After the HBV attaches to the host membrane, cell fusion occurs through a process that is not fully understood. It is assumed that membranes fuse together; releasing the HBV into the cytoplasm. In the cytoplasm the nucleocapsid unbounds and releases the HBV genome (RC-dsDNA) which is transported into the host nucleus (Figure 2.15). Once inside the nucleus, the viral RNA primer and viral polymerase attached to the 5´end of the (Pawlotsky) DNA strands are removed from the RC-dsDNA. Allowing completion and ligation of the (+) DNA strand into ccc dsDNA (Lee and Locarnini, 2004).

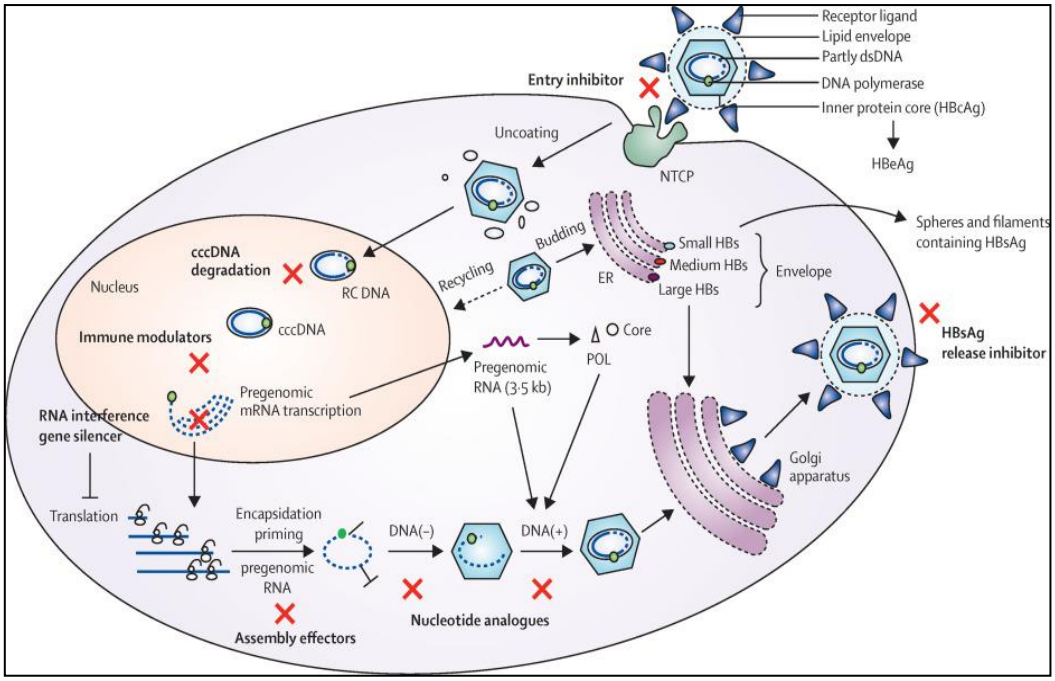


Figure 2.15: Stages of the HBV infection cycle, Adapted from (Brahmania *et al.*, 2016).

2.2.11 HBV Transcription, Replication and Translation

The ccc dsDNA acts as a template for transcription of three subgenomic mRNAs (2.4 Kb, 2.1 Kb and 0.7 Kb) and a supergenomic pgRNA (3.5 Kb) molecules. The supergenomic it is unique because it is greater than genome length, has two copies of direct repeat 1 (DR1) element and two copies of ε-signal elements. The pgRNA also acts as a template during the reverse transcription into RC-dsDNA and a template for translation of HBcAg , HBeAg and viral polymerase. Transcripts, including the pgRNA are transported into the cytoplasm where they are translocated into proteins (Figure 2.15).

During translocation, the TP domain of the polymerase protein attaches to the 5´end of the pgRNA ε-signal resulting in the initiation of ε-signal encapsidation. The core protein homodimers cause the formation of capsid encompassing the pgRNA and polymerase (pgRNA-P) complex (Nassal, 2008). The encapsidated particle matures and the pgRNA undergoes reverse transcription to form RC-dsDNA.

Reverse transcription of pgRNA is induced by a protein-priming method; the polymerase attached to the protruding sequence on the ε structure, using this site to synthesize four-base DNA primer. The 5´end of the DNA primer binds to the TP domain of the polymerase. The four-base DNA primer is translocated to the 3´end of the pgRNA DR1, where it base pairs with the complementary sequence and initiates completion of the (Pawlotsky) strand synthesis (Wang and Seeger, 1993).

During elongation of the (-) strand, the pgRNA is digested by the polymerase RNase H domain retaining only the 18 bp (DR1 and CAP) of the 5´end pgRNA. The 18 bp is translocated to the direct repeat 2 (DR2) element sequence acts as a primer for initiation of the (+) strand synthesis (Seeger and Mason, 2000). The (+) strand elongation process continues to the 5´end of the (-) strand and a nine bases terminal repeat sequence is formed which is complementary to the 3´end of the (-) strand. The 5´end of the (+) strand is transferred to the 3´ end of (-) strand, forming a circular genome and allowing the elongation of the (+ and -) strand synthesis resulting into a RC-dsDNA molecule (Seeger and Mason, 2000). RC-dsDNA contained in the nucleocapsids, can either be transported back into the host cell nucleus, allowing for further cccDNA synthesis nor can they bind with envelope proteins in the endoreticular (ER) organelle and mature into infectious HBV particle. The HBV lifecycle is summarized in Figure 2.15.

2.2.12 HBV Mutations

The core promoter directs the transcription of the 3.5 Kb mRNA into pgRNA and pre-C mRNA. The precore/ core ORFs contains two start codons for the overlapping proteins HBeAg and HBcAg depending on the start codon. Start of translation at the precore/core ORF, there is 87 nucleotides upstream of initiation site result in the production of core protein HBeAg whilst initiation of translation at the pgRNA results in the production of HBcAg.

The precore/core gene contains the core promoter and a basal core promoter. The core promoter is 1814 to 1901 nucleotide (nt) which consists of ε-signal site, DR1 and priming site for pgRNA synthesis. The ε-signal encapsidation stability is vital for initiation of genomic replication and packaging of pgRNA (Kreutz, 2002). The BCP is 1724-1849 nt and consists of DR1, cis-acting elements directing the transcription of pre-C mRNA.

The common variant occurring in the ε-signal region of the core promoter involves G-to-A base change in the DNA position 1896 nt (G1896A); G1898A is a premature stop codon terminates the translation and expression of HBeAg. The G1896A is dependent on the presence of T1858C complementary occurrence of G1896A with opposite T1858C causes the stability of ε-signal resulting in the increase of DNA synthesis and pgRNA stability (Buti *et al.*, 2005). In contrast, the complementary occurrence of G1896A with opposite T1858C destabilises the ε-signal which causes a decrease of DNA synthesis.

A silent mutation, G188A has been reported common in subtype A1 South Africans and Indians can account for reduced viral loads in individuals infected with subgenotype A1 (Kimbi *et al.*, 2012). The BCP region is vital for HBV replication and morphogenesis by directing the transcription of preC-mRNA and pgRNA. Variants present in this area have an effect on the viral replication and HBeAg production (Buckwold *et al.*, 1996).

The common mutations within this region are A1762T together with G1764A, this pair mutations decrease levels of HBeAg expression and has been reported in T1858C and induces the development of G1896A mutation (Tacke *et al.*, 2004). Variants in the core and BCP may be associated with higher serum HBV DNA levels in HBeAg negative but not HBeAg positive, prevalence of variants is related to HBV genotypes. Mutations in the core promoter have been reported in many HBeAg negative patients with chronic hepatitis infection (Ozasa *et al.*, 2006).

Hepatitis surface antigen (HBsAg) is encoded by the envelope ORFs, the HBV has three surface envelope proteins LHBs, MHBs and SHBs. The major SHBs (HBsAg) contains 226 amino acids, contains the cysteine-rich ”ä” determinant located in the second hydrophilic domain of HBsAg, about 99 to 169 nt (Sheldon *et al.*, 2006). Another HBsAg characteristic is a threonine or isoleucine nucleotide base at position 126 used to differentiate HBV serotypes d/y and w/r (Kramvis *et al.*, 2008).

The “ä” determinant region is able to induce neutralizing antibodies after immunization, however, the mutations in this region causes the viral propagation in the presence of neutralizing immune response. HBV mutations on the HBsAg coding gene causes challenges with antigenicity and immunogenicity resulting in immune escape variants and may affect the efficiency of diagnosis causing false HBsAg negatives in some immunoassays (Iacomi *et al.*, 2009).

The most common vaccine escape mutants identified are G145R, Q129, P120L, T123N, M133L, and D144 (Szomor *et al.*, 2008). Understanding immunoassays reactivity with HBsAg mutants is vital to establishing appropriate testing methods. HBV mutants found within the polymerase region are characterized by the amino acid substitution in the highly conserved tryosine-methionine-aspartate-aspartate (YMDD) motif part of the catalytic site of the polymerase (RT) (Firnhaber *et al.*, 2009).

The variant result from isoleucine to valine substitution (I-V) in the YMDD, this mutation may develop resistance during therapy with nucleoside analogue 2´, 3´-dideoxy-3-thiacytide (3TC), famciclovir. These therapy agents change the properties of HBV RT resulting in impaired viral replication within the cell. RT variants common on the YMDD include F501L, and L515M and common mutations in the triple region are rtM240r, M204I, rtV173L, rtL180M and L18M and W196S.

2.3 The Virology of Hepatitis C Virus (HCV)

2.3.1 Classification

HCV is a small, enveloped RNA virus belonging to the *Flaviviridae* family, genus *Hepacivirus*. Formerly termed as non-A, non-B hepatitis (NANBH) by (Feinstone *et al.*, 1975). According to the Baltimore classification, the *Flaviviridae* are Class IV ssRNA viruses, with a positive-sense RNA genomes, they are directly read by ribosomes to translate into proteins.

2.3.2 HCV Virion and Genome Organisation

HCV virion (Figure 2.16) consists of a single stranded RNA in a nucleocapsid surrounded by a lipid envelope with spike-like glycopreotein (E1 and E2) projections (Robertson *et al.*, 1998). HCV genome (Figure 2.16) entails a positive-sense, single-stranded 9.6 kb RNA (+ssRNA) molecule with a single open reading frame (ORF) that encodes three structural (core, E1, and E2) and seven non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins flanked by 5´ and 3´ untranslated regions (UTRs).

2.3.3 Epidemiology and Heterogeneity

HCV has a high propensity for establishing a chronic infection and it is estimated that 130–170 million people globally are infected and an estimated 350,000 people die annually from HCV-related liver diseases (Lavanchy, 2011). Acute HCV leads to chronic HCV and 90% of the case can progress to cirrhosis and hepatocellular carcinoma (HCC) (Gededzha *et al.*, 2014)

Genotyping and phylogenetic analysis of the HCV core/E1, NS5B, and/or complete genome sequences are essential for HCV classification and molecular epidemiology (Smuts and Kannemeyer, 1995).

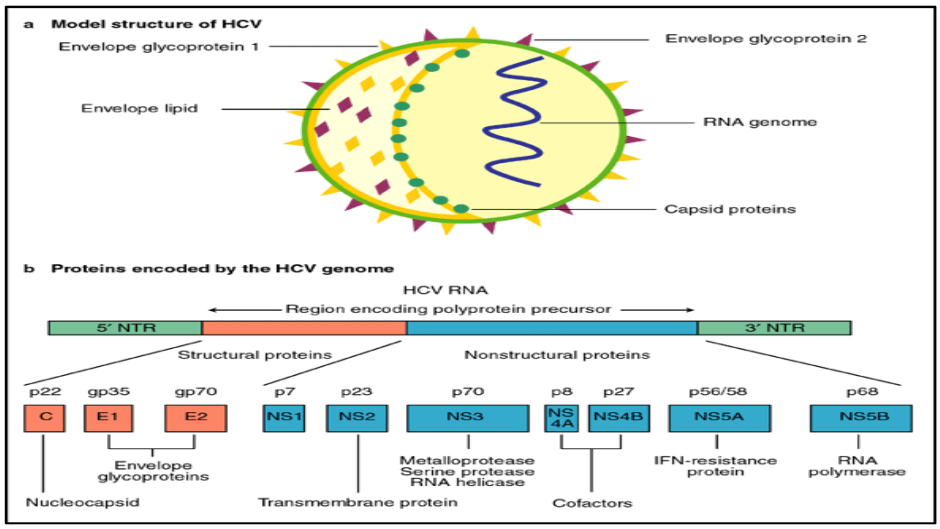


Figure 2.16: Schematic of HCV viral structure and genome organization. A. Cross-sectional diagram of HCV virion. B. The genome of HCV is encoded on a 9.6 kb single-stranded RNA with a polyprotein; adapted from [(Roe and Hall, 2008), 20 July 2015]

HCV genotypes are classified into genotypes 1 to 7 and 82 subtypes globally (Choo *et al.*, 1989). Genotypes 1, 2, 4, and 5 are endemic in Africa with the highest prevalence (Figure 2.17). Genotype 3, 6 evolved in Asia and 7 in Democratic Republic of Congo (Simmonds, 2013; Murphy *et al.*, 2015). Genotype 5 with only one subtype (5a) is predominant in South Africa (Figure 2.17), although genotypes 1–4 have also been reported (Gededzha *et al.*, 2014; Prabdial-Sing *et al.*, 2008). Genotype 5 is not indigenous to South Africa; it has been reported in other regions (Henquell *et al.*, 2004; Verbeeck *et al.*, 2006). Genotype 5 was reported in 1995 in South Africa and there are only two complete genomes of HCV genotype 5a available in GenBank (Drexler *et al.*, 2009) (Drexler *et al.*, 2009; Robertson *et al.*, 1998).

2.3.4 Transmission

The major routes of HCV transmission are through contact with contaminated blood and sporadically through the sharing of needles (Sithebe *et al.*, 1996). The route to exposure

includes intravenous drug use, sharing of drug paraphernalia, and reuse of injection needles (Thorpe *et al.*, 2002). Drug use is critical risk behaviour for HCV infection and it is reported in 40% of all identified cases.

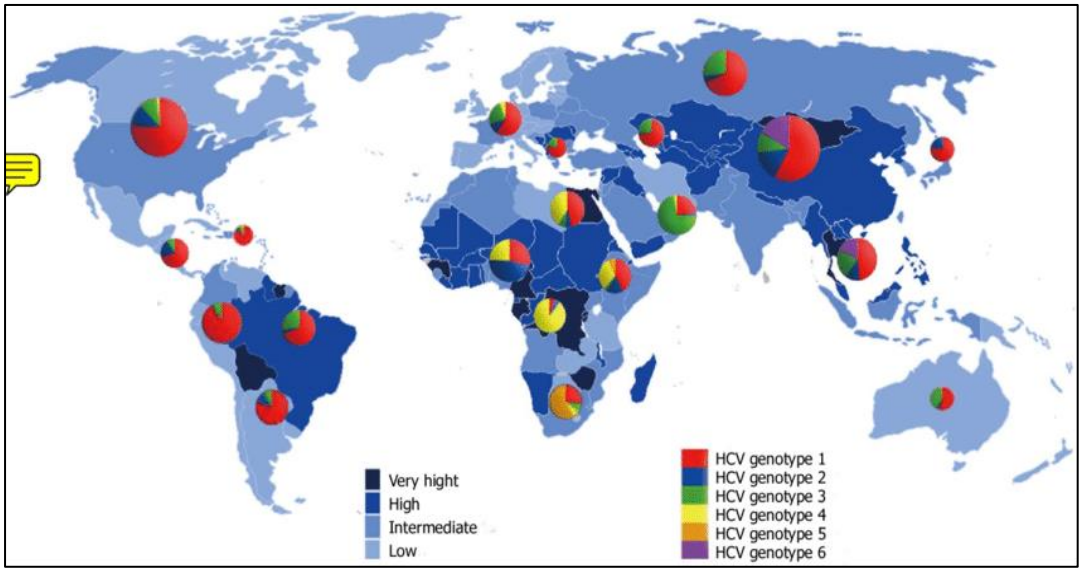


Figure 2.17: HBV prevalence and genotypes distribution globally. Adapted from (Murphy *et al.*, 2007).

Acute HCV infections in HIV-positive men who have sex with men (MSM) are high risk behaviour and the immuno-suppressed except in monogamous heterosexual couples. Perinatal transmission of is poorly understood, with 8% of transmission reported (Cornberg *et al.*, 2011) and that hinders effective methods for prevention. And perinatal transmission might be high in those co-infected with HIV.

2.3.5 Diagnosis

HCV-specific antibodies are detected by an enzyme immunoassay or chemiluminescence immunoassay which uses core, NS3/4/5 proteins to indicate past or present infection. Although development of detectable antibody can be delayed as a result HCV RNA quantification is essential for accurate diagnosis. For characterization of HCV genotypes; sequencing and phylogenetic analysis of 5´UTR less conserved regions, core and/or the NS5B is the successful standard method which is highly specific and sensitive for all

genotypes (Smuts and Kannemeyer, 1995). But sequencing of these regions is a challenge and for routine diagnosis the common assays performed are enzyme immunoassay, hybridisation assays and qualitative HCV RNA detection especially in the developing countries (Richter, 2002).

2.3.6 Treatment

There are no effective vaccines available for the control of HCV infection. However, there were two therapeutic peptide vaccines on trails (Franciscus, 2010). Previously treatment for chronic HCV was achieved with a combination of pegylated interferon (pegIFN α) and ribavirin (RBV). Ribavirin can be used to increase rates of virologic response however, there has been a challenge with affordability and unfavourable tolerability profile and low potency leading to success rates of less than ~50% (Franciscus, 2010).

The use of IFN is associated with side effects that can be serious. The results of IFN-based therapies depend mainly on the patients' responsiveness to IFN, which is determined genetically, the absence or presence of cirrhosis, and the HCV genotype however, pegIFN /RBV remained the only option of therapy for many patients in the world (Franciscus, 2010). However, there are advances in hepatitis C treatments since the introduction of antiviral drugs such as inhibitors of HCV polyprotein maturation and inhibitors of HCV RNA synthesis (Pawlotsky, 2014).

These drugs are already on the market or in clinical development. They include host-targeted agent (HTA) and DAA (direct-acting antivirals) such as NS3-4A protease inhibitors, non-nucleoside inhibitors of the HCV RdRp, Nucleoside/nucleotide analogues, NS5A inhibitors, cyclophilin inhibitors, antagonist of miRNA-122 these drugs differ in their mechanism of action and their barrier to resistance shown in (Table 2.2) (Smith *et al.*, 2014). The direct-acting antiviral drugs (DAAs), with two protease inhibitor (PI) drug names has increased the number of patients who respond to treatment, with less barrier to resistance.

Table 2.2: HBV antiviral agents and their functions

Antiviral drug class	Function	Drug	Reference
NS3-4A protease inhibitors	Blockpost-translational processing	Telaprevir, vertex-Boceprevir,	(Bacon *et al.*, 2011)
Nucleoside/nucleotide analogues	Chain termination of viral RNA	Mericitabine Sofosbuvir	(Sheng *et al.*, 2012)
Non-nucleoside inhibitors of the HCV-RNA–dependent RNA polymerase	Blocking RNA replication	Lomibuvir Setrobuvir Dasabuvir,	(Gentles *et al.*, 2014)
NS5A inhibitors	Inhibit assembly and release of viral particles	Daclatasvir Ledipasvir Ombitasvir	(Guedj *et al.*, 2013)
HTAs	Inhibit replication by blocking the peptidyl-prolyl cis-trans isomerase activity of cyclophilin A	Daclatasvir	(Pawlotsky, 2014)

DAA (direct-acting antiviral), HTA (host-targeted agent), IFN (interferon), RdRp (RNA–dependent RNA polymerase), SVR (sustained virologic response).

2.3.7 Life Cycle

The HCV lipoproteins recognise specific receptors on the livers cells (CD81, LDL, SR-B1, CDL1-1 and OCLN-1) (Dustin *et al.*, 2016). This association leads to entry of the viral particle into the cell, endosome fusing, and release of the positive + ss RNA molecule (Shepard *et al.*, 2005). The +ss RNA serves as a template for replication into many copies of negative and positive stranded RNA and direct translation into polyprotein (Dustin *et al.*,

2016). Assembly and packaging of new virions occurs on the rough ER (Andersson *et al.*, 2013). HCV life cycle is important as it can guide in the development of vaccines and treatment of HCV infections (Franciscus, 2010).

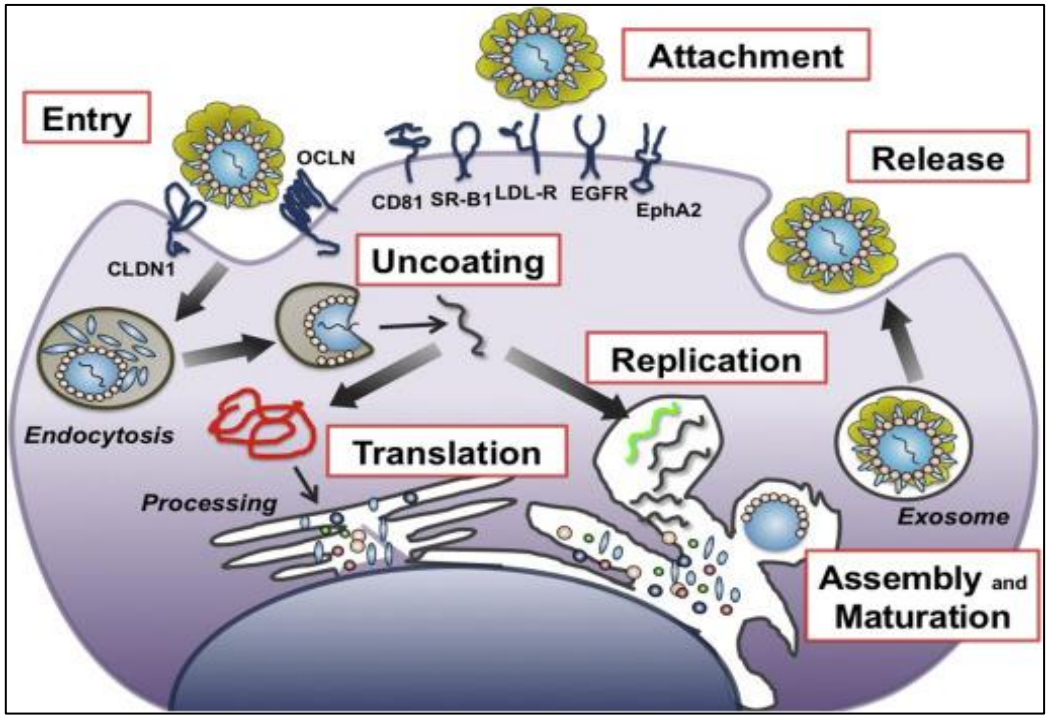


Figure 2.18: Schematic diagram of the HIV-1 life cycle, adapted from (Dustin *et al.*, 2016).

2.4 Co-infections

2.4.1 HBV/HIV Co-infection

The co-infection of HIV with HBV is very common due to shared mode of blood-born transmission (Audsley *et al.*, 2010). The common routes of transmission vary from place to place depending on the endemicity of HBV. In areas of low endemicity e.g. in North America, Australia, and in Europe; HBV and HIV are usually acquired in adulthood. In countries with intermediate and high HBV endemic status, the main route of transmission of HBV and HIV are vertical in children and through sexual intercourse in adults.

The prevalence of chronic co-infection among HIV-infected individuals is around 7% in low endemic countries and 10-20% in countries with intermediate to high HBV endemic (Thio *et al.*, 2002). Chronic HBV is common in HIV-1-infected people with a prevalence range from

5-20% in various studies of HIV-infected individuals (Diop-Ndiaye *et al*., 2008; Nyirenda *et al*., 2008; Alter, 2006). The prevalence of HBV infection among HIV+ individuals is significantly higher than in HIV- negative (HIV-) individuals and globally it is reported that the prevalence of HBV infection among the HIV+ individuals is significantly higher than in HIV- individuals (Burnett *et al.,* 2005).

South Africa is highly endemic for both HBV and HIV infections; HBV/HIV co-infections are common in this region because of shared transmission routes. There are limited studies on the burden and impact of HBV/HIV co-infection in South Africa. The HBV/HIV prevalence has not been well characterized; there are differences in geographic distribution of HBV, HIV and HBV/HIV co-infections. Close to 63% South Africans are living with HIV have been exposed to HBV (Firnhaber *et al.*, 2009; Lukhwareni *et al.*, 2009).

HBV/HIV co-infection has been reported at approximately 20% among male mine workers in South Africa; 39% in HIV+ women in Gauteng and close to 7.1% was reported from rural and urban areas and antenatal study from Gauteng province reported a prevalence of 39% among HIV+ women (Firnhaber *et al.*, 2009; Boyles and Cohen, 2011; Mayaphi *et al.*, 2012).

The impact of HBV/HIV co-infection on the HBV disease progression has been well documented. Most studies reported a negative effect of HIV on chronic HBV associated with reduced rate of Hepatitis B e antigen (HBeAg); increase HBV replication as manifested by higher HBV DNA levels (Sulkowski, 2008). HIV also increase the risk of chronic cirrhosis and serum ATL may be lower in HBV/HIV chronic co-infected patients than in HIV mono-infected patients (Colin *et al.*, 1999). HIV also accelerates the risk for HCC and liver-related deaths in HBV co-infection (Thio *et al.*, 2013) . HCC are associated with evidence of lower CD4+ T cell counts in HBV/HIV co-infected people (Clifford *et al.,* 2008). Occult HBV individuals co-infected with HIV may escape diagnosis in poor resources areas as where it is

not possible to measure HBV DNA viral load and use nucleic acid tests (Mphahlele *et al.*, 2006).

2.4.2 HBV/HIV Co-infection Treatment

HBV therapy is recommended for all HIV/HBV co-infected patients with abnormal ALT values or HBV DNA level of >2000 Iu/ ml (threshold for treatment of HBeAg-positive) (Degertekin *et al.,* 2009). However, experts recommend treatment of HBV for all HIV co-infected patients whom HBV replication is present.

Most HIV treatment contains one or more HBV-active agents e.g. 3TC, FTC and TDF. Patients with indication for HBV treatment should be started on fully active ART that contains HBV-active nucleoside/nucleotide analogues regardless of CD4 cells to ensure HIV is completely treated (Benson *et al.*, 2009). However, HIV- infected individuals should not receive 3TC or FTC monotherapy for HBV infection because of development of drug resistance with 4 years of single-drug treatment (Di Martino *et al.*, 1999). Resistance to 3TC or FTC is characterized by the development of mutation rtm 204 (YMDD mutation). 3TC and FTC should be used only for patients on fully suppressive ART.

The National HIV treatment guidelines by the South Africa Department of Health recommended by WHO includes HBsAg screening and ART initiation of co-infected patients but it is not clear on whether HIV+ should be vaccinated against HBV (WHO, 2016a). Prevalence of HBV vaccinated individuals co-infected with HIV has been reported from 60% patients in Limpopo and 8.3% in pregnant women (Ayuk, 2013). Understanding HIV/HBV prevalence is vital for patients' management.

2.5 HCV/HIV Co-infection

The HCV prevalence in Sub-Saharan Africa prevails from 4.3% to 55% demonstrating a huge geographic variation of HCV prevalence. HCV prevalence has been reported uncommon in

South Africa, suggesting low prevalence of injecting drug use (Manasa *et al*., 2013). But in South Africa the HCV prevalence is reported to range from 0.16 to 1.8% (Gededzha *et al.*, 2010). The HCV is reported higher in high-risk individuals, e.g. 39.4% in haemophiliacs and 4.8% in chronic dialysis patients (Soni *et al.*, 1993). However, insufficient data exist on the HCV prevalence in HIV-infected people with a study reporting 1.9% low prevalence rate and another reporting a high prevalence of 13.4% (Parboosing *et al.*, 2008). HCV prevalence among the HIV-infected pregnant women has been reported uncommon.

CHAPTER 3

3. MATERIALS AND METHODS

3.1 KwaZulu-Natal Cohort

3.1.1 Study Design

This was a cross-sectional laboratory-based study and a total of 50 archived HIV positive plasma samples were used. The plasma was collected from patients around Durban. A total of 50 stored plasma samples from HIV-infected patients were donated by the National health laboratory services (NHLS) in May 2017. The patients were not on ART at that time.

3.1.2 Processing and collection of Samples from Kwa-Zulu Natal Cohort

Processing of samples at NHLS: Plasma samples were collected from participants between February to October 2016. The fifty plasma samples of both male and females had been confirmed to be HIV-positive from previous serological tests at NHLS. The plasma samples were subjected to HIV genotyping at NHLS, all samples were HIV-1 subtype C and there were no drug resistance mutations detected. The plasma was donated to us by the National health laboratory services (NHLS) in May 2017.

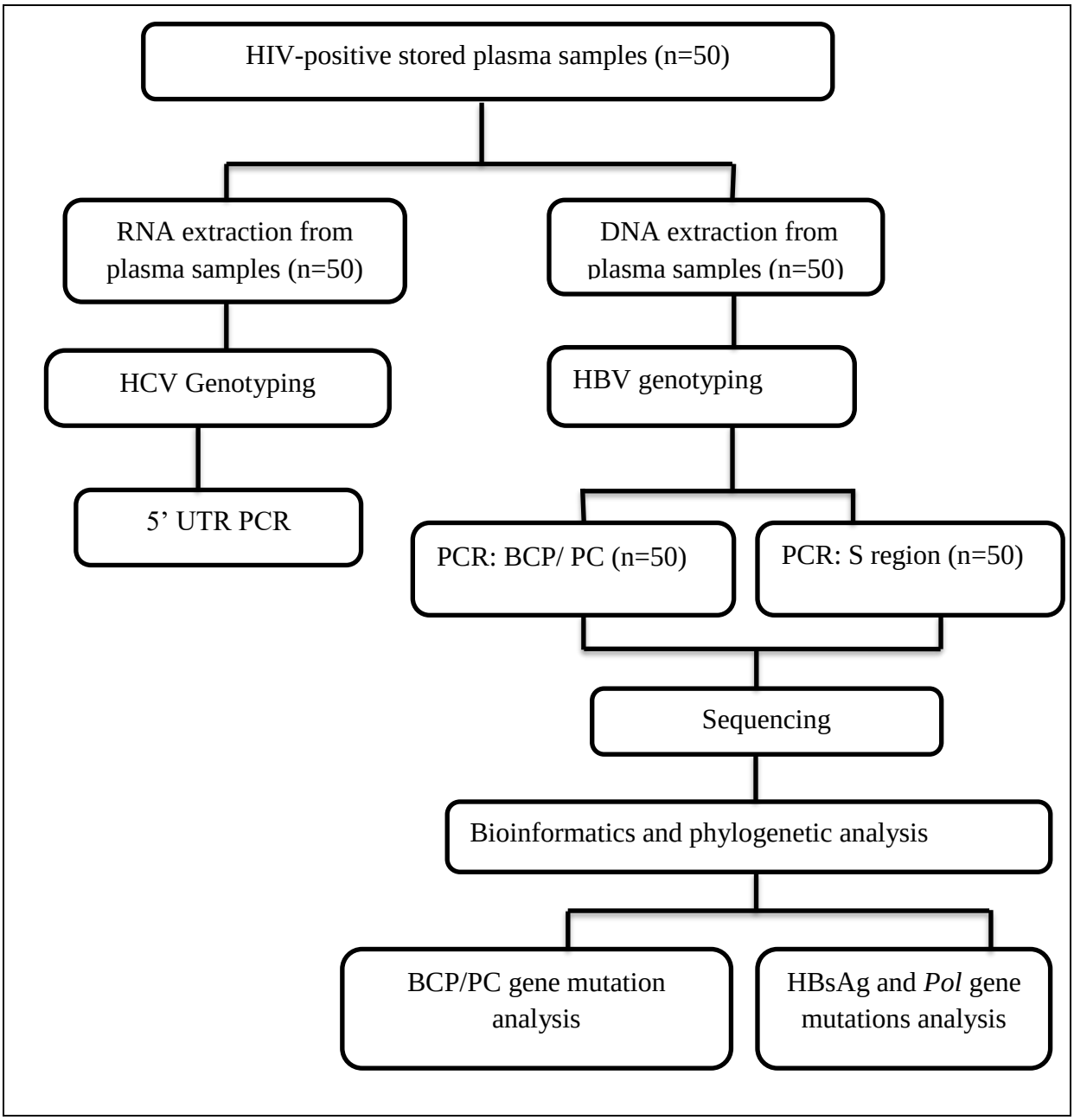
3.1.3 Ethical Clearance

Ethic clearance for processing samples from KwaZulu-Natal cohort study was obtained and granted by the North-West University Research Ethics Regulatory Committee (NWU-RERC).

3.1.4 Methods Overview

Overview of methods used to study HBV/HCV and HIV co-infections in the HIV infected KwaZulu-Natal cohort is shown in (Figure 3.1).

Figure 3.1: Study overview of the methods used in the KwaZulu-Natal cohort.



3.2 Mahikeng Cohort

3.2.1 Study Design

A cross-sectional design was used in this cohort and contained of 23 participants. Study participants were pregnant females from surrounding villages in Mafikeng and were attending antenatal care at public clinics around Mafikeng. Patients included first time antenatal care bookers who were tested for the first time and revisiting patients who have been confirmed to be HIV-positive from previous antenatal serological tests. Written informed consent was obtained from all study participants prior to their recruitment.

3.2.2 Collection of Samples from the Mahikeng Cohort

Five millilitres of venous blood was collected once from each consenting HIV positive adult individual into Ethylenediaminetetraacetic acid (EDTA) purple vacutainer tubes between April to November 2015 and placed on ice by the nurse. Once the samples were collected they were immediately transported to the laboratory. Upon arrival at the laboratory the blood was centrifuged for 5 min at 3000Xg. Following centrifugation, plasma was aspirated aseptically and aliquoted into sterile labelled Eppendorf tubes and stored at -80°c.

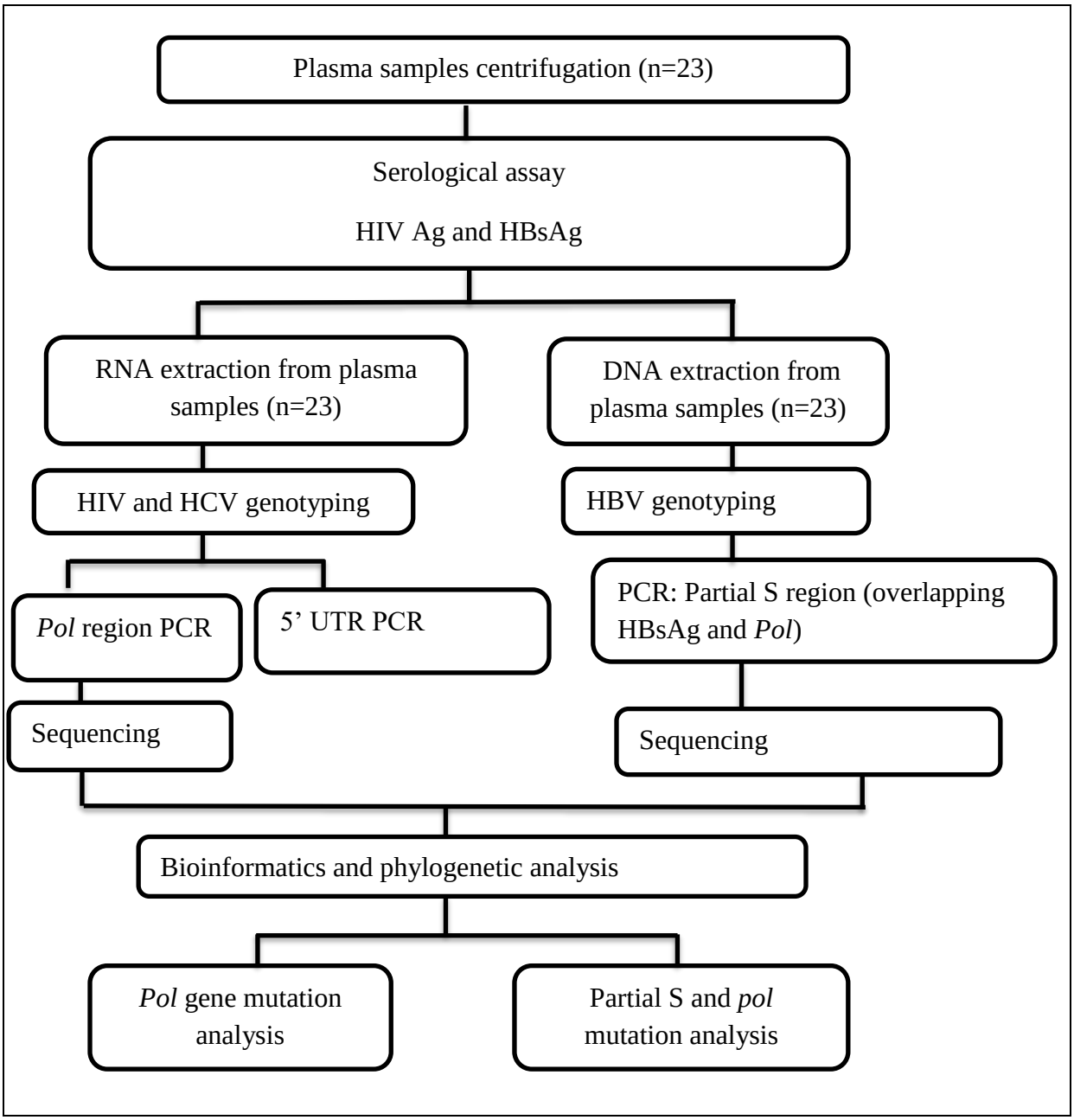
3.2.3 Ethical Clearance

Ethic clearance for this study was obtained and granted by the North-West University Research Ethics Regulatory Committee (NWU-RERC) (ref. NWU-00068-15-A9) for Mahikeng cohort. To collect fresh blood samples from the local clinics, permission was granted by the North-West Provincial Department of Health, Policy, Planning, Research, Monitoring and Evaluation section.

3.2.4 Methods Overview

Overview of methods used to study HBV/HCV and HIV co-infections in the HIV infected Mahikeng cohort is shown in (Figure 3.2).

Figure 3.2: Study overview of the methods used in the Mahikeng cohort (public clinics).



3.3 Serological Assays used to identify Viruses (HBV, HCV and HIV)

3.3.1 Identification of HIV-1 and HIV-2 Antigen

The test was done to differentiate HIV-1 from HIV-2 antigens (HIV-Ag) and to confirm the HIV ELISA tests done at the clinics. Plasma was tested for HIV-1 or HIV-2 antigen using Genscreen ULTRA HIV-Ag according to manufacturer instructions. Genscreen ULTRA HIV-Ag is an enzyme immunoassay based on the principle of the sandwich technique for the detection of HIV antigen.

Briefly, 25 µl of conjugate 1 (biotinylated polyclonal antibody to p24 HIV-1) was dispensed into microplate wells, 75 µl of undiluted plasma was pipetted into wells of the microplate and incubated at 37 degree Celsius (°C) for 60 minutes. The contents were aspirated, microplates were washed 2x with 0.370 ml of washing solution (1:20 Tris NaCl) and plates were air dried. An aliquot of 100 µl of the conjugate 2 (polyclonal antibodies + skimmed milk solution) was dispensed into the wells and incubated for 30 minutes at 30 °C. The plates were washed 5x and subjected to 80 µl of peroxidase substrate buffer with chromogen and 100 µl of stopping solution (IN sulphuric acid). After 2 minutes the microplate wells were placed in a plate reader.

The quantity of HIV Ag in the specimens was determined by comparing the rate of fluorescent formation by the test specimens. Optical density (O.D.) index of specimens was measured at 450 nm and compared to an O.D. cut-off rate mean index calibrator of a negative control HIV Ag. Samples with an index greater or equal to the cut off rate were considered reactive for HIV-1 or HIV-2 positive.

3.3.2 Hepatitis B Surface Antigen (HBsAg) Assay

Briefly, 100 µl of undiluted plasma samples were used to detect presence and expression of HBsAg using Monolisa HBsAg ULTRA confirmatory kit following the manufacturer

instructions (Bio-Rad, Raymond Poincare, Marnes-la-Coquette, France). This was performed using neutralization by excess of antibodies to anti-HBs (anti-HBs diluent: neutralization reagent) of the HBsAg found in specimens. The presence or absence of HBsAg in the specimens was determined by comparing the rate of fluorescent formation by the test specimens. The O.D. index of specimens was measured at 450 nm and compared to an O.D. cut-off rate mean index calibrator of a negative control HBsAg rate. Samples with an index greater or equal to the cut off rate were considered reactive for HBsAg.

3.3.3 Hepatitis C Virus antigen-antibiotic (Ag-Ab) Assay

Briefly, 200 µl of undiluted plasma samples were used to detect presence of HCV Ag-Ab using Monolisa HCV Ag-Ab ULTRA confirmatory kit following the manufacturer instructions (Bio-Rad, Raymond Poincare, Marnes-la-Coquette, France). HCV Ag-Ab ULTRA assay is an enzyme immunoassay on the combination of an indirect test for the antibodies and a sandwich test for antigen detection of HCV. It is based on the detection of capsid antigen and antibodies associated with an infection by Hepatitis C virus in patient plasma. The presence or absence of HCV Ag-Ab in the specimens was determined by comparing the rate of fluorescent formation by the test specimens. The O.D. index of specimens was measured at 450/620-700 nm and by comparing for each sample the recorded absorbance with that of the calculated O.D. cut - off value. Samples with an index greater or equal to the cut off rate were considered reactive for HCV Ag-Ab.

3.4. Genomic Material Extraction

The genomic HIV and HCV RNA were extracted from the patient's fresh and stored sera samples using automated Nuclisens easyMAG extraction system and QIAamp MinElute Virus Spin Kit following the manufacturer's instructions. This technique contains four steps (lyse, bind, wash and elute of the genomic material) and allows the purification of total

viruses (HIV and HCV) RNA and DNA from contaminants, inhibitors and nucleases from the plasma.

3.4.1. DNA and RNA Extraction of HCV and HIV

QIAamp MinElute Virus Spin Kit was used to extract and purify viral RNA, An aliquot of 25 µl of the QIAGEN protease was added into 1.5 ml Eppendorf tubes, to which 200 µl of plasma and 200 µl buffer AL (containing 28 µg/ ml carrier RNA) was added. The mixture was pulse-vortexed for 15 seconds to homogenise the mixture followed by 15 minutes incubation at 56 °C. An aliquot of 250 µl of absolute ethanol was added into the tube, pulse-vortexed for 15 seconds, then incubated for 5 minutes at room temperature and briefly centrifuged to homogenise the mixture.

The mixture was then transferred to a QIAamp MinElute column and centrifuged for 1 minute at 8 000 revolutions per minute (rpm). The column was placed into a clean collection tube then 500 µl buffer AW1 was added and centrifuged for 1 minute at 8 000 rpm. The solution was aspirated, 500 µl buffer AW2 was added followed by centrifugation for 1 minute at 8 000 rpm. A total of 500 µl of absolute ethanol was added onto the column, centrifuged at 8 000 rpm for 1 minute. The column was placed into a clean 2 ml collection tube and centrifuged at 14 000 rpm for 3 minutes to dry the membrane. The QIAamp MinElute column was placed in a sterile 1.5 ml Eppendorf tube, 50 µl of elution buffer (provided by the kit as buffer AVE) was added directly into the column and incubated at room temperature for 5 minutes. The RNA was eluted by centrifugation at 8 000 rpm for 1 minute and stored at −20 °C until further analyses were performed. The negative control consisting of nuclease free water was included in the extraction procedure to detect possible contamination.

3.4.2. DNA Extraction of HBV

Plasma obtained from patients was used to extract HBV DNA using QIAamp DNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. This technique allows the purification of total DNA from contaminants, inhibitors and nucleases from the plasma.

An aliquot of 200 µl of the plasma sample was added into 1.5 ml Eppendorf tubes, to which 20 µl of proteinase K and 200 µl Buffer AL (binding buffer mixed with poly [A] carrier RNA) was added. The mixture was pulse-vortexed for 15 seconds to homogenise the mixture followed by 10 minutes incubation at 56 °C. The mixture was then transferred to a QIAamp spin column and centrifuged for 1 minute at 8 000 rpm. The column was placed into a clean collection tube then 500 µl buffer AW1 was added and centrifuged for 1 minute at 8 000 rpm. The solution was aspirated, 500 µl buffer AW2 was added followed by centrifugation for 3 minutes at 14 000 rpm. The QIAamp spin column was placed in a sterile 1.5 ml Eppendorf tube, 50 µl of elution buffer (provided by the kit as buffer AE) was added directly into the column and incubated at room temperature for 5 minutes. The DNA was eluted by centrifugation at 8 000 rpm for 1 minute and stored at −20 °C until further analyses were performed. The negative control consisting of nuclease free water was included in the extraction procedure to identify contamination.

3.5. Polymerase Chain Reaction (PCR) Amplification

3.5.1. PCR Amplification for HIV

The extracted RNA samples were thawed at room temperature and briefly centrifuged to collect samples at the bottom of tube. The negative control (nuclease free water) and a positive control were included in the PCR amplification assays. Samples and master mix were kept on ice during preparations.

3.5.2 Reverse Transcriptase (RT)-PCR

Sample preparation was as follow: an aliquot of 10 µl extracted RNA was introduced into 0.2 ml PCR tubes and heated at 65 °C for 10 minutes. The tube was placed on ice for 1 minute and briefly centrifuged. Master mix was prepared using Superscript III one step RT-PCR with Platinum *Taq* high fidelity (Invitrogen, Massachusetts). For each sample the following were prepared: 10 µl nuclease free water, 25 µl 2x reaction mix, 2 µl primer PrtM-F1 (10 µM), 2 µl primer RT-R1 (10 µM), 1 µl SSIII RT-PCR enzyme. PrtM-F1 is a 1:1 (wt/wt) combination of two forward (F1a) and reverse (F1b) primers Table 3.1. An aliquot of 40 µl of the PCR master mix was added to 10 µl of extracted RNA and subjected to RT-PCR amplification on a thermal cycler CFX96 (Bio-Rad, Raymond Poincare, Marnes-la-Coquette, France). The RT-PCR amplification conditions are shown in Table 3.2.

3.5.3 Nested-PCR

Nested PCR was done using Ampli*Taq* Gold (Applied Biosynthesis) using inner forward primers PRT-F2 and inner reverse primer RT-R2. Primer sequences used in nested PCR are shown in (Table 3.1). For each sample the following were prepared: 71 µl nuclease-free water, 10 µl 10x gold buffer, 8 µl $MgCI_2$, 2 µl dNTP (10 µM), 2 µl primer Prt-F2 (10 µM), 2 µl primer RT-R2 (10 µM) and 1 µl ampli*Taq* gold. A total of 96 µl of the master mix was aliquoted into 0.2 ml PCR tube and 4 µl of first round RT-PCR products was added and subjected to nested-PCR amplification on a thermal cycler CFX96 (Bio-Rad, Raymond Poincare, Marnes-la-Coquette, France). The nested-PCR amplification conditions are shown in **(**Table 3.2).

Table 3.1: HIV *pol* gene primers sequence and location

Primer sequence	**Nucleotide site in HXBII genome**	Reference
PrtM-F1a 5'-TGA ARG AIT GYA CTG ARA GRC AGG CTA AT- 3'	2057 to 2085	(Inzaule *et al.*, 2013)
PrtM-F1b 5'-ACT GAR AGR CAG GCT AAT TTT TTA G-3'	2068 to 2092	(Inzaule *et al.*, 2013)
RT-R1 5'-ATC CCT GCA TAA ATC TGA CTT GC-3'	3370 to 3348	(Bredell *et al.*, 2002)
PRT-F2 5´-CTT TAR CTT CCC TCA RAT CAC TCT-3´	2243 to 2266	(Hunt *et al.*, 2012)
RT-R2 5´-CTT CTG TAT GTC ATT GAC AGT CC-3´	3326 to 3304	(Hunt *et al.*, 2012)

Table 3.2: HIV RT-PCR and nested PCR cycling conditions

RT-PCR				Nested-PCR	
Cycling condition	Temperature °C	Time	Cycling condition	Temperature °C	Time
Denaturation 1x	94 °C	2 minutes		94 °C	4 minutes
Amplification	40 x cycles			40 x cycles	
Denaturation 1x	94 °C	15 seconds		94 °C	15 seconds
Annealing	50 °C	20 seconds		55 °C	20 seconds
Elongation	72 °C	2 minutes		72 °C	2 minutes
Final elongation	72 °C	10 minutes		72 °C	10 minutes

3.5.4 PCR Amplification for HCV

Some cDNA synthesis and first-round PCR were carried out using the TITAN One Tube RT-PCR system (Roche Diagnostics, Germany); the outer sense strand primer (Sn1) and antisense strand primer (Asn1) were used and primer sequences are shown in Table 3.3. Each 50 µl of the sample reaction mix contained: 26.25 µl distilled water; 2 µl Sn1 primer (10 µM); 2 µl Asn1 primer (10 µM); 1 µl each dNTP (25 mM); 2.5 µl of 5 mM dithiothreitol (DTT); 0.45 µl of 40 U/ ml ribonuclease (RNAse) inhibitor (Roche Diagnostics, Germany); 1 µl enzyme mix (AMV); 10 µl of 1x concentration of RT-PCR buffer (with 1.5 mM $MgCl_2$); and 5 µl extracted RNA. The cDNA synthesis was carried out at 50 °C for 30 minutes; followed by the first-round PCR that was incubated at 94 °C for 30 seconds, 55 °C for 30 seconds, 68 °C for 50 seconds x 35 cycles and a final elongation step at 68 °C for 7 minutes (Table 3.4).

For the nested PCR assay, inner sense strand primers (Sn2) and antisense strand primer (Asn2) were used and primer sequences are shown in Table 3.3. The master mix volume and concentration for each sample made up of 5 µl of the first-round product, 400 nM of each primer, 200 µM of each dNTP, 2.5 U/ ml of *Taq* polymerase and 1x concentration of PCR reaction buffer. The nested reaction was incubated at 94 °C for 60 seconds, 50 °C for 60 seconds, 72 °C for 60 seconds, and a final extension step at 72 °C for 5 minutes (Table 3.4).

Table 3.3: HCV 5'UTR region primer sequences and location

Primer sequence	**Nucleotide site in 5´UTR region**	Reference
SN1- 5´CTGTGAGGAACTACTGTCTT-3´	297 to 277	(Sithebe *et al.*, 1996)
Asn1- 5´GCACTCGCAAGCACCCTA-3´	45 to 28	
Sn2- 5´TTCACGCAGAAAGCGTCTAG-3´	278 to 258	
Asn2- 5´CCTATCAGGCAGTACCACAA-3´	60 to 42	

Table 3.4: HCV RT-PCR and nested PCR cycling conditions

RT-PCR			Nested-PCR		
Cycling condition	Temperature °C	Time	Cycling condition	Temperature °C	Time
	50 °C	30 minutes			
Amplification	35 x cycles			35 x cycles	
Denaturation 1x	94 °C	30 seconds		94 °C	60 seconds
Annealing	55 °C	30 seconds		50 °C	60 seconds
Elongation	68 °C	50 seconds		72 °C	60 minutes
Final elongation	68 °C	7 minutes		72 °C	5 minutes

3.5.5 PCR Amplification Assay- HBV

First Round and Nested-PCR

Outer sense strand primer, S1, and antisense strand primer, S2Na, were used. Primer sequences are shown in Table 3.5. For each sample the following reagent volumes and concentration were prepared: 18.5 µl nuclease free water, 2.5 µl 10x PCR buffer with $MgCl_2$, 0.5 µl 10 mM dNTP mix, 0.5 µl (50 uM) forward primer S1; 0.5 µl (50 uM) reverse primer S2Na anti-sense primer, 0.125 *Taq* DNA polymerase. A total of 22.5 µl of master mix was aliquoted into a 0.5 ml thin-walled PCR tube and 3 µl of DNA template was added. The PCR reaction mixtures (25.5 µl) was subjected to amplification of HBV DNA, carried out in an automated touch down thermal cycler CFX96 (Bio-Rad, Raymond Poincare, Marnes-la-Coquette, France). The HBV DNA amplification conditions are shown in Table 3.6.

For the second round (nested) PCR assay, sense strand primers S6E and antisense strand primer S7B were used and primer sequences are shown in Table 3.5. An aliquot of 3 µl of the first round PCR reaction was subjected to a nested PCR, the master mix volume and concentration were prepared as same for the first round PCR. The nested PCR conditions used were the same as first round PCR protocol (Table 3.6). The negative control consisting of nuclease free water and a positive control were included in the PCR amplification assays.

3.5.6 Real-Time PCR for HBV DNA Load Detection

Positive control of an HBV genotype A was used to make serial dilutions to concentrations from 500,000; 50,000; 5,000; 500; 50 to 5 IU/ ml, corresponding to 1,400,000 copies/ ml to 14 copies/ml. Each sample was tested in triplicate during the quantification test. The threshold cycles (Ct) were set manually after PCR was completed to generate the standard curve; the unknowns were then calculated based on the standard curve. $Y= 3.5x +41.41$, $Ct= 3.5 \log X+ 41.41$ and $R^2= 0.9918$. These dilutions were extracted, and PCR was performed and the quantity of HBV DNA was determined.

The reaction was carried out using a commercial SYBR Green I master (Roche Diagnostics GmbH, Sandhofer Strasse, Mannheim, Germany). The 20 µl reaction mixture for each sample was prepared as follows: 7µl nuclease free water, 10 µl SYBR green, 1 µl (50 µM) of forward and reverse inner primers and 2 µl of first round PCR product. The final master mix was run on a thermacycler CFX96 Touch Real-Time PCR detection System (Bio-Rad, Raymond Poincare, Marnes-la-Coquette, France) under the qRCR thermal cycling conditions shown in Table 3.6.

Table 3.5: HBV surface gene primers sequences and location

Primer sequence	**Nucleotide site in HBV genome**	Reference
S1 5´-CCT GCT GGT GGC TCC AGT TC-3´	56–76	(Mphahlele *et al.*, 2006)
S2Na 5´-CCA CAA TTC KTTGAC ATA CTT TCC A-3´	980–1003	
S6E 5´-GAGAAT TCCGAGGACTGG GGA CCC TG-3´	130–146	
S7B 5´-CGG GAT CCT TAG GGT TTA AAT GTATAC C-3´	823–842	

Table 3.6: HBV amplification first round PCR and nested PCR cycling conditions

First Round PCR and nested PCR				Real-time PCR	
Cycling condition	Temperature °C	Time	Cycling conditions	Temperature °C	Time
Denaturation 1x	95 °C	4 minutes		95 °C	4 minutes
Amplification	40 x cycles				
Denaturation 1x	95 °C	1 minute	40 x cycles	95 °C	1 minutes
Annealing	55 °C	45 seconds		55 °C	45 seconds
Elongation	72 °C	1 minute		72 °C	1 minutes
Final elongation	72 °C	10minutes		72 °C	10 minutes

3.6 PCR Products Verification (HBV, HCV and HIV)

PCR products were qualitatively verified using 1% agarose gel (ThermoFischer,Waltham, Massachusetts) stained with 0.15 U/ µl ethidium bromide (Biorad, California, USA). Aliquot of 10 µl PCR product was mixed with 2µl 10x loading buffer. The mixtures were run on 1% gel along with a molecular weight maker (based on the viral gene of interest) as a band size reference. The agarose gel was run at 100 V for 45 minutes. Gel was placed inside the UV transilluminator to visualise and image capturing.

3.7 Sequencing

The PCR products were sent for direct sequencing at the Inqaba Biotechnological Industry, PTY, LTD, Pretoria, South Africa. The amplicons were prepared for direct sequencing using the BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems., Foster City, USA) and sequencing was done using the ABI 3130XL Genetic analyser (Applied Biosystems, Foster City, CA).

3.7.1 Sequencing Reaction

Sequencing reaction mix for each sample was prepared as follows: 1.5 µl 5X sequencing buffer, 1.0 µl BigDye terminator, 1.0 µl primer (5 pmol/ µl), 2.0 µl template DNA, DEPC-treated water and primer sequences are shown in Table 3.7. A total of 10 µl mixture was added into 96-well plate and subjected into sequencing under the cycling condition shown in Table 3.8.

3.7.2 Sequence Reaction Clean-up

An aliquot of 50 µl of the 1:1 ratio of sodium acetate: ethanol (NaAc:EtOH) was added into samples and centrifuged at 2 000 g for 30 minutes. The well plates were inverted and centrifuged at 150 g for 1 minute. Pre-chilled 70% ethanol was added into the wells, and then centrifuged at 2000 g for 5 minutes. The samples were dried at 65 °C for 5 minutes and

loaded into the sequencing machine ABI 3130XL Genetic analyser (Applied Biosystems, Foster City, CA). An aliquot of 10 µl of the Hi-Di formamide was added into the sample for 5 minutes and loaded into a sequencing machine.

3.7.3 Sequence Editing

Nucleotide sequences of HBV, HCV and HIV were edited and contiguous sequences were formed using sequencher v4.5 (Gene Codes Coorporation, USA). The chromatograms were edited by removing unwanted and mixed nucleotides character we removed spaces within in the sequences. The contiguous sequences were formed by joining overlapping DNA sequences of a gene.

3.8 Bioinformatics and Phylogenetic Analyses

The consensus sequences were compared with complementary genotype sequences from the GenBank using Basic local alignment search tool (BLAST). Representative sequences belonging to distinct genotypes were redeemed from the GenBank to make comparison with study sequences. Multiple sequence alignment of the sequenced nucleotides region was performed with CLUSTALW within the MEGA software package version 7.0.; the aligned nucleotide base sequences were subjected into phylogeny inference on MEGA 7.0. The neighbour-joining method was used to generate dendograms and the evolutionary relationship was performed using pairwise genetic distance with 1000 bootstrap replicates (Felsenstein, 1985; Saitou and Nei, 1987; Kumar *et al.*, 2004). Frequency estimates of evolutionary divergence between nucleotide sequences were then estimated using the Kimura 2-parameter model (Kimura, 1980).

Table 3.7: Sequencing primers for HBV and HIV

Primer name	Primer sequence	Reference
PRT-F2	5'- CTT TAR CTT CCC TCA RAT CAC TCT-3'	(Hunt *et al.*, 2012)
RT-R2	5'- CCT CTG TAT GTC ATT GAC AGT CC-3'	
SeqF3	5'- AGT CCT ATT GAR ACT GTR CCA G- 3'	
PolM1	5'-GTT AAA CAA TGG CCA TTG ACA GA- 3'	
PolM8	5'-CTG TAT ATC ATT GAC AGT CCA G- 3'	
PolM1-R	5'- TCT GTC AAT GGC CAT TGT TTA AC- 3'	
PolM	5'- TCC CTC AGA TCA CTC TTT GGC A- 3'	
RTC-2R	5'- TGT CAA TGG CCA TTG TTT AAC CTT TGG- 3'	
S1	5´-CCT GCT GGT GGC TCC AGT TC-3´	(Mphahlele *et al.*, 2006)
S2Na	5´-CCA CAA TTC KTTGAC ATA CTT TCC A-3´	
S6E	5´-GAGAAT TCCGAGGACTGG GGA CC TG-3´	
S7B	5´-CGG GAT CCT TAG GGT TTA AAT GTATAC C-3´	

Table 3.8: Sequencing cycling conditions

Cycling condition		Temperature (°C)	Time
Denaturation 1x		94 °C	1 minute
Amplification	25 cycles		
Denaturation 1x		94 °C	10 seconds
Annealing		50 °C	5 seconds
Elongation		60 °C	4 minutes
Final elongation		60 °C	1 minutes

3.9. Mutations Analyses

3.9.1 Nucleotide Bases and the Translated Amino Acids Mutations Analysis

The aligned sequences nucleotide bases and the translated amino acids sequences were analysed for mutations using BioEdit. Mutations on the nucleotide and amino acids sequences were compared with previously identified mutations from Geno2Pheno database, GenBank and literature.

3.9.2 Drug Resistance Mutations Analysis

The HIV consensus sequences were deposited into Standford HIV resistance database for interpretation according to the available Stanford genotypic resistance interpretation algorithm (http://hivdb.standardford.edu/). The nucleic acid sequences quality was assessed using the calibrated population resistance (CPR) tool version 6.0. The HBV consensus sequences were deposited into the Geno2pheno database (hppt://hbv.geno2pheno.org.index.php) to identify the sequence genotype and for prediction of phenotypic drug resistance and escape mutations within the reverse transcriptase (RT) domain and HBsAg gene frame, respectively (Cento *et al.*, 2013).

3.10 Statistical analyses

Data were analysed using GraphPad Prism (version.6.0.) to calculate whether the serological markers (HBsAg) expression of the samples concentrations differed significantly from each other with regard to optical density measured.

CHAPTER 4

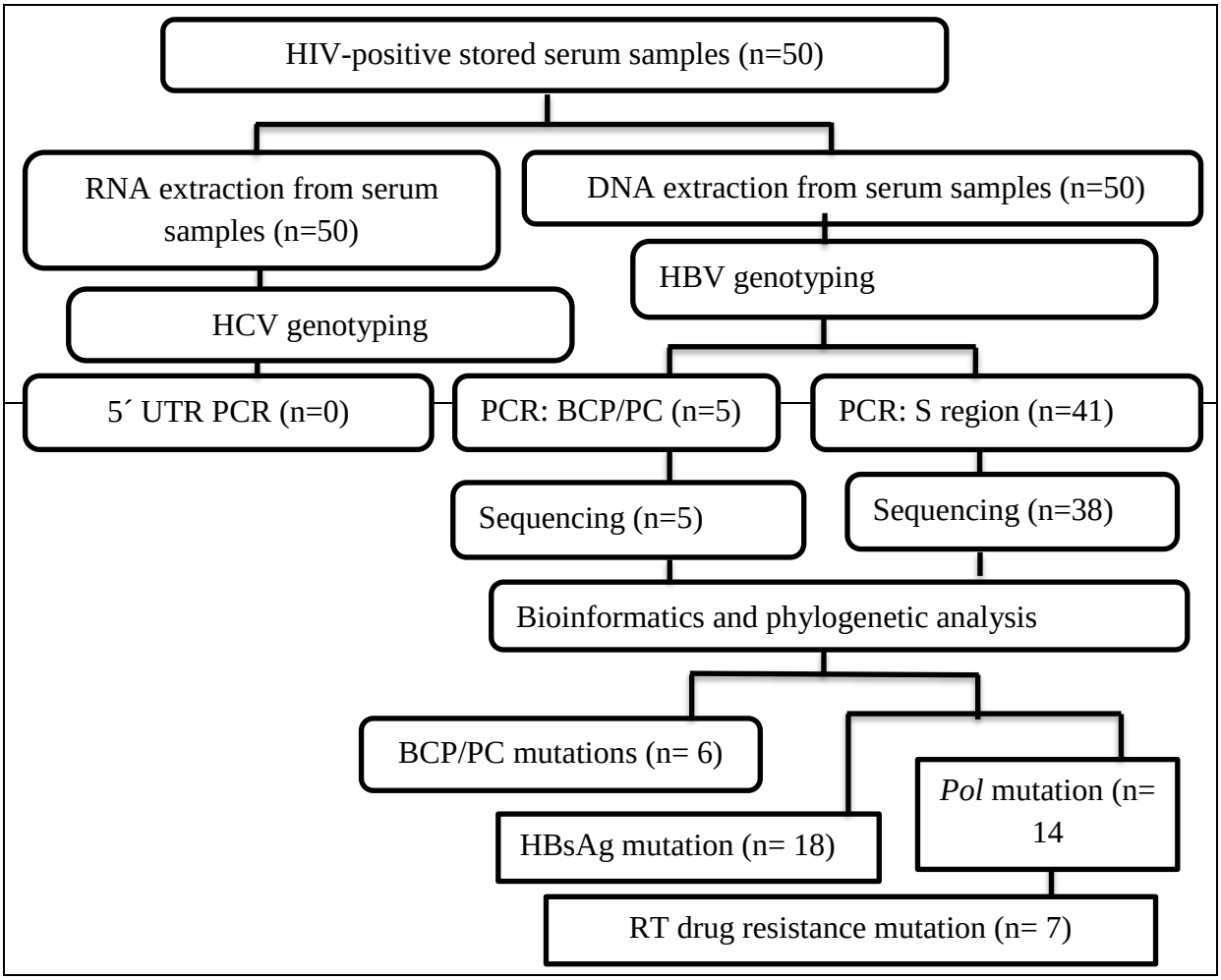
4. RESULTS

4.1 HBV/HIV co-infection from KwaZulu-Natal cohort

4.1.1 Detection of HBV Amplified Products: BCP/PC Region Amplicons

The basic core promoter/precore region of HBV was successfully amplified by nested PCR at 10% (5/50) out of DNA extracted from HIV infected patients (Figure 4.1). HBV BCP/PC amplicons are shown as 307 bp bands below (Figure 4.2).

Figure 4.1: Summary of the results on the analysis of BCP/PC and partial surface regions from the Kwa-Zulu Natal isolates infected with HIV.



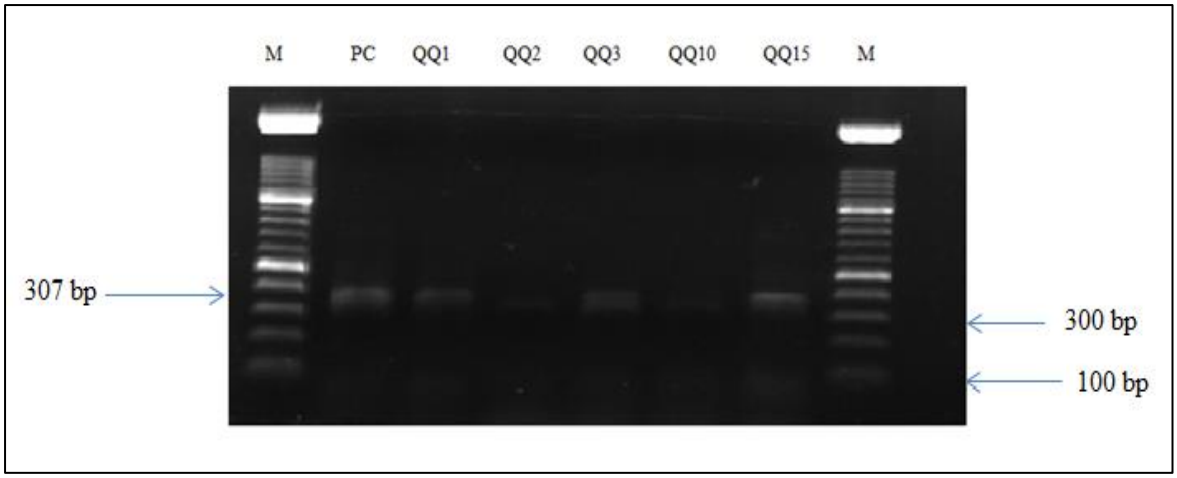


Figure 4.2: Amplified BCP/PC region of HBV genome (1659-19741 *EcoRI* site) shown as 307 bp amplicons on a 1% ethidium bromide stained agarose gel. Blue arrows on the left indicate the size of MW (100 bp) and targeted band size ~ 307 bp. M: molecular weight, PC: positive control, bp: base pair. M= 100 bp molecular weight marker; PC= positive control BCP/PC region amplicons (~ 307 bp) = QQ1, QQ2, QQ3, QQ10 and QQ15.

4.1.2 Partial Surface Region (overlapping surface/polymerase) Amplicons

The overlapping surface/polymerase gene of HBV was successfully amplified by nested PCR at 78% (41/50) from extracted DNA samples from stored sera of HIV infected patients. HBV overlapping surface/polymerase region amplicons are shown as 547 bp bands below (Figure 4.3). PCR amplification could not be obtained for the other 11 (22%) samples.

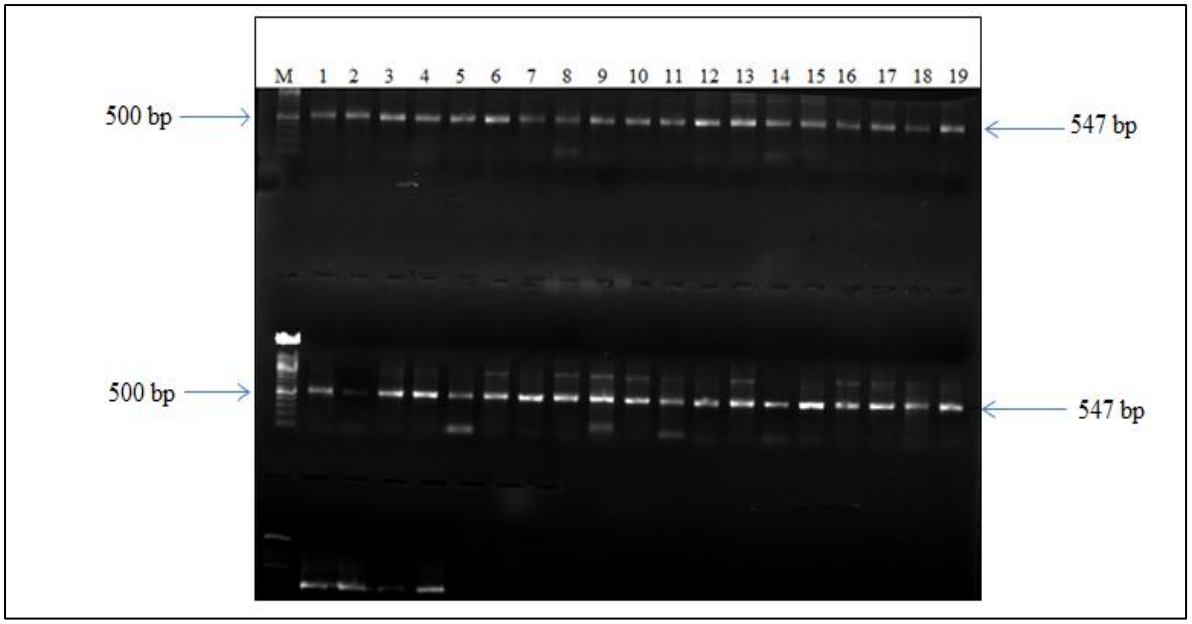


Figure 4.3: Amplified overlapping surface/polymerase region of HBV genome (256 to 796 *EcoRI* site) shown as 547 bp amplicons on a 1% ethidium bromides-stained agarose gel obtained from the HIV infected KZN cohort. Top and bottom lane samples: Well M = 1000 plus bp molecular weight marker, well 1 sample = positive control, from well 3 to well 19 = sample amplicons of targeted band size ~ 547 bp.

4.2 Sequence Analyses of BCP/PC Gene Region

All 5 BCP/PC individuals' sequences were identified as HBV genotype A (Figure 4.4a). This study sequences clustered with subgenotype A1 sequences previously isolated from South Africa (Figure 4.4b). sequence QQ10 clustered with serological HBV subtype *adw4* (red) sequence and QQ1, QQ2 and QQ3 sequences clustered with *ayw2* and *ayw3* HBV serotype sequences (Figure 4.4c). The study nucleotide sequences did not cluster with reference sequences for genotypes B, C, E, F, G and H.

The genotype and subgenotype of sequences were confirmed by depositing the BCP/PC nucleotide sequenced into the Genotype2pheno database. The isolated sequences were identified as genotype A and subgenotype A1 based on the results retrieved from the Geno2Pheno database with the percentage of similarity to subgenotype profile of 98% - 99.0%.

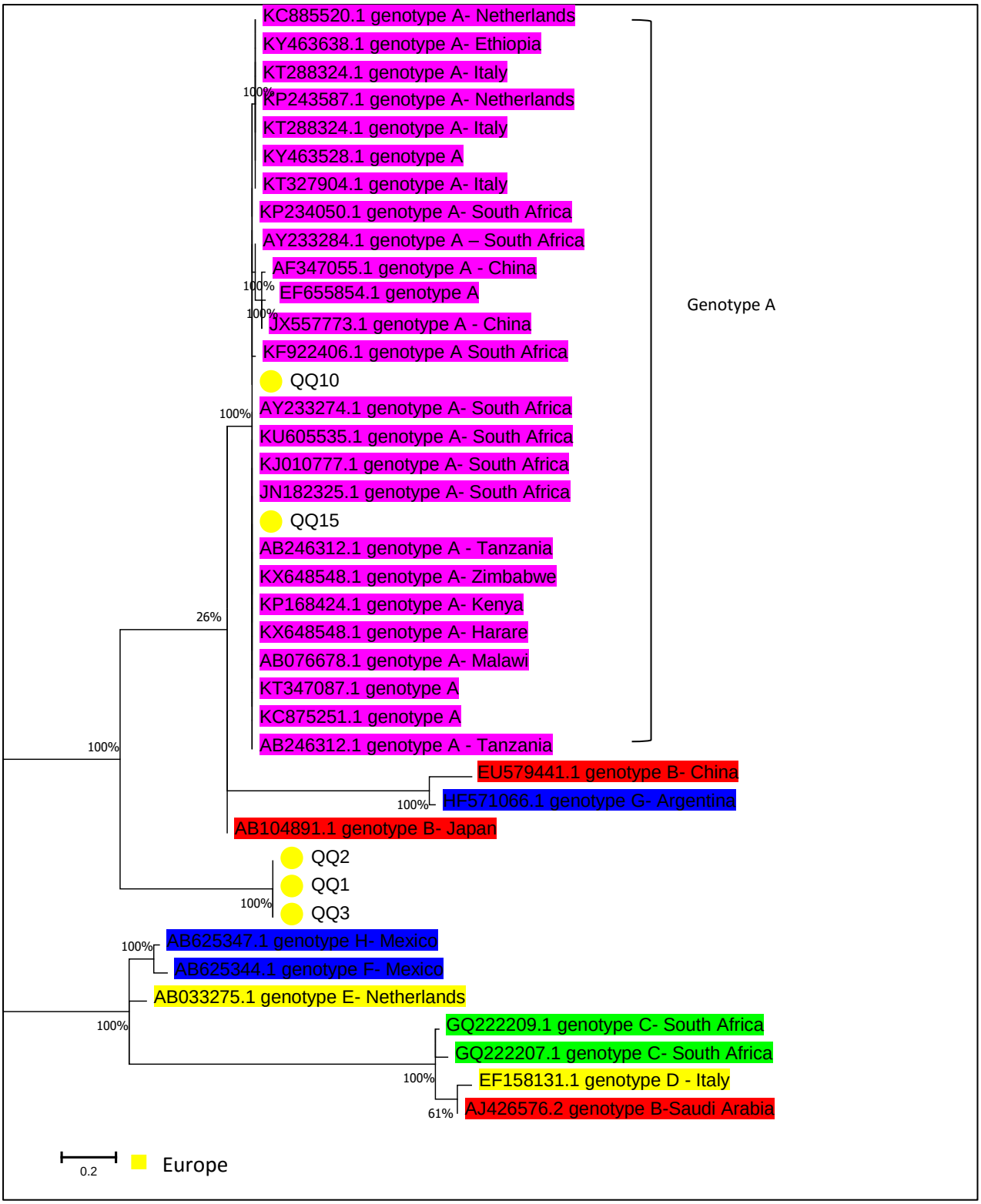


Figure 4.4a: Phylogenetic tree identification of the BCP/PC sequences from the KZN cohort by comparing to genotypes sequences obtained from the GenBank (designated by accession numbers and location). Study sequences are labelled ● while reference sequences from GenBank are highlighted with pink, red, blue, purple, yellow and green.

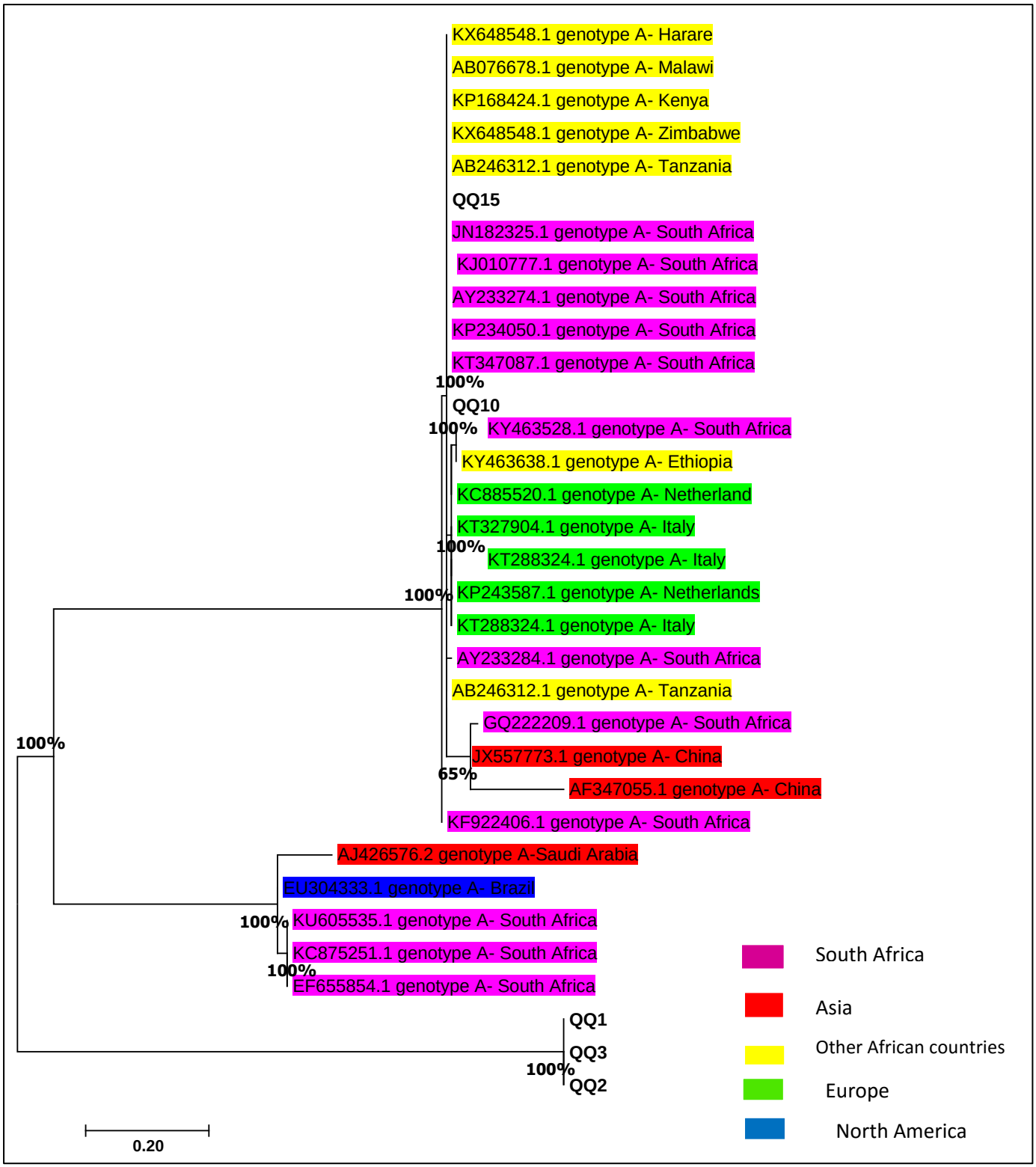


Figure 4.4b: Phylogenetic relationship of the BCP/PC sequences of KZN cohort with representative subgenotype A1 sequences obtained from the GenBank (designated by accession numbers and location). Study sequences are unlabelled while Genotype A reference sequences from GenBank are highlighted with pink, red, blue, yellow and green.

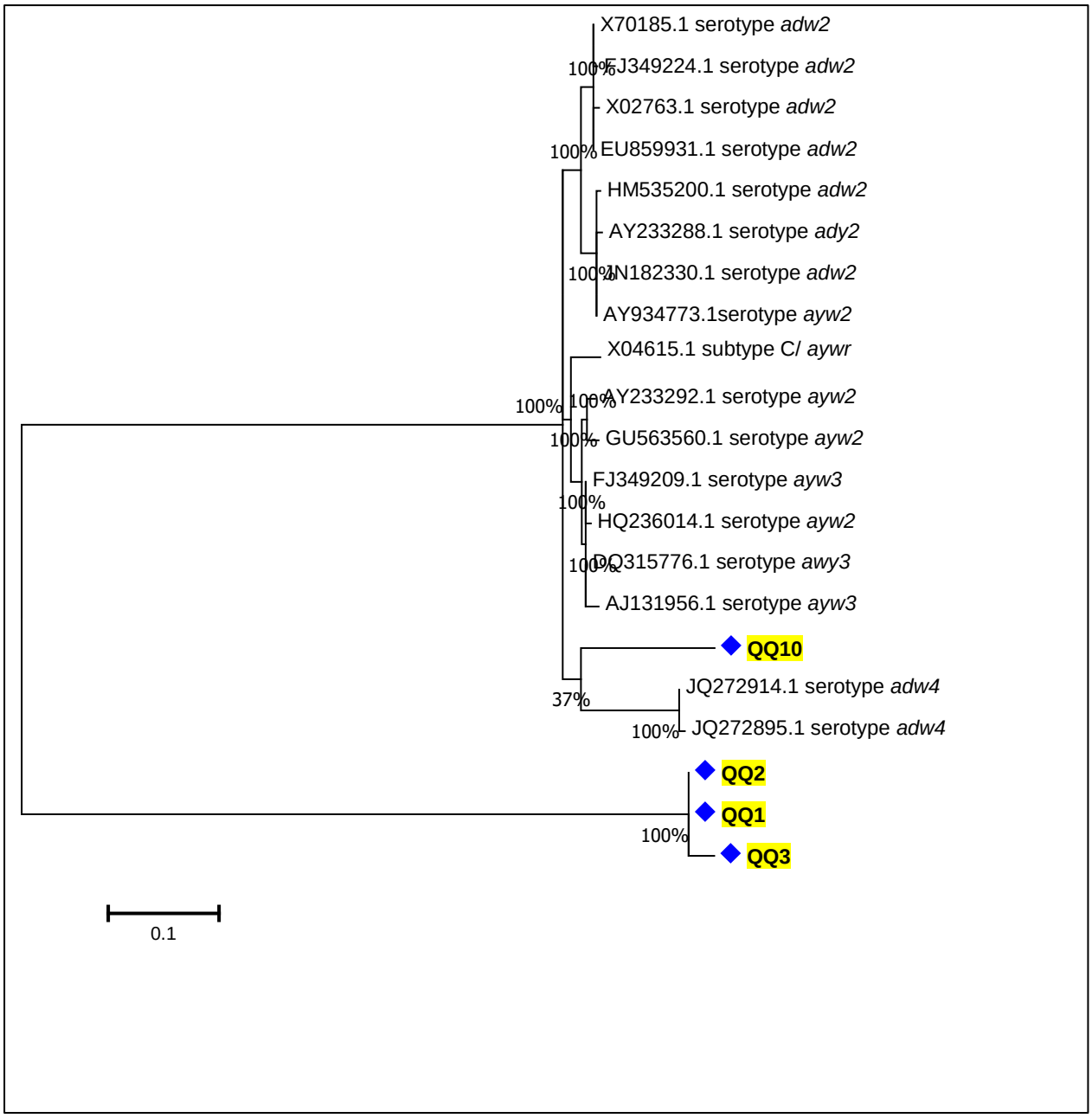


Figure 4.4c: Phylogenetic tree comparing the BCP sequences of this study with representative serological serotypes sequences obtained from the GenBank (designated by accession numbers). Study sequences are labelled ◆while reference sequences from GenBank are unlabelled.

4.3 Sequence Analyses of overlapping Surface/ Polymerase Gene Region

A total of 38 out of 41 HBV overlapping surface/polymerase amplicons were successfully sequenced, a phylogenetic tree was generated with all 26 nucleotide sequences as shown in (Figure 4.5). Isolated sequences clustered with genotype A sequences previously deposited in GenBank. The study nucleotide sequences did not cluster with reference sequences for genotypes B, C, E, F, G and H.

The genotype and subgenotypes of sequences were confirmed by depositing all the surface gene nucleotides sequenced into the Genotype2pheno database. The 38 sequences were identified as genotype A and subgenotype A1 based on the results retrieved from the Geno2Pheno database with the percentage of similarity to subgenotype profile of 96.85% - 99.0%.

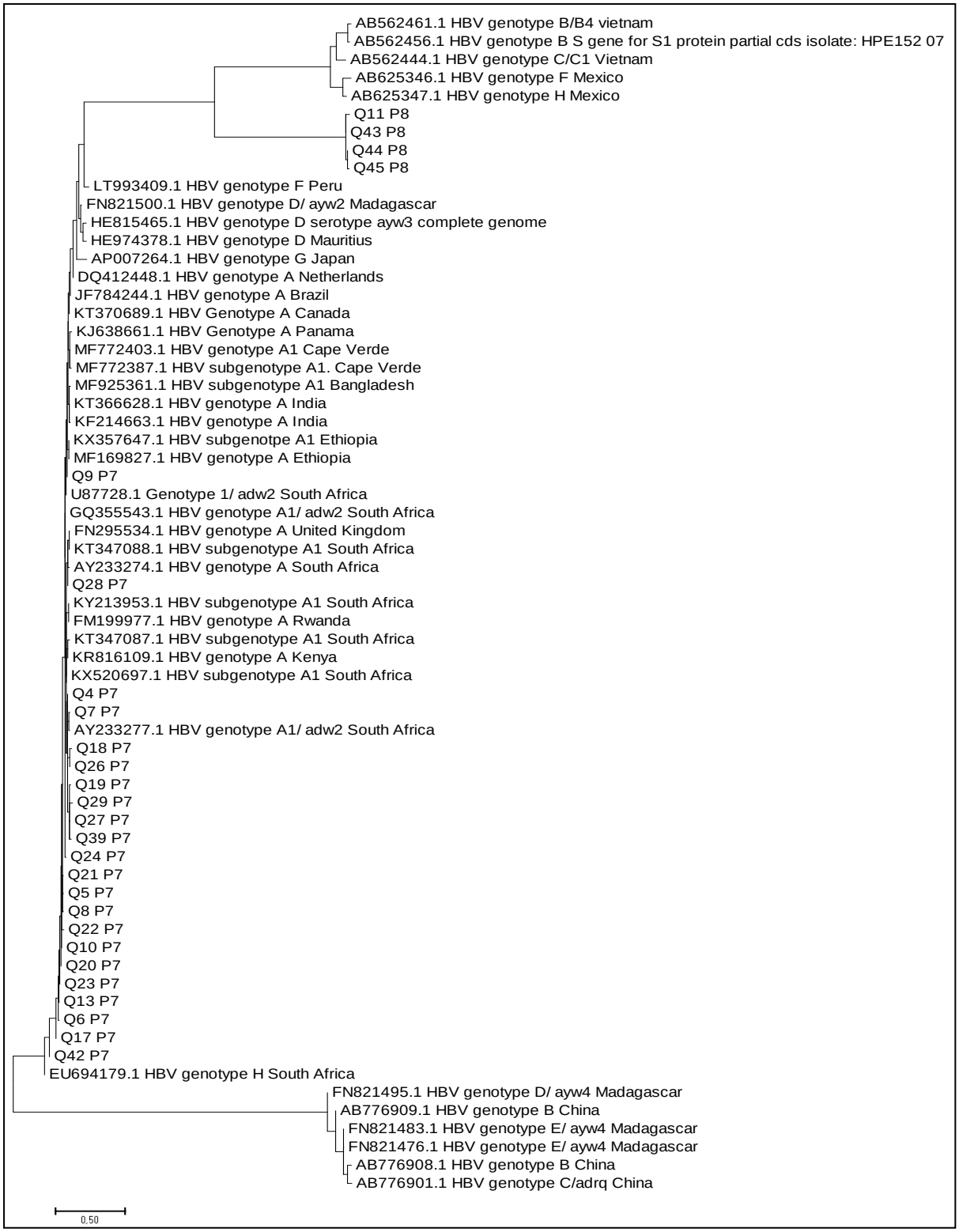


Figure 4.5: Phylogenetic tree comparing the S gene sequences of this study with representative sequences obtained from the GenBank (designated by accession numbers). Study sequences *are represented by letters Q.

4.4 HBV Mutations Analysis

4.4.1 Analysis of BCP/PC Mutations

BCP/PC nucleotide sequences were aligned with a wildtype (Wt) reference sequence (X70185.1) which is genotype A to identify the presence of any nucleotide variations.

A total of five BCP/PC DNA sequences were analysed for mutations at loci 1659-1974 and the aligned sequences are shown in (Figure 4.6). Three nucleotide sequences of five individuals had wild type BCP sequences and two sequences had mutations when compared to the wild type reference sequence (X70185.1). The prevalence of mutations on the BCP/PC region of HBV from Durban cohort 16% (6/38), The BCP/PC sequences had mutations; A1752G, T1753C, A1762T, G1764A, C1766T, G1862A and GCAC Kozak sequence. QQ10 had the A1752G, T1753C, A1762T, G1764A nucleotide variants; QQ3 had C1766T and G1862A mutations (underlined and highlighted in yellow (Figure 4.6). QQ15 had the GCAC Kozak sequence (1809-1812) preceding the precore start codon (highlighted in red and underlined). Other 4 sequences had with Kozak sequence TCAT (1809-1812). Overall, a total of 16% (6/38), nucleotide sequence substitutions were detected within the QQ10 and QQ3 sequences, this include A1752G, T1753C, A1762T, G1764A, C1766T, G1862A and GCAC the Kozak sequence (1809-1812). This mutations cause immune escape HBV. All nucleotide variations detected for BCP/PC sequences are summarized and indicated by highlighted areas (Table 4.1).

Figure 4.6: BCP/PC aligned nucleotide sequence and mutations of the KZN cohort, BCP/PC nucleotide sequence alignment with wild type (Wt) reference sequence (X70185.1). Underlined and highlighted in yellow indicate regions with mutations.

Table 4.1: Nucleotide variations sequences of aligned BCP/PC region with reference wild type sequence (X20185.1). Region highlighted in yellow indicate with nucleotide variations and region highlighted with red represent the Kozak (1809- 1812)

	Nucleotides position Loci position (1659-1899)														
Sample	1659	1752	1753	1758	1762	1764	1766	1768	1809	1810	1811	1812	1862	1896	1899
Wt	A	A	T	T	A	G	C	T	T	C	A	T	G	G	G
QQ1	A	A	T	T	A	G	C	T	T	C	A	T	G	G	G
QQ2	A	A	T	T	A	G	C	T	T	C	A	T	G	G	G
QQ3	A	A	T	T	A	G	T	T	T	C	A	T	A	G	G
QQ10	A	G	C	G	T	A	C	T	T	C	A	T	G	G	G
QQ15	A	A	T	T	A	G	C	T	G	C	A	C	G	G	G

4.4.2 Nucleotides and Amino Acids Mutations within Overlapping Surface/Polymerase Gene

A total of 41 amplicons were generated by PCR amplification of the overlapping surface/polymerase region and 38 nucleotide sequences were generated. Since the 547 bp fragment sequenced in this study was too short to identify mutations when compared to referenced wild type, the Geno2Pheno database was used for mutation predictions. The 38 generated sequences were deposited into the Geno2Pheno database to determine nucleotide and amino acid variations within overlapping surface/polymerase gene region.

4.4.3 Amino Acids Mutations within HBsAg

Mutations within the HBsAg of the overlapping surface/polymerase gene were determined from the 38 sequences, 18 amino acid variations were detected within the HBsAg region in all 38 partial S sequences as shown in (Table 4.2). The most common mutation was S207N found in 27 individual sequences at 71%, followed by L216V and A194V at 23%, P70H at 21% L209V at 18%, P217L at 8%, F134L, E164D and T189I at 5% and S204R, S117N, T143S, G145R, Y206H, P127T, Y200T, F129T and K122R all at 3% (Table 4.2).

Table 4.2: Amino acids mutations within the HBsAg region

	Amino acid variation	Frequency	Function
"α" epitope			
	K122R		Sub-serotype change (d/y)
	F134L		Unclear
	S117N		Unclear
	T143S		Reflect the genotype variations
β-cell epitope			
	S207N		Unknown in genotype A
	Y200T		Unknown in genotype A
	G145A		Vaccine-escape mutant
T-helper **epitope**	P127T		Vaccine-escape mutation
			lower reactivity in HBsAg assay
outside **the "a" epitope**	E164D		Vaccine-escape mutation
			Cause V173L mutation in the overlapping pol gene
	L209V		
	Y206H		
	L216		
	A194V		
	P70H		Overt HBV infections
	P217L		
	T189I		
	S204R		Unknown/genotype
	F129T		

4.4.4 Amino Acids Mutations within Polymerase Region

Within the RT domain of the polymerase, a total of 38 nucleotide mutations were identified with the amino acid substitutions being identified at 36% (14/38) different positions within the RT region (Table 4.3). The M129L mutation was the most common in this study, accounting for 84% (32/38), followed by V163I at 78%; I253Y at 50% and S105T at 40%. Other amino acid variations identified included L217R, A233S, Q125E, T128A, V214A, V204I, M204V, L180M, V173L and S202K at (21%, 16%, 13%, 8% and 3%). Multiple mutations were also identified in an individual sequences, with QQ31 containing the highest number of multiple amino acids variants being A233S, V163I, I213Y and M129L.

Table 4.3: Distribution of amino acids substitution in reverse transcriptase region of Polymerase of HBV positive patients coinfected with HIV

	Amino acids position	Significance	Frequency
Amino acids substitutions	Drug resistance associated		
	M129L		84%
	M204V		3%
	L180M		3%
	V163I		78%
	V173L		3%
	A223S		16%
	Compensatory mutation		
	Q125E		13%
	S202K		3%
	L217R		21%
	V214A		3%
	V204I		3%
	I253Y		50%
	T128A		8%
	S105T		40%

4.4.5 Drug Resistant Mutations

The prevalence of mutations associated with drug resistance was 50% (7/14) within the RT region (Table 4.4). Primary mutations within the RT domain of the polymerase associated with drug resistance were identified and included L180M, M204V, V163I, S202K, M250I, I223V and I253Y. Drug resistance mutations included LMV resistance at 57% (4/7), LdT at 57% (4/7), 14% for ETF and 43% for ADV resistance. Mutations causing resistance to LMV and LdT were M204V, L180M, V163I, and S202K; with S202K also being resistance to EFT and ADV resistance mutation were I253Y, I223V and M250I. Multiple drug resistance mutations within a single sample were identified from sample QQ46 containing L180M, M204V, S202K and M250I mutations. All the mutations associated with drug resistance were identified in the polymerase (RT) were identified from genotype A sequences.

Table 4.4: Distribution of drug resistant mutations in reverse transcriptase region of Polymerase of HBV positive patients coinfected with HIV

Amino acids	Frequency (n/ 38)	Drugs level of susceptibility				
		LMV	LdT	ETF	ADV	TNF
M204V	31	Resistance	Resistance	Susceptible	Susceptible	Susceptible
L180M	1	Resistance	Resistance	Susceptible	Susceptible	Susceptible
M129L	1	Unclear	Unclear	Unclear	Unclear	Unclear
V163I	1	Resistance	Resistance	Susceptible	Susceptible	Susceptible
L180M + M204V	1	Resistance	Susceptible	Susceptible	Susceptible	Susceptible
S202K	1	Resistance	Resistance	Resistance	Susceptible	Susceptible
M250I	1	Susceptible	Susceptible	Susceptible	Resistance	Susceptible
I223V + I253Y	1	Susceptible	Susceptible	Susceptible	Resistance	Susceptible

LMV- lamivudine; LdT- telbivudine; ETF- entecavir; ADV- adefovir; TDF-tenofovir

Discussion

A total of 34 million people were living with HIV at the end of 2010, with 10% of this population being infected with HBV. Both HBV and HIV are highly endemic in South Africa with an estimated 0.4%- 23% of HBV infections reported in HIV-positive patients (Burnett *et al.,* 2005; Firnhaber *et al.,* 2009; Lukhwareni *et al.,* 2009; Boyles and Cohen, 2011; Mayaphi *et al.,* 2012). HBV/HIV co-infections are common in the region because of shared transmission modes and risk factors.

High DNA sequence heterogeneity has led to HBV being phylogenetically classified into nine genotypes (A–I) with a presumed 10^{th} genotype, 'J', isolated first from a Japanese patient (Tatematsu *et al.,* 2009). HBV sequence heterogeneity is caused by the error-prone nature of HBV replication; the lack of proofreading mechanisms of the viral polymerase (reverse transcriptase) has also led to high mutation rates. HBV sequence heterogeneity is also caused internally by selective immune system pressure and externally by vaccination and antiviral treatment.

The HBV sequence heterogeneity may have an impact on the progression of the hepatitis and HIV infection; they may influence the HBV/HIV coinfected genotypes currently, prevailing in South Africa. Understanding the HBV genotypes in HIV infected individuals is vital, because it helps in diagnosis, prognosis, management and treatment of HBV and HBV/HIV coinfection.

Therefore this study was conducted to determine HBV genotypes; mutations occurring at BCP/PC, partial surface gene and polymerase overlapping the HBsAg of HBV isolated from KwaZulu-Natal individuals with HIV infection.

From 50 donated stored samples from HIV-positive Durban cohort, HBV DNA was extracted and subjected to nested PCR amplification of the BCP/PC region. The 307 bp BCP/PC region

(Figure 4.2) was successfully amplified by a nested PCR for the 5 DNA sample out of 50 HBV DNA samples.

The 50 HBV DNA samples were also subjected to nested PCR of the partial surface gene region (consist of the overlapping HBsAg/polymerase region). The 547 bp partial surface gene region was successfully amplified by a nested PCR for the 78% (41/50) samples (Figure 4.3). More amplicons (41) were generated by PCR amplification of partial S region as compared to the BCP/PC suggesting that PCR amplification with DNA *Taq* amplification is more efficient with long DNA fragment as compared to short DNA fragment.

From the KwaZulu-Natal cohort, HBV PCR amplification of the BCP/PC region identified that 5 individuals were HBV/HIV co-infected; whilst the HBV partial surface gene amplification identified that 41 individuals were HBV/HIV co-infected. HBV/HIV co-infection was 10% and 78% based on PCR amplification of the BCP/PC and partial surface gene regions.

To identify the HBV genotypes in HIV co-infected individuals and to determine their mutations; the BCP/PC and partial surface gene regions were subjected to sequencing and phylogenetic analyses. Identification of HBV and their mutations was largely dependent on direct sequencing which is considered to be the "gold" standard. All 5 BCP/PC amplicon sequences were identified as genotype A and subgenotype A1 based on the phylogenetic analysis of the BCP/PC amplicon sequences (Figure 4.4b).

A total of 38 sequences were obtained by direct sequencing of the HBV partial surface gene as compared to BCP/PC region. The 38 partial surface gene region sequences were subjected to genotype and subgenotype identification using a neighbour joining phylogenetic tree which identified all 38 sequences as genotype A and subgenotype A1 (Figure 4.5).

However, the ability to distinguish subgenotype A1 from A2 sequences was not as obvious by phylogenetic tree analyses because of the small size of the BCP/PC (~307 bp) and partial surface (547 bp) gene fragments amplified. The identity of the individuals was further confirmed by comparing the study sequences to reference sequences from the Geno2Pheno HBV database. The Geno2Pheno sequence analyses confirmed all 5 BCP/PC and 38 partial surface gene sequences as genotype A and subgenotype A1; these results are in agreement with previous studies from South Africa, which reported HBV genotype A and subgenotype A1 to be prevailing (Kramvis *et al.*, 2005; Kramvis and Kew, 2007; Lukhwareni *et al.*, 2009).

Phylogenetic tree analysis was also used to determine the serotype of BCP/PC sequences; QQ10 sequence was closely related to a serological HBV subtype *adw4*, The subtype *adw4* sequence has previously been associated with genotypes A, B, F, G, and H which is in agreement with our results (Lukhwareni *et al.*, 2009; Kramvis, 2014). QQ1, QQ2 and QQ3 sequences were closely related to *ayw2* and *ayw3* subtypes (Figure 4.4c), these results are contrary to previous report where the *ayw2* and *ayw3* were associated with genotype D and E (Kramvis, 2014). The serological HBV subtypes of this study still need to be confirmed using HBsAg sequences because previous studies have reported serological subtypes identification based on HBsAg heterogeneity to be more effective as compared to using BCP/PC region (with amino acid substitutions on the antigenic determinant) (Kramvis *et al.*, 2005).

The BCP and precore regulatory sequence controls the production of the 3.5 Kb pregenomic RNA (pgRNA) and preCore (preC) mRNA. The pgRNA is translated into core and polymerase whilst precore mRNA is translated into HBeAg. Mutations within the BCP region have been reported to affect the precore start codon (preC mRNA) and the translation of preC mRNA into HBeAg. BCP/PC region of 5 individuals coinfected with HIV were analysed for mutations at 1659-1974 loci (Figure 4.6).

Mutations identified included BCP mutations (A1752G, T1753C and A1762T) and PC mutations (C1766T and G1862A). In this study dual BCP mutation A1762T and G1764A were identified in QQ10. The double BCP mutations A1762T and G1764A in one sample have been previously detected in South Africa studies (Baptista *et al.*, 1999; Alfaresi *et al.*, 2010). But combination of these double BCP mutations was also identified previously from genotype B and C (Chen *et al.*, 2014). Suggesting that these variants are not restricted to genotype A only.

The BCP mutations A1762T and G1764A are reported to reduce transcription of preC-mRNA which suppresses HBeAg expression (Song *et al.*, 2005; Tong *et al.*, 2007). The resultant decrease of HBeAg expression might trigger down-regulating toll-like receptors expression on hepatocytes leading to reduced cytokine expression which causes immune escape virus (Buckwold *et al.*, 1996). These mutants were reported to cause 20% reduction on HBeAg expression by double BCP mutation (A1762T and G1764A), 30% reduction on HBeAg expression by triple BCP mutation (Parekh *et al.*, 2003); suggesting that the reduction on HBeAg expression by BCP mutation depends on the frequency of BCP variants on a patient.

The dual BCP mutations A1762T and G1764A are present in QQ10 which is also co-infected with HIV. However, there is controversy about the effect of A1762T and G1764A mutations in HBV/HIV co-infected individuals (Song *et al.*, 2005; Tong *et al.*, 2007). Previous studies report increased replication of both viruses, others indicated reduced replication and no effect on the viral replication (Scaglioni *et al.*, 1997; Tong *et al.*, 2007). Some studies reported lower detection of A1762T and G1764A in HIV coinfected patients while other studies reported higher detection of A1762T and G1764A in HIV coinfected patients (Scaglioni *et al.*, 1997; Parekh *et al.*, 2003). In this current study there is also low detection of these

mutations, however, the frequency of these mutations may be influenced by the small sample size in this study.

The dual BCP mutations A1762T and G1764A were previously identified in patients with chronic hepatitis, fulminant HCC and associated with high potential risk for hepatocarcinogenesis (Takahashi *et al.*, 1995; Baptista *et al.*, 1999). However, our study findings were not sufficient to support the idea that BCP A1762T and G1764A mutations effect on the stages of hepatitis (chronic hepatitis, fulminant, HCC and hepatocarcinogenesis) since we did not identify the patients stage of hepatitis.

QQ10 had T1753C mutation in addition to A1762T and G1764A mutations, the presence of these triple mutation (A1762T, G1764A and T1753C) has been previously reported as a marker for HCC (Liu *et al.*, 2010; Yin *et al.*, 2011).

QQ3 had C1766T and G1862A preCore mutations. The G1862A missense mutation has been reported to cause the substitution of valine (Val) for phenylalanine (Phe); the Phe substitution may abrogate signal peptide cleavage of HBeAg thus affecting the expression of HBeAg (Kramvis *et al.*, 1997). The G1862A mutation has also been reported to interfere with reverse transcription. The G1862A in the precore region has been identified previously in patients with hepatocellular carcinoma, fulminant hepatitis, cirrhosis and has been reported less in HIV-co-infected patients (Carman *et al.*, 1995).

Four sequences (QQ1, QQ2, QQ3 and QQ10) as compared to the wildtype sequence had with Kozak sequence (TCAT) at (1809-1812) and QQ15 had the GCAC at the Kozak sequence (1809-1812). The Kozak sequence mutants affect HBeAg translation and were previously identified from South African HBV subgenotype A1; supporting that this study individuals containing the Kozak sequence TCAT are subtype A1 (Baptista *et al.*, 1999; Kimbi *et al.*, 2004). The identification of this study HBV as subtype A1 using TCAT Kozak sequence is in

agreement with the phylogenetic analysis results (Figure 4.4a and 4.4b) whereby these 4 isolates clustered with previously identified subgenotype sequences. QQ15 had the GCAC at the Kozak sequence, which has been identified in other subgenotypes other than subgenotype A1, suggesting that isolate QQ15 is not a subgenotype A1. This is contrary to the phylogenetic analysis results (Figure 4.4a and 4.4b) where QQ15 clustered with previously identified subgenotype A1 sequences.

The PreS/S ORFs codes for 3 different surface proteins LHBs, MHBs and SHBs that forms the HBV surface antigen (HBsAg). The HBsAg is a foreign toxin which induces the immune system response to the HBV infection. HBsAg is integral part to HBV attachment to hepatocyte and HBV particle assembly (Prange and Streeck, 1995).

The surface antigen serves as an epitope to which the antibody attaches; hence it is used in current recombinant vaccines. The HBsAg contains the alpha (α), beta (β) and T –cell epitopes, the "α" epitope is the main region targeted by antibodies and it contains the (β) and T –cell epitopes (Caligiuri *et al.*, 2016). The "α" epitope is referred to as the major hydrophilic region of HBsAg, which is between the amino acids 124- 150 region (Liu *et al.*, 2017). Amino acid substitution within the "α" epitope domain of HBsAg may cause structural and functional changes in the S protein (Zuckerman and Zuckerman, 2003).

We determined the mutations on the partial surface gene of HBV/HIV co-infected individuals from the Durban cohort. A total of 18 amino acid substitutions were observed within the HBsAg region in all 38 partial surface sequences. The sequences contained mutations at α, β and T –cell epitopes (Table 4.2). Individuals sequences showed the following prevalent mutations at the "α" epitope (aa 79 to aa 150) K122R, F134L, S117N, T143S. However, the role of F134L and S117N mutations in HBV infection still need to be explored.

Mutation K122R was identified in QQ1 (subgenotype A1), this result is in agreement with a previous study by (Kwange *et al.*, 2013); whereby the K122R mutation was identified from subgenotype A1. K122R mutation has been reported to be associated with determining sub-serotype change from "d" to "y" (d/y) (Cooreman *et al.*, 2001). Previous studies have failed to detect HBsAg in containing the K122R using certain ELISA diagnostic tests (Malik *et al.*, 2012). T143S was another mutation detected in the "α" epitope; it was identified and has been previously reported to be responsible in reflecting the genotype variations (Soussan *et al.*, 2001).

Mutations outside the "a" determinants were frequent, with mutations identified within the β-cell and T-cell epitopes. A vaccine-escape mutation within the T-helper epitope identified from this study was P127T; this mutation was identified in QQ16 and QQ17 and has been reported to cause diagnostic escape within anti-HBc positive patients (Kuzin *et al.*, 2012). Mutations within the β-cell epitope included S207N, Y200T (aa 160 to aa 270) and G145R (aa 122 to aa 148). The S207N mutation was detected in 27 individuals whilst Y200T was detected in 1 patient, these mutations were detected within the β-cell epitope however, their role in S protein and HBV infection still need to be elucidated further. However, S207N has been reported to affect HBsAg expression.

G145R was detected in QQ41, this mutation has previously been identified by (Colson *et al.*, 2007; Yan *et al.*, 2017). G145R mutation is a classical mutation because it was the first vaccine-escape mutation identified from Italian vaccinated children (Zanetti *et al.*, 1988). G145R mutation is a major vaccine escape mutation in the β-cell epitope of the "α" epitope of HBsAg, because it changes amino acid sequence of the "α" epitope structure hence it interferes with the antibody binding causing the HBsAg antigenicity to be reduced (Kao and Chen, 2002; Su *et al.*, 2008). Its occurrence has increased in recent years with an increase of HBV endemicity and use of universal immunization (Caligiuri *et al.*, 2016). This mutation is

crucial in causing failure on the detection of HBV by serological routine assays (Cooreman *et al.*, 2001). G145R has become more dominant with vaccine selection, and it is recommended to be incorporated into future vaccine design (Yan *et al.*, 2017).

Other vaccine-escape mutation identified from this study where E164D, L209V and Y206H. E164D was detected in 2 individuals; it was reported outside the "a" determinants epitopes. The E164D causes substitution change in the overlapping polymerase region creating the V173L mutation which is associated with lamivudine resistance therapy and causes the reduction of anti-HBs (Torresi, 2002). Y206C mutation was identified in an individual and causes the substitution in the overlapping polymerase. P70H mutation was found in 8 individuals and this mutation has previously been identified in vaccinated infants due to vertical transmission in China (Su *et al.*, 2013). In South Africa, this mutation was reported in overt HBV infections (Amponsah-Dacosta *et al.*, 2015). Mutations L209V, L216V and L209V were also identified in this study; however, there is limited literature on their role in surface protein and HBV infection still needs to be explored.

Polymerase gene product is needed for encapsidation of the pre-genomic viral RNA particle and conversion of the pre-genomic viral RNA molecule into viral DNA. The polymerase ORF codes for the reverse transcriptase domain of the HBV polymerase (Singla *et al.*, 2013). The HBV polymerase is vital to the replication cycle, however, the polymerase (RT) lacks the proofreading mechanism causing high nucleotides substitution error rate of approximately close to $1X10^{7}$ per site annually (Miller *et al.*, 1989). The HBV polymerase is the target of antiviral agents which are used to suppress the DNA replication of the HBV by blocking the reverse-transcription process. Antiviral agents used against HBV polymerase include the nucleoside/nucleotide analogues such as LMV, adefovir (ADV), TDF, telbivudine (LdT), and entecavir (ETF). In South Africa, lamivudine treatment is still the preferred treatment among the HBV/HIV co-infected patients, regardless of the introduction of TNF.

In addition, to the development of high mutation rate by the HBV error prone, antiviral drugs may also cause the development of drug resistance as a result of selection pressure during long-term use of the antiviral treatment (Fares and Holmes, 2002). The mutations associated with drug resistance within the RT region are classified into primary and compensatory mutations. Primary mutations directly reduced the susceptibility of the antiviral drugs whilst the compensatory mutations are reported to increase HBV replication (Lok *et al.,* 2007).

The RT region of the overlapping surface and polymerase gene was amplified and analysed for mutations. Mutation M129L was the common mutation identified in 84% of isolate sequences and the effect of this mutation on drug resistance has not been explored.

Mutation M204V was identified from QQ46 (3%), this mutation has been found in the (YMDD) tyrosine-methionine-aspartate-aspartate region in the polymerase. This mutation has been reported to cause resistance to LMV and LdT (Singla *et al.*, 2013), additionally it leads to production and intracellular retention of truncated HBsAg forms. M204V has been associated with cirrhosis and HCC (Caligiuri *et al.,* 2016); previous studies reported this mutation in therapy naïve South Africans who are co-infected with HIV (Lukhwareni *et al.*, 2009). QQ46 had mutation L180M in addition to M204V, L180M is associated with resistance LMV and LdT, a combination of these mutations in a single patient have been reported previously (Zoulim and Seeger, 1994). These mutations have been reported in treatment-naïve patients (Jin *et al.*, 2012). A combination of L180M and M204V gives cross-resistance to other nucleosides and reduces sensitivity to EFV but not ADV and may cause vaccine escape mutations in the overlapping S-region and prevent the secretion of HBsAg (Sheldon *et al.,* 2005). QQ28 had V173L mutation causing resistance to LMV. The occurrence of V173L could be caused by the presence of the E164D vaccine-escape mutation found within the “α”epitope as indicated earlier that the E164D mutation causes substitution

change in the overlapping polymerase region creating the V173L mutation which is associated with lamivudine resistance therapy.

Compensatory mutations S202K and I223V were identified within the RT region; these mutations were reported to be associated with ADV resistance, with I233V reported to be causing ADV failure (Tan *et al.*, 2008). Other compensatory mutations identified included S202K, Q125E, L217R, V124A, V204I, I253Y, T128A and S105T moreover. The effects on these mutations on the RT functions still need to be explored. However, it has been speculated that compensatory mutations within the RT region increase HBV replication (Lok *et al.*, 2007).

RT region of the polymerase overlaps with HBsAg ORF and HBsAg mutations arising on the RT domain may cause vaccine-escape mutations. Analysis of the RT region was able to identify mutations associated with resistance to LMV, LdT and ADV. Detection of LMV resistance mutation from HBV individuals co-infected with HIV was not unique, since LMV is used as a backbone for both HIV and HBV therapy in South Africa. However, the individuals were infected with a LMV-resistant strain. This suggests a need for HBV drug resistance screening among HBV/HIV co-infected individuals before and during therapy.

Conclusion

In conclusion, the subgenotype A1 showed to be prevailing in HBV/HIV co-infected individuals from Durban cohort in KZN. HBV had mutations within the BCP/PC that may affect HBeAg-expression at the transcriptional level and account for the HBeAg being non-reactive (Ahn *et al.*, 2003). In this study, we identified mutations in the HBsAg region which are known to allow escape from neutralizing antibodies. These mutations in the HBsAg region may cause less reactivity with serological assays leading to failure in the detection of HBV by commercial ELISA assays. We also detected mutations within the RT region which

are associated with antiviral drug resistance; hence mutations within this region may lead to treatment failure. Based on the identified RT mutations from this study, it is suggested that the RT sequence analysis is done before initiation and during therapy in order to identify drug resistance mutations, so that appropriate antiviral drugs can be prescribed and to identify treatment failure so that patients can be switched to the effective treatment

Our study showed that understanding HBV the genotype and mutations in HIV-infected individuals is significant, since the information could be useful to aid in the diagnosis of HBV/HIV co-infection, management of antiviral therapy in patients with HBV/HIV co-infections.

In conclusion, this study has added to the limited South African data on HBV genotypes and mutations associated with pathogenesis and drug resistance in HBV/HIV co-infected individuals.

CHAPTER 5

5. RESULTS

5.1 HBV/HIV co-infection from Mahikeng antenatal cohort

5.1 HIV ELISA Assay

HIV-1 p24 antigen expression was positive in all 23 patients resulting in 100% seroprevalence (figure 5.1). All samples were positive for p24 antigen expression shown by the p24 concentrations in (O.D) which were greater than the negative control cut of value (0.148) (Table 5.1). HIV-2 antigens were not detected indicating that all participants were infected with HIV-1.

Figure 5.1: Summary of the results of HBV/HIV co-infection from the Mahikeng public clinic isolates.

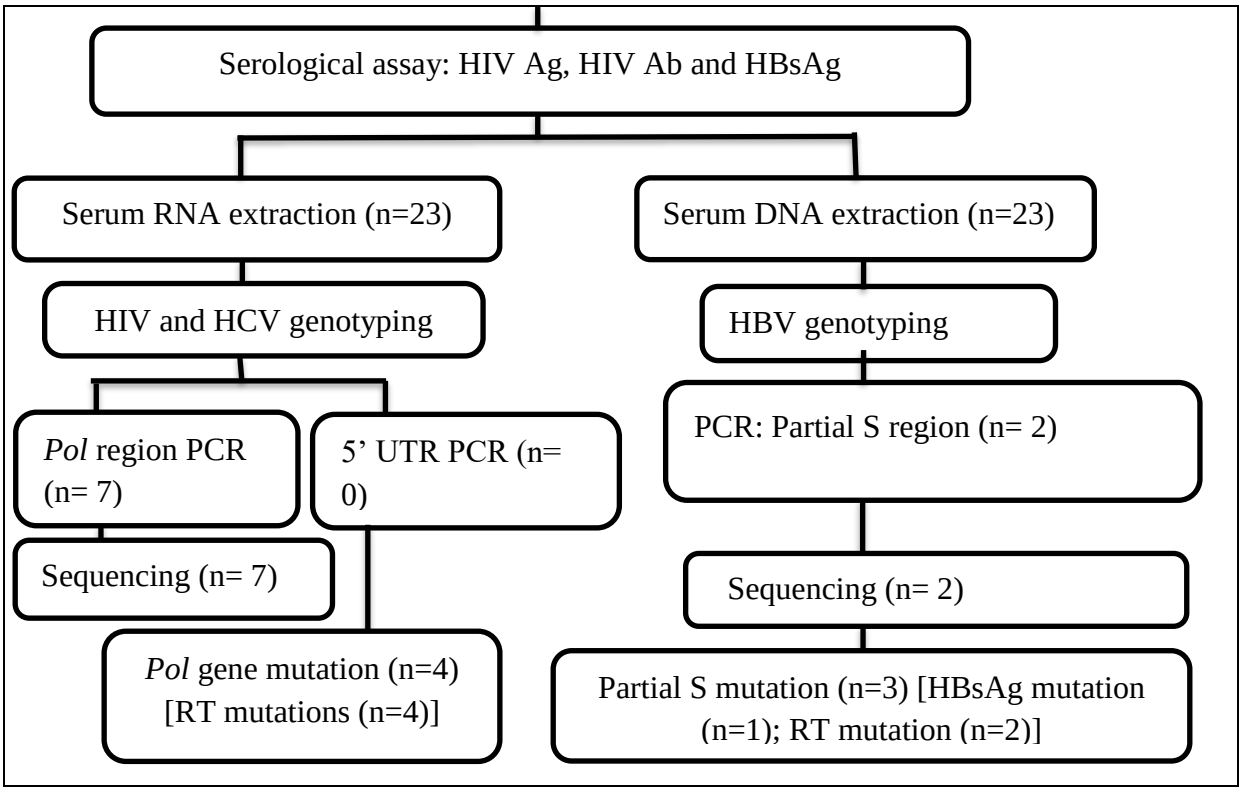


Table 5.1: HIV-1 P24 antigens expression rate

Sample code	HIV-1 p24 antigen	Mean OD	Interpretation
STD1	+	3.467	+
STD2	+	3.500	+
STD3	+	3.478	+
STD4	+	3.967	+
RBS2	+	3.366	+
RBS1	+	3.608	+
RBS3	+	0.615	+
RBS5	+	2.073	+
RBS6	+	3.472	+
LP1	+	3.500	+
LPS2	+	3.266	+
LP3	+	3.113	+
LPP4	+	3.300	+
CIS11	+	3.008	+
CIS12	+	3.354	+
CIS13	+	3.239	+
CIS15	+	3.098	+
CIS16	+	3.355	+
CIS17	+	3.344	+
CIS18	+	3.478	+
CIS19	+	3.429	+
CIS20	+	2.988	+
CIS21	+	3.012	+
Positive control	+	1.298	+
Negative control	-	0.072	-

OD: Optical density; Cut off value= Mean value of negative control O.D X 2.1= 0.148; positive samples O.D ≥ 1.298; negative samples O.D.≤ 0.148.

5.2 Amplified PCR product of HIV Polymerase Gene

The *pol* gene of HIV was successfully amplified by nested PCR for 7 (30%) of the 23 individual samples (figure 5.1), after amplifying the HIV DNA, PCR products of 1, 84 Kb for the 7 samples were visualised (Figure 5.2). PCR amplification could not be obtained for the other 16 (70%) samples.

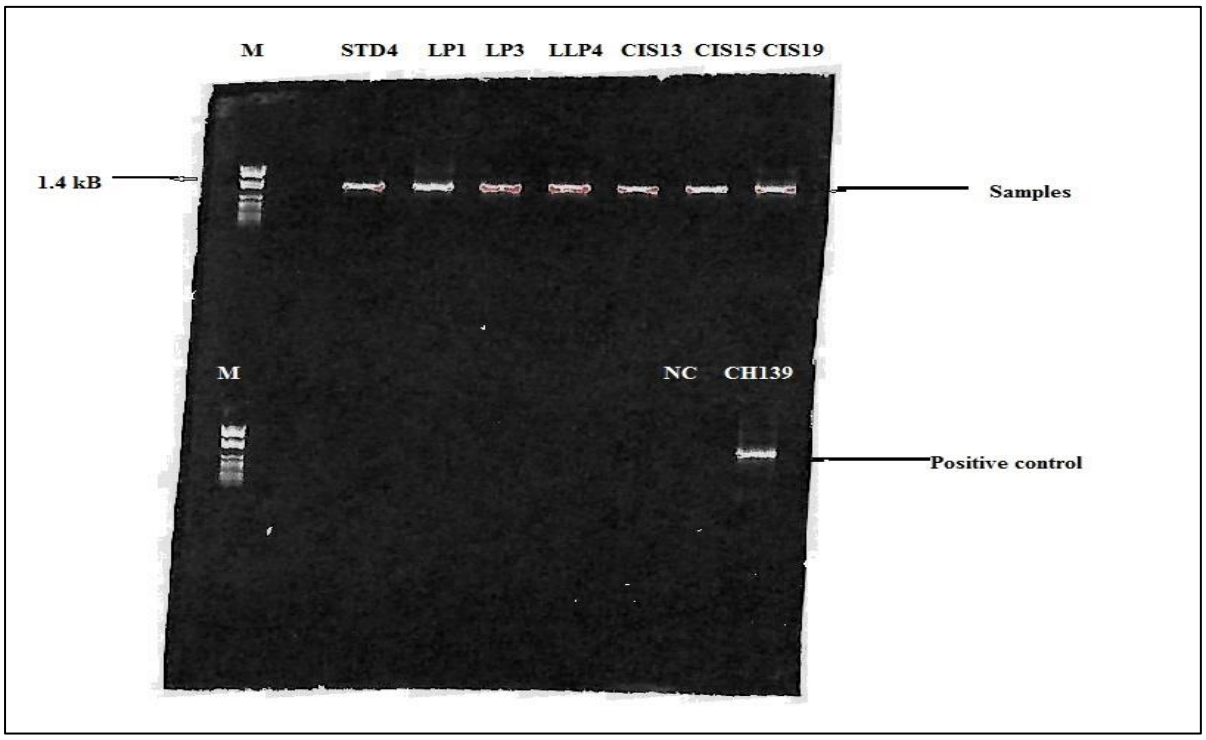


Figure 5.2: Amplified *Pol* region of HIV-1 shown as 1, 84 Kb amplicons on a 1% ethidium bromide stained agarose gel obtained from antennal women at Mahikeng public clinics.

Top wells lane, from top left to right: Top lane M = 1000 plus bp molecular weight marker. Lane 1 to lane 7 represents samples (STD4, LP1, LP3, LPP4, CIS13, CIS15 and CIS19) Bottom lane 6 is a negative control (nuclease-free water) and bottom lane 7 is a reference positive sample (CH139).

5.3 DNA Sequencing Analyses

5.3.1 HIV-1 Genetic Subtyping

The sequences were deposited into the NCBI and GenBank to determine their identity and the accession numbers of samples are shown in (Table 5.2). Samples sequences were identified belonging to the HIV-1 subtype C (Table 5.2).

Table 5.2: Identity of sample subtypes and designated accession numbers

Sample ID	Phylogenetic tree codes	Accession number	GenBank sequence no	Nucleotide similarity (%)
CIS13	KKCIS1388	KY827008	KT737133.1	98
CIS15	KKCIS1588	KY827009	AF411966.1	97
CIS19	KKCIS1988	KY827010	JF960530.1	99
LP3	KKLP388	KY827011	FJ199573.1	98
LP1	KKLPP188	KY827012	AF254776.1	99
LPP4	KKLPP488	KY827013	KT735618.1	99
STD4	KKSTD488	KY827014	FJ956787.1	97

5.3.2 HIV-1 Genetic Subtyping by Phylogenetic Analysis

All sample sequences were identified as HIV-1 subtype C (Figure 5.3). Sample STD4 (KKSTD488), LPP4 (KKLPP488) and LP3 (KKLPP388) clustered with subtype C sequences from South Africa. Sample LP1 (KKLPP188) clustered with a subtype C from Malawi; CIS13 (KKCIS1388) with subtype C from Botswana and sample CIS19 (KKCIS1988) with subtype C from Zambia.

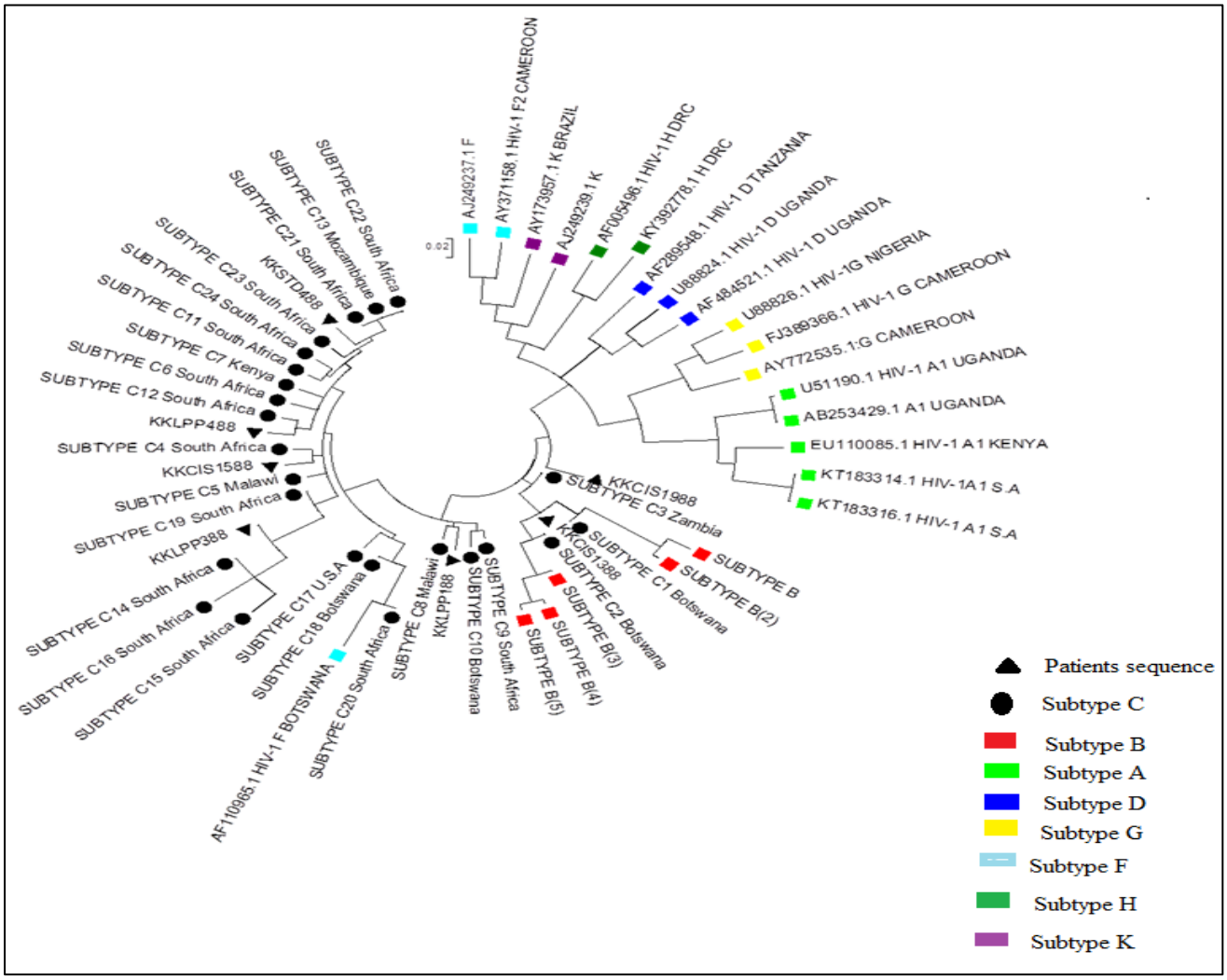


Figure 5.3: Maximum likelihood phylogenetic tree with 1000 bootstrap test replicates of HIV sequences of antenatal cohort from Mahikeng. The 7 HIV individuals sequence from HIV-infected antenatal women in this study represented with a black triangle (▲). Reference nucleotide sequences from GenBank are designated by its genotype/subgenotype and country.

5.4 Mutations on HIV Polymerase

According to the Stanford HIV resistance, sequence STD4 had 4 amino acid variants on the RT region of the polymerase; these nucleotide variants included 2 NRTIs (M184I and K65R) and 2 NNRTIs (K103N and M230L) giving a total of 4 (14%) drug resistance mutations on genotyping (Table 5.3).

Table 5.3: Drug resistance mutations identified on the reverse transcriptase nucleotide sequence of HIV- infected pregnant women

Sample ID	RT Subtype	NRTIs	Resistance profile	NNRTIS	Resistance profile
STD4	C	M184I	3TC	K103N	NVP
			FTC		EFV
			ABC		
			ddI	M230L	EFV
		K65R	TDF		NVP
			ABC		
			d4t		
			ddI		
LPP4	C	None	None	None	None
LP3	C	None	None	None	None
CIS15	C	None	None	None	None
LP1	C	None	None	None	None
CIS19	C	None	None	None	None
CIS13	C	None	None	None	None

NRTIs, nucleoside reverse transcriptase inhibitors, NNRTIs, non-nucleoside reverse transcriptase inhibitors, lamivudine (3TC), emtricitabine (FTC), Abacavir (ABC), didanosine (ddI), TDF, Stavudine (d4T), Nevaripine (NVP), Efavirenz (EFV).

5.5 HBsAg Assay

The presence of HBsAg amongst the 23 samples was determined (Figure 5.1), 4 out of the 23 samples were positive for HBsAg and sample STD4 had the highest HBsAg concentration (Figure 5.4).

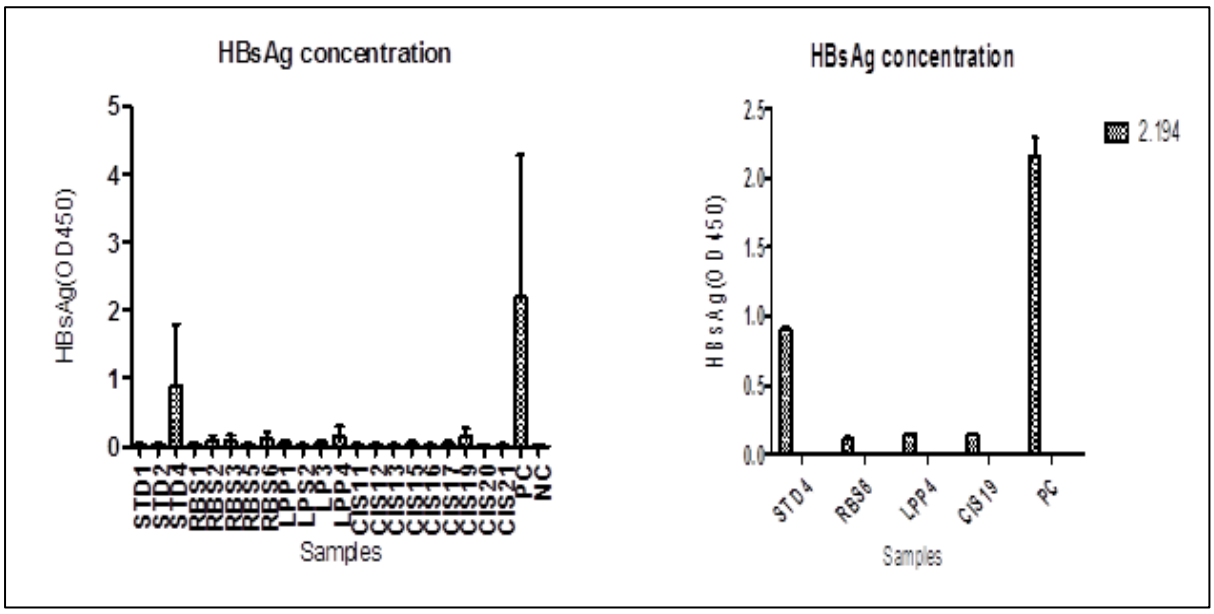


Figure 5.4: HBsAg expression levels prevalence. The HBsAg concentration was measured as O.D. values at 450 nm. HBsAg concentrations ranged from 0.01-2.194 O.D. The HBsAg prevalence was 17% (95% Cl (0.89-2.194).

5.6 Qualitative and Quantitative Viral DNA

5.6.1. Qualitative Detection of HBV

The partial surface gene region of the 4 HBsAg positive samples was successfully amplified by nested PCR. After PCR amplification of the HBV DNA, a PCR product of ~ 700 bp was visualised (Figure 5.5).

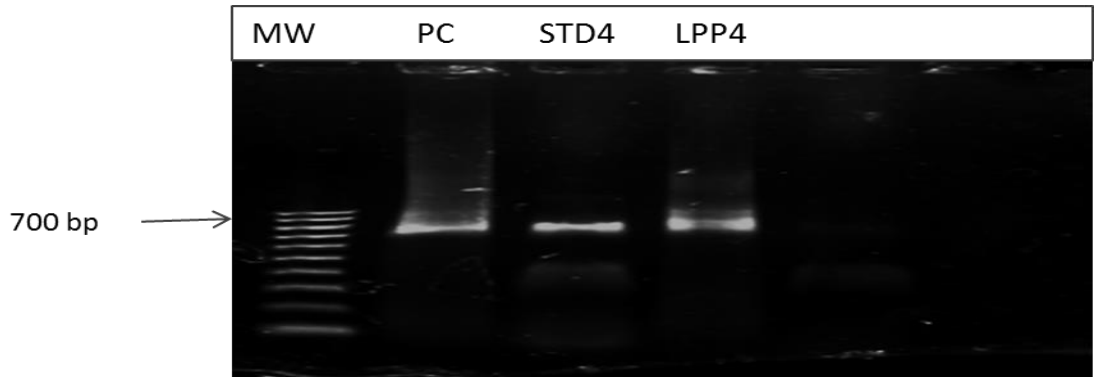


Figure 5.5: Amplified HBV overlapping HBsAg and *pol* gene obtained from HBV DNA on a 1% ethidium bromide agarose-stained gel. Black arrows on the left indicate the size of MW and targeted band size ~ 700 bp. MW: molecular weight, PC: positive control, bp: base pair. Lane1 = MW is a 100 bp molecular weight marker, Lane 2 = Positive control Lane 3 = sample STD4 Lane 4 = sample LPP4

5.6.2. Quatitative Detection of HBV

Real-time PCR amplification of the overlapping of the HBsAg and *pol* gene was successful for 2 out of 23 samples (STD4 and LPP4) (Figure 5.1). Real-time PCR amplification of HBV successfully determined the copy number for STD4 and LPP4 from a standard curve (Figure 5.6).

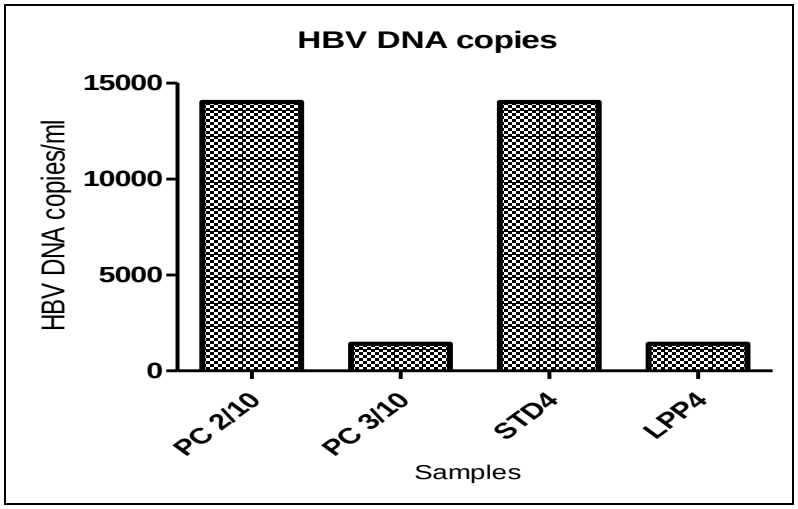


Figure 5.6: DNA copies of the diluted HBV positive control PC 2/10 with 10^{2} dilutions (14,000 copies/ ml), PC 3/10 with 10^{-3} dilution (1,400 copies/ ml) as compared to samples STD4 (14,000 copies/ ml) and LPP4 (1,400 copies/ ml).

5.6.3. Confirmation of qPCR Amplification Product

Samples STD4 and LPP4 overlapping polymerase and HBsAg region was successfully amplified by real-time PCR. After qPCR amplification of the HBV DNA, a PCR product of ~ 700 bp was visualised (Figure 5.7).

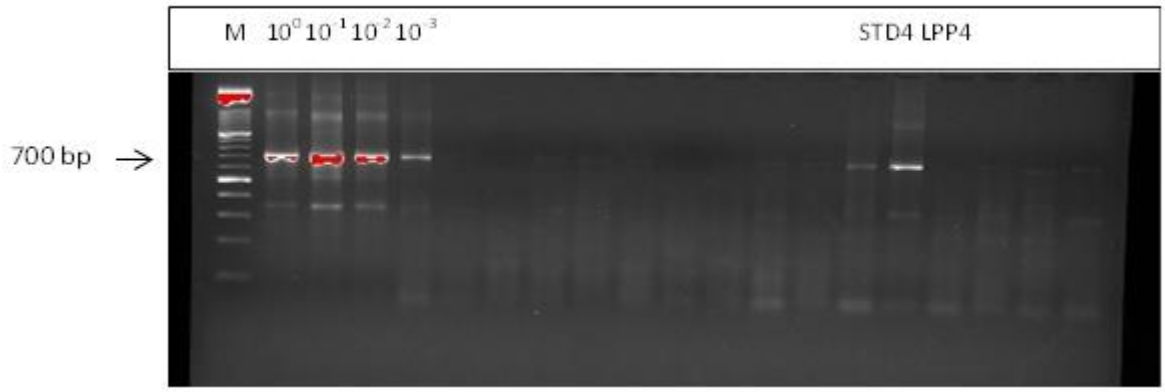


Figure 5.7: Amplified partial surface gene of diluted HBV positive control and positive amplified samples.

M- 100 bp molecular weight marker,

Lane 1= Positive control stock 100 represents 10^0 dilution (1,400,000 copies/ ml), lane 2 = 10^{-1} (140,000 copies/ ml), lane 3= 10^{-2} (14,000 copies/ ml), lane 4= 10^{-3} (1,400 copies /ml),

Lane 14 = SDT4 HBV detected at Ct= 28 (correlating with HBV at 14,000 copies/ml)

Lane 15 = LPP4 HBV detected at Ct= 31(correlating with HBV at 1,400 copies/ml).

5.7. Sequence Analyses

5.7.1 Phylogenetic Analyses

Two samples were positive for HBV DNA amplification, however, only 1sample was successfully sequenced, the STD4 sequence clustered and intermingled with referenced genotype A sequences from the 66 sequences representatives of different genotypes (Figure 5.8). The database Geno2Pheno confirmed the SDT4 sequence as genotype A and subtype A1 with percentage similarity of 99%.

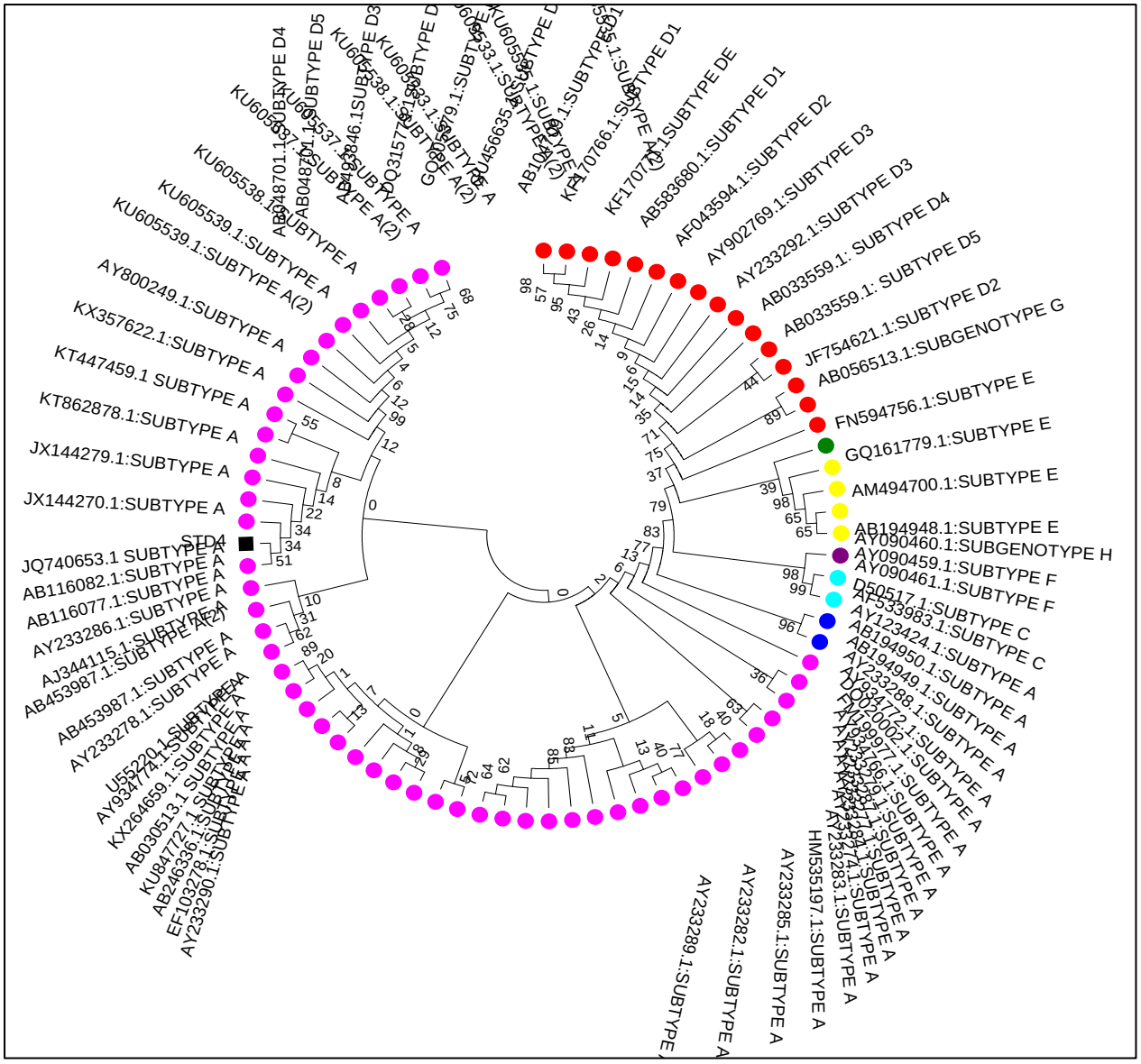


Figure 5.8: A circular phylogenetic tree comparing the identity relationship of this study sample STD4 HBsAg and *P* overlapping gene sequence with distinct genotypes sequences obtained from the GenBank (designated by accession numbers and genotype type). Pink: Genotype A sequences; Red: Genotype D, Blue: Genotype C, Yellow: Genotype E, Aqua: Genotype F, Olive: Genotype G, Maroon: Genotype H: Trees established using statistical method of neighbour-joining method with statistical analysis performed using Bootstrap statistical of 1 000 replicates of nucleotide substitution to test the phylogeny

5.7.2 Clinical Mutations on Overlapping Surface/ Polymerase HBV Gene

Essential clinical mutations associated with HBsAg antigenicity and reverse transcriptase drug resistance were determined using Geno2Pheno. Geno2Pheno indicated that the STD 4 sequence contained S207N, M129L, I253Y and V163I mutations within the overlapping HBsAg and polymerase region (Table 5.4.).

Table 5.4: Mutations in the HBV overlapping *S* and *P* regions of sample STD4

	Surface gene region		Polymerase region			
Sample code	SHB	HBsAg antigenicity	RT	Drugs	Resistance	Interpretation
STD 4	S207N	None	M129L	3TC ADV ETV TDF	None	Susceptible
			V163I	3TC ADV ETV TDF	None	Susceptible

SHB: lamivudine (3TC), adefovir (ADV), entecavir (ETV), tenofovir (TDF).

Discussion

In South Africa, prenatal HIV testing is conducted during the first antenatal clinic visit, every 3 months and at 34 weeks a retest is done. Furthermore, HIV tests are done during delivery, after birth, six weeks postpartum and during breastfeeding period. However, this is not a case for detection of HBV/ HIV co-infection. Hence we conducted this study to identify HBV prevalence in HIV-infected antenatal women and characterize the genotype and mutation associated with HBV/HIV co-infections. But first we had to identify the prevalence of HIV infection in the samples provided during routine antenatal phase from local clinics.

All 23 specimens were positive at point of care HIV rapid testing was performed by health professionals at Mahikeng public clinics. The specimens were further confirmed for presence of HIV-1 specific p24 antigen and the results were concurred with point of care HIV rapid testing (Table 5.1). In all specimens, p24 antigen was detected presumably proposing that specimen's viral nucleic acids were replicating and that all 23 participants were infected with HIV-1.

The results of this study indicated a high seroprevalence of 100% of HIV p24 antigen in pregnant women from this study cohort. To rule the possibilities of low sensitivity of rapid ELISA and to indicate that our specimens were indeed true positive of HIV p24 antigen, specimens were subjected to confirmatory PCR amplification of the *pol* region. PCR amplifications are reported to be exceptional for HIV detection and diagnosis since they are more sensitive and specific unlike the rapid and ELISA testing (Boyle *et al.*, 2013).

DNA PCR amplification was repeatedly successful for 30% (7/23) of the samples (Figure 5.2). DNA amplification could not be obtained from the other 70% (16/23) samples. We suggest that, the samples that tested negative should be tested for HIV viral load and reanalysed with PCR with optimization for low viral loads.

However, unsuccessful PCR amplification of HIV-1 DNA did not propose that specimens had minimal *pol* gene expression or that proviral viral replication was not occurring and that the viral reached latent phase because HIV actively replicate throughout the course of the infection even during latent phase (Piatak *et al.*, 1993). The 7 samples that were positive for PCR amplification are indeed true HIV-positive supported by the fact that nucleic acid is the first marker to appear during HIV infection, detected up to 8 days prior to antibody production (Busch and Satten, 1997).

PCR amplification of HIV is vital in HIV screening, but has limited use in predicting species identity, prognosis in patients with established HIV infection and management of the infection. Hence the PCR amplicons were subjected to sequence analysis which is considered the "golden standard" to determine the genotypes and drug resistance variants which will aid in better diagnosis and treatment.

A total of 7 nucleotide sequences were generated and deposited into the NCBI and GenBank to determine their identity; sample sequences were identified belonging to the HIV-1 subtype C because they had 97-98% nucleotides similarity with reference HIV-1 subtype C sequences deposited at the NCBI (Table 5.2).

Based on phylogenetic analysis, all sample sequences were identified as HIV-1 subtype C because they clustered with other reference HIV-1 subtype C sequences (Figure 5.3). Sample STD4 (KKSTD488), LPP4 (KKLPP488) and LP3 (KKLPP388) clustered with subtype C sequences from South Africa. Sample LP1 (KKLPP188) clustered with a subtype C sequence from Malawi; sample CIS13 (KKCIS1388) with subtype C sequences from Botswana. Sample CIS19 (KKCIS1988) clustered with subtype C sequences from Zambia. These study sequences belong to HIV-1 subtype C, the prevailing subtype in South Africa associated with heterosexual transmission (Marconi *et al.*, 2008; El-Khatib *et al.*, 2010; Wallis *et al.*, 2010a;

Van Zyl *et al.*, 2013). However, the clustering based on replication bootstrap showed that sequences from Mahikeng were distantly related to other South African HIV-1 subtype C sequences (Figure 5.3). Phylogenetic analysis of sequences from this cohort was suggestive of multiple introductions of these HIV-1 subtype C strains within the HIV- infected antenatal women in this cohort and not through transmission of a single strain.

To determine the prevalence of HBV in HIV infected individuals from this cohort, a total of 23 samples infected with HIV were subjected to HBV serological and molecular assays. The HBsAg seroprevalence for this study was 17% (95% CI 0.89-2.194) with specimen STD4 having the highest HBsAg concentration among the HBsAg positive specimens in (Figure 5.4). The HBV/HIV co-infection based on serological assay was 42% (3/7) as compared to previous studies on HIV-infected pregnant women in South Africa. The rate is high as compared to 2.1% - 9.7% from previous studies (Andersson and Van Rensburg, 2011; Matthews *et al.*, 2015; Diale *et al.*, 2016).

The high seroprevalence of HBV/HIV co-infection as compared to previous reports in South Africa may be attributed to small sample size and could have influenced the bias on the results. We subjected samples to subsequent HBV genotyping to confirm HBV/HIV co-infection, because HBsAg ELISA assays alone may not be sensitive enough. HBV genotyping including: HBV PCR amplification, qPCR, sequencing, and phylogenetic analyses are sensitive and accurate in diagnosis compared to serological ELISA tests.

STD4 and LPP4 were confirmed to be HBV positive by PCR amplification of ~ 700 bp overlapping HBsAg and *pol* gene indicating HBV/HIV co-infection at 9% (Figure 5.5). Two samples (RBS6 and CIS19) expressing HBsAg were negative for PCR amplification of HBV. PCR amplification was done on all HBsAg negative specimens and they were negative for

HBV DNA. The 2 PCR amplicons (STD4 and LPP4) were further subjected to qPCR amplification to determine the active replicating HBV by detection of their copy numbers.

The specimens were scored positive for active replicating HBV when the HBV DNA levels were equal to or greater than 200 copies/ ml. Correlation between the STD4 Ct=28 and the HBV positive control 10^{-2} dilution with 14,000 copies/ ml was observed, indicating that STD4 at Ct=28 had ~14, 000 DNA copies/ ml. Correlation between the LPP4 (Ct=31) and the HBV positive control 10^{-3} dilution with 1,400 copies/ ml was observed, indicating that LPP4 at Ct=31 had 1,400 copies/ ml DNA (Figure 5.4).The DNA copies/ml for both STD4 and LPP4 were above the recommended level cut-off value (200 copies/ ml). The other 21 samples did not amplify and HBV DNA was not detected from them.

From all the 23 specimens, 2 samples (STD4 and LPP4) were positive for active replicating HBV DNA quantification 7% (2/23) and 21 samples were negative for HBV DNA quantification 91% (21/23). We also confirmed if the DNA copies detected during SYBR green qPCR had our gene of interest (overlapping HBsAg and *Pol*). The qPCR products were run on a 1% agarose gel to indeed confirm the presence of HBV DNA and 2 samples were positive for ~ 700 bp overlapping HBsAg and *pol* gene by qPCR (Figure 5.7).

Out of the 23 specimens from the Mahikeng antenatal cohort only 2 samples (STD4 and LPP4) were co-infected with HBV and HIV since they presented active replicating HBV DNA and HIV RNA. RBS6 and CIS19 which were positive for ELISA HBsAg, yet negative for PCR amplification of HBV DNA, suggesting that either they were false positive since ELISA assays are not sensitive and accurate enough. The results also may suggest that the patient might have cleared acute HBV infection and was not infected with chronic HBV. Since HBV DNA can be detected approximately 21 days before HBsAg which typically appears in the serum. Patients with chronic HBV infection fail to clear the virus and remain

HBsAg-positive with HBV DNA detection. Furthermore, the presence of HBV DNA greater than 200 copies/ ml in the specimens indicated that the patients are infected by active replicating HBV.

We further characterized HBV, HIV genotypes and mutations in samples STD4 and LPP4 coinfected with HBV/HIV.

According to the Standford HIV resistance, STD4 sequence had 4 amino acid variants on the RT region of the polymerase gene which included; M184I, K65R, K103N and M230L variants. These variants confer resistance to drug in two classes NRTIs (M184I and K65R) and NNRTIs (K103N and M230L) giving a total of 14% drug resistance mutations (DRM) on genotyping (Table 5.3).

Amino acid variants associated with drug resistance identified in sample STD4 were similar to those reported previously by other studies from South African regions; involving individuals infected with HIV-1 subtype C except for M230L (Baptista *et al.*, 1999; Manasa *et al.*, 2013). Amino acid substitutions on STD4 included; K103N mutation which has selected resistance in patients receiving nevirapine and efavirenz (Bacheler *et al.*, 2000).

K103N mutation in this study has been reported as one of the clinically significant mutation in the RT region. Similarly this mutation has been associated with HIV-1 subtype C in South Africa, however, K103N has been identified in HIV-1 subtype A1, B and D in other regions of the world such as Tanzania, Israel, Jamaica and Brazil (Grossman *et al.*, 2004; Amarakoon *et al.*, 2014). Suggesting that, K103N is not genotype specific.

The K103N mutation is frequent in causing NNRTI failure and it causes cross-resistance in the older NNRTIs (Lecossier *et al.*, 2005). This mutation has been studied extensively, and directed the discovery of etravirine (ETR). The K103N was notable especially in single dose

nevirapine treatments, where mothers or infants receive only one dose of nevirapine and were not necessarily followed by HAART (Jackson *et al.*, 2000; Flys *et al.*, 2006). However single dose nevaripine is no longer available in South Africa. The rare M230L was also detected, even though are limited studies done on this variant but it is reported to be selectively resistance to EFV and NVP (Tang and Shafer, 2012; Kuhn *et al.*, 2014). The sample STD4 harboured M184I variant that originate from the M184V wild type with ATG sequence. Because the ATG of the wildtype prostage to form GTG on M184I easily as compared to ATA of M184IV, hence M184I emerge before M184V (Keulen *et al.*, 1999; Frost *et al.*, 2000). M184V has selected resistance to lamivudine and emtricitabine (Harrigan *et al.*, 2000; Staszewski *et al.*, 2001; Das *et al.*, 2012). Contrarily, M184V has been reported to have high susceptibility to zidovudine, stavudine and tenofovir (Maguire *et al.*, 2000; Mouroux *et al.*, 2001; Averbuch *et al.*, 2006).

Another NRTI resistance variant detected in STD4 was K65R, reported to commonly emerge frequently in HIV-1 subtype C (Brenner *et al.*, 2006; Invernizzi *et al.*, 2009). This mutation confers less resistance for lamivudine and high-level resistance for tenofovir among patients administering a tenofovir-containing regimen (García-Lerma *et al.*, 2008; Coetzer *et al.*, 2013). It has been isolated in patients receiving ART regimen lacking zidovudine (Svarovskaia *et al.*, 2008).

The presence of dual M184I and K65R in a single sample STD4 detected in this study were similar to those reported previously by another study in pregnant women infected with HIV-1 subtype C (Bennett *et al.*, 2008). These two NRTI drug resistance mutations have been reported previously in South African patients failing the first-line ART drugs from Durban, Cape Town and Johannesburg (Orrell *et al.*, 2009; Wallis *et al.*, 2010b; El-Khatib *et al.*, 2011; Van Zyl *et al.*, 2013). Phylogenetic sequence analysis indicated that STD4 is closely related to KC424181 (GenBank accession number) which also had K65R and M184V

mutations associated with resistance to NRTI in patient failing first-line ART regimen (Van Zyl *et al.,* 2013). Suggesting that patient STD4 was infected with a strain that is resistant to first-line ART regimen.

The mutation substitutions at the following regions M184I, K103N and K65R are considered drug mutations for reverse transcriptase usually used during first-line regimen suggesting that patient STD4 was not susceptible to reverse transcriptase inhibitors on first line regimen drugs. Supported by findings by (Marconi *et al.*, 2008; Manasa *et al.*, 2013), who similarly identified M184I, K103N and K65R as the most common acquired HIV-1 mutations for resistance to drugs classified in the first line regimen.

The presence of mutation associated with drug resistance to first line regimen harboured by patient STD4 sequences suggest that the patients was either previously exposed to first line regimen or were infected with a strain which is resistant to ART first line regimen.

However, there have not been reports on the presence of these 4 combined variants associated with drug resistance (M184I, K65R, K103N and M230L) in a single patient among the HIV-infected pregnant women in South Africa. Combination of these 4 variants may limit the efficiency ART.

HBV genotyping of STD4 and LPP4 amplicons generated STD4 consensus sequence (350 bp) and the LPP4 consensus sequence (192 bp). STD4 and LPP4 consensus sequences were edited and assessed for phylogenetic and mutations analyses. The 2 consensus sequences were too short; however, the STD4 sequence was sufficient enough to determine genotype identity on a phylogenetic tree analysis.

The phylogenetic analysis included 66 sequence representatives of different genotypes and subgenotypes retrieved from the GenBank as well as a partial overlapping HBsAg and *pol*

sequence from this study sample (STD4). The STD4 clustered and intermingled with referenced genotype A sequences from the GenBank, hence it was classified as genotype A (Figure 5.8). Geno2Pheno confirmed STD4 co-infected with HIV as genotype A, subgenotype A; this was consistent with previous reports from South Africa (Kimbi *et al.*, 2004; Lukhwareni *et al.*, 2009; Makondo, 2012; Mayaphi *et al.*, 2013; Kramvis, 2014).

However, HBV Genotype C and D have been reported in HBV/HIV co-infected South Africans. HBV genotype D is second in prevalence after genotype A and HBV genotype C is rare in South Africa (Kimbi *et al.*, 2004; Firnhaber *et al.*, 2009; Amponsah-Dacosta *et al.*, 2015). Mutations associated with HBsAg antigenicity and drug resistance were determined. Geno2Pheno indicated that the STD4 sequence contained S207N, M129L and V163I mutations. The S207N mutation was identified on the HBsAg domain; M129L and V163I were detected in the reverse transcriptase (RT) domain of the polymerase encoding protein sequences.

Previous studies have also reported the presence of S207N and M129L and V163I variants on the overlapping HBsAg and *pol* gene regions within HBV/HIV co-infected sample (Tuteja *et al.*, 2014; Amponsah-Dacosta *et al.*, 2015). These findings were in agreement with our results. Amino acid substitution S207N mutation has previously been reported in HIV-infected patients (Taffon *et al.*, 2014). This mutation has similarly being identified in HBV subgenotype A2, D1 and D3 (Abdelnabi *et al.*, 2014; Taffon *et al.*, 2014). Indicating that, S207N is not a unique variant to subgenotype A1.

The S207N mutation is found in the MHR in the ‘‘a’’ determinant epitope of the S region, mutations found in this region have been reported to affect HBsAg expression. However, there is limited information on the effect of this variants on the HBsAg antigenicity and its impact on the surface protein has not been explored. Unlike the previously identified

mutations associated with HBsAg antigenicity (V168A, S45P, P217, E164D, V184A, F23L and D14N) (Makondo *et al.*, 2012; Pollicino *et al.*, 2013). The M129L and V163I variants detected from this study RT domain are in accordance with previous reports (Ismail *et al.*, 2012). However, these amino acid variants have been reported present in genotype B, C and D (Singla *et al.*, 2013). The M129L and V163I mutations on the RT domain were not associated with drug resistance to the RT inhibiting drugs.

Despite the presence of the M129L and V163I variants within the RT domain, phenotypic drug resistance indicated M129L and V163I were susceptible to NRTIs such as lamivudine adefovir, entecavir, tenofovir and telbivudine which are included in both HBV treatment regimens. Similar results were reported, where M129L and V163I did not confer drug resistance in a study by (Silva-Pinto *et al.*, 2015) . The RT mutations such as rtM204V, rtL180M, rt, F135Y, rtS78T, rtS202I, rtM250V, rtQ215S, rtI233N and rtW236I were reported to be associated with proliferation of liver disease treatment failure and drug resistance to 3TC, ADV, ETV, TDF drugs. These variants were not detected in our study as compared to previous studies (Cento *et al.*, 2013).

Secondly, the STD4 susceptibility to 3TC based regimen suggests that the patient never had exposure to anti-HBV drugs or was briefly exposed to 3TC regimen; because the previous study reported long exposure to 3TC therapy has been associated with the emergence of drug resistance RT mutation (Fischer *et al.*, 2001; Lok *et al.*, 2002). S207N, M129L, and V163l effects towards HBsAg antigenicity and drug resistance has not been elucidated and explored in greater extend; we assume that they might affect the encoded proteins (surface and polymerase) functions. There is limited information on the effect of the identified mutations in HBV/HIV coinfected individuals; however, we suggest that the effect may impact the pathogenicity of these viruses as well as diagnosis and treatment.

CHAPTER 6

6. RESULTS

6.1 HBV/HCV and HCV/HIV co-infection in individuals from KwaZulu-Natal

6.1.1 Detection of HCV 5´URT Region Amplicons

The HCV 5´URT was not successfully amplified by nested PCR for all the samples from Mahikeng and KwaZulu-Natal cohorts (Figure 6.1).

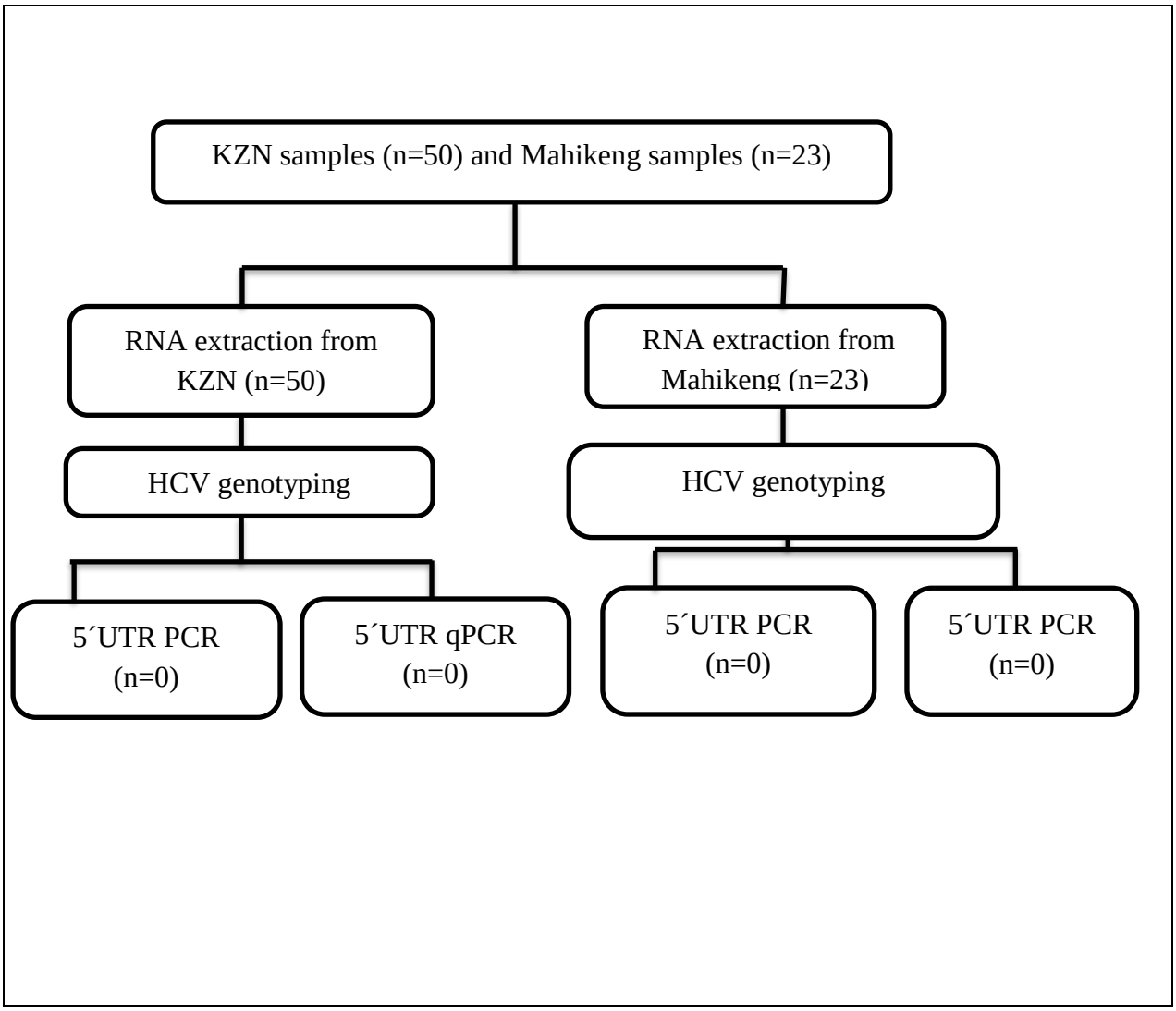
6.1.2 HCV Copies Numbers

The HCV viral loads were not successfully detected by real-time PCR amplification for all the samples from Mahikeng and KwaZulu-Natal cohorts (Figure 6.1).

6.1.3 HCV Sequence Analysis

HCV nucleotide and amino acid sequences could not be detected because of failed HCV PCR and real-time PCR amplifications (Figure 6.1).

Figure 6.1: Summary of the results on the analysis of HCV 5´UTR region from the Mahikeng and KwaZulu-Natal individuals.



Discussion

HBV, HCV and HIV, are morphological and physiologically different viruses that can be transmitted concurrently, even though at different efficiencies (Burnett *et al.*, 2005). HCV is a common infection in HBV and HIV-positive patients because of shared routes of transmission and common risk factors. Risk factors include blood and blood products and sharing of needles for injecting drugs; however, it is less clear whether HCV, like HIV is sexually transmitted which is common in HBV/HIV co-infected individuals.

The effects of HBV and HCV on HIV infection and their clinical implications have not been adequately described. However, HIV has been associated with an increased progression of hepatic disease and its effect on HCV is known to affect the epidemiology, transmission, pathogenesis and natural history of HCV infection (Blackard *et al.*, 2006). Treatments of HBV/ HIV and HCV/HIV co-infections are more complex in co-infected patients because of liver toxicity and drug interactions (Bonacini and Puoti, 2000; Gonzalez and Talal, 2003; Mehta *et al.*, 2003). The geographical variation in HCV prevalence is well known and reflects the relative contribution of the risk factors to the epidemiology of HCV in different parts of the world.

In this study HCV-HIV infection was absent being when compared to 1– 11% found in previous South Africa studies (Parboosing *et al.*, 2008; Barth *et al.*, 2010). The HCV seroprevalence in South Africa includes: HCV antibodies found in 1% of AIDS patients at a Hospital in Johannesburg; 1.2% in HIV/AIDS patients enrolling for HAART in Pretoria; in 1.8% of healthcare workers and 11% of HIV-positive patients on antiretroviral drugs who experienced hepatoxicity (Karim and Tait, 1993; Vardas *et al.*, 2002; Sanne *et al.*, 2005). In South Africa, there is a low prevalence of HCV in people infected with HBV and HCV in heterosexual cohorts; contrary, hepatitis C prevalence was reported in a high risk-defined population with 39.4% found in haemophiliacs and 4.8% in chronic dialysis patients.

HCV antibodies were not detected in HBV and HIV- infected individuals in this study. Indicating that there were no HBV/HCV and HCV/HIV co-infections, because of the sensitivity of HCV antibody testing, we doubt that we failed to identify a significant number of individuals with chronic HCV infection. The absence in detection of HCV in HBV and HIV- infected individuals suggests absence of circulating HCV within the population, low rates of injection drug use and that, sexual transmission of HCV is not a major factor (Lodenyo *et al.*, 2000). Given our finding of no HCV infection, routine HCV screening among HBV and HIV- infected individuals must be continued (Chasela *et al.*, 2012; Hoffmann *et al.*, 2012).

CHAPTER 7

CONCLUSIONS, RECOMMENDATIONS AND LIMITATIONS

7.1 CONCLUSION AND RECCOMENDATION

This study confirmed HBV/ HIV co-infection detection by PCR amplification and sequencing as effective in identifying active viral replication and co-infection of HBV/HIV as compared to standard commercial ELISA kits.

The study data showed that HIV subgenotype A1 predominates amongst the HBV/HIV co-infected prenatal women in Mahikeng. This study showed that HBV subgenotype A1 prevails in HBV/HIV co-infected individuals from both KwaZulu-Natal and Mahikeng cohorts. HBV Subgenotype A1 had mutations within the BCP/PC that may affect HBeAg-expression at the transcriptional, translational and post translational level and account for the HBeAg being non-reactive (Ahn *et al.*, 2003).

In this study, from the KZN cohort, we identified mutations in the HBsAg region which are known to allow escape from neutralizing antibodies. These mutations in the HBsAg region may cause less reactivity with serological assays leading to failure in the detection of HBV by commercial serological ELISA assays being used in South Africa. Whilst from the Mahikeng cohort HBsAg mutations detected were not associated with HBsAg antigenicity.

We identified variants in the reverse transcriptase (RT) region that were not associated with drug resistance. Even though these mutations are reported to have no resistance towards the NRTIs, these mutants are more replicative due the lack of proofreading activity of the RT and the replicative mutants can be transmitted and may have vital effects causing liver disease which might compromise the immune system can cause rapid progression of HIV among HBV-HIV coinfected individuals.

We also detected mutations within the RT region which are associated with antiviral drug resistance; hence mutations within this region may lead to treatment failure. Based on the identified RT mutations from this study, it is suggested that the RT sequence analysis is done before initiation and during therapy in order to identify drug resistance mutations, so that appropriate antiviral drugs can be prescribed and to identify treatment failure so that patients can be switched to the effective treatment.

Our study showed that understanding the HIV and HBV genotypes and mutations could be useful to aid in the diagnosis of HBV/HIV co-infection, and management of antiviral therapy in patients with HBV/HIV co-infections.

In conclusion, this study dataset has added to the limited South African data on HBV and HIV genotypes and mutations in HBV/HIV co-infected individuals associated with pathogenesis and drug resistance.

7.2 LIMITATIONS

1. There was absence of patient's history on viral load, CD4 count, and treatment data due to ethical issues around patient confidentiality, this information could have given us a good estimation of immunologic and virus status of patients.

2. Small sample size limited the overall prevalence and molecular characterization of viruses from both cohorts.

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