GENOTYPING AND MOLECULAR CHARACTERIZATION OF HEPATITIS B VIRUS (HBV) FROM HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTED INDIVIDUALS IN SOUTHERN AFRICA

Euphodia Makondo

Dissertation submitted to the Faculty of Health Sciences,

University of the Witwatersrand, Johannesburg, in fulfilment of

the requirements for the degree of

Master of Science in

Medicine

Johannesburg, 2011

DECLARATION

I, Euphodia Makondo declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

……

…….. day of …… 2011

PRESENTATIONS

GENOTYPING AND MOLECULAR CHARACTERIZATION OF HEPATITIS B VIRUS (HBV FROM HUMAN IMMUNODEFICIENCY VIRUS -INFECTED S IN SOUTH AFRICAN PATIENTS

- International Meeting on Molecular Biology of Hepatitis B Viruses October 9 -13, 2010, International Conference Hall, Humanities and Social Sciences Building, Academia Sinica Taipei, Taiwan.

- Third Cross Faculty Postgraduate Symposium: 25 - 29 October, 2010. University of the Witwatersrand, Johannesburg, South Africa.

ACKNOWLEDGEMENTS

To my Heavenly Father, I would not have made it without you.

To my parents, family and friends, thank you for your encouragement, support, love and advice.

My earnest appreciation goes to my supervisor, Prof. A. Kramvis for sharing her immense knowledge in this field with me, for providing support and assistance constantly throughout the duration of this project and for your endless patience.

Further thanks goes to all HVDRP members, thank you so much, for always being willing to provide your expertise, assistance and advice

The University of the Witwatersrand, the Belgium Technical cooperation fellowship, the National Research Foundation and the Poliomyelitis Research Foundation for the financial assistance.

ABSTRACT

Hepatitis B virus (HBV) and human immunodeficiency virus (Thakur et al.) are endemic in South Africa. There no data on the HBV genotypes prevailing in HIV-infected South Africans. The aim this study was to determine the HBV genotypes in HIV-infected patients and to identify mutations occurring in the basic core promoter/preccore (BCP/PC) and complete S regions. Twenty six HBV isolates from HBV-HIV co-infected individuals from Helen Joseph urban hospital prior to and at six month after initiation of HAART were analyzed. Three hundred HBV isolates from the rural Shongwe Hospital were recruited prior to treatment. Restriction fragment length polymorphism (RFLP) together with sequencing of the BCP/PC and complete surface region amplicons were employed for genotyping and analysis. There was no significant difference in the HBV genotypes from both cohorts. Subgenotype A1 was the predominant genotype. HBV DNA was detected in 13/26 (50 %) Helen Joseph patients and 72/300 (24%) HBV DNA was detected in patients from Shongwe cohort: 28/300 (9.3%) HBsAg-positive and 44/300 (14.7%) HBsAg-negative. The BCP/PC region of HBV isolates from both cohorts showed mutations that could account for the HBeAg negativity, although in the case of the Helen Joseph cohort the HBeAg status was unknown because of depletion of serum. The HBeAg negativity in 44/49 Shongwe patients (89,7%) could be accounted for by the following mutations: 1) the basic core promoter mutations T1753C A1762T G1764A, which down regulate transcription of precore mRNA; 2) the Kozak sequence mutants that affect HBeAg translation, 3) the G1862T mutation, which interferes with post-translational modification of the HBeAg-precursor, and 4) the classical G1896A stop codon mutation with C1858T. The G1862T mutation occurred in HBV from 24.1% HBsAg-positive Shongwe patients but in none of the HBsAg-negative patients ($p<0.05$). PreS deletion mutants were found in isolates from both cohorts. These have previously been described in hepatocellular carcinoma patients. The Helen Joseph HBV

isolates had the "*a*" determinant and immune/vaccine escape mutants. In addition to these, Shongwe isolates had HBV reactivation markers mutations V168A + S174N in 23.8% of the HBsAg-negative and in none of the HBsAg-positive infections. Drug resistant mutations were found in three Shongwe HBV isolates from ART naïve individuals and in none Helen Joseph HBV isolates. In conclusion, South African HIV-infected individuals were predominantly infected with subgenotype A1 of HBV. Mutations in the BCP and precore region of HBV isolates could account for the HBeAg negativity in the majority of patients. A number of HBV isolates displayed both immune escape and drug resistance mutations that were responsible of the HBsAg-negativity in patients from which they were isolated.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

aa	Amino acid
ALT	Alanine aminotransferase
Anti-HBe	Hepatitis B e antibodies
BCP	Basic core promoter
bp	Base pair(s)
BQW	Best quality water
cccDNA	Covalently closed circular DNA
CHB	Chronic Hepatitis B
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate

EDTA	Ethylenediamine tetra-acetic acid
EtBr	Ethidium bromide
EtOH	Ethanol
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HBx	Hepatitis B x antigen
HCC	Hepatocellular carcinoma
HIV	Human immunodeficiency virus
IFN	Interferon
LHBs	Large surface proteins
MHBs	Medium surface protein
mRNA	Messenger ribonucleic acid
NEIGHBOR	Neighbor-joining
nt	Nucleotide(s)
ORF	Open reading frame
PCR	Polymerase chain reaction
pgRNA	Pregenomic RNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RT	Reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
SHBs	Small surface protein
TBE	Tris Boric acid EDTA

TM	Annealing Temperature
UV	Ultraviolet
WHO	World Health Organization
YMDD	Tyrosine, Methionine, Aspartic acid, Aspartic acid

UNITS

µl	Microlitres Litres
µM	Micromolar
bp	Base pairs
g	Grams
kb	Kilobase(s)
L	Litre
Mg	Milligrams
ML	Millilitres
mM	Millimolar
sec	Second(s)
V	Volts

CHAPTER 1: INTRODUCTION

Hepatitis B virus (HBV), with a genome of 3 200 base pairs, is the smallest DNA virus infecting man, yet it is one of the most important human pathogens, causing major health problems globally. HBV is endemic in several parts of the world (Alter, 2003, Hou *et al*., 2005) and causes chronic and acute infections associated with severe liver diseases, including hepatitis, cirrhosis, hepatic fibrosis and hepatocellular carcinoma (HCC) (Mahoney, 1999, Quarleri *et al*., 2007) (Salisse and Sureau, 2009) Although there is a safe and effective vaccine against HBV and great progress has been made in the treatment of the infection (Francois, 2008), an estimated 380 million people in the world are chronically infected with the virus (Soriano *et al*., 2008).

Thirty four million people globally are infected with the human immunodeficiency virus (Thakur *et al*., 2002). As a result of shared blood-borne transmission routes such a s sexual and injection drug use (IDU) (Shire *et al*., 2007), HBV and HIV have been shown to cause major public health problems in the world. An estimated four million people worldwide are coinfected with HBV-HIV (Burnett *et al*., 2005). Moreover, the prevalence of the HBV-HIV co-infection is very high in Africa especially in e Sub-Saharan Africa, where both viruses are endemic (Selabe *et al*., 2007, Soriano *et al*., 2008, Thio, 2003). HIV infection leads to the acquired immunodeficiency syndrome, opportunistic infections and premature death (UNAIDS, 2009, Hazenberg *et al*., 2003). Under certain conditions, HIV can have serious and harmful effects on the clinical outcomes of hepatitis B (Nunez *et al*., 2006).

1.1 THE HEPATITIS B VIRUS

1.1.1 Classification

HBV belongs to the family of enveloped DNA viruses, the *Hepadnaviridae,* which consists of two genera: the *Orthohepadnavirus,* infecting mammals and *Avihepadnavirus,* infecting birds (Seeger and Mason, 2000). All hepadnaviruses share a similar structural and genomic organization containing a partially double-stranded DNA genome. They all replicate via an RNA intermediate, the pregenome, which encodes an error-prone polymerase enzyme with both DNA polymerase and reverse transcriptase activity (Nassal and Schaller, 1993, MacDonald et al., 2000b) and the capsid protein (HBcAg). Human HBV is the prototype member of the family *Hepadnaviridae* and has a narrow host range, infecting humans and chimpanzees only (Lai, 2002). The other members of the family include the chimpanzee hepatitis virus B (ChHBV) (Hadler *et al*.), woolly monkey hepatitis B virus (WMHBV) (MacDonald et al., 2000a), gibbon hepatitis B virus (GiHBV) , orang-utan hepatitis virus (OuHV), woodchuck hepatitis virus (WHV) and ground squirrel hepatitis B (GSHV) (Scaglioni *et al*., 1996).

1.1.2 Transmission

HBV, like HIV, is transmitted through percutaneous and mucous membrane exposure to infectious blood and other body fluids that contain blood (Alter, 2003). Modes of transmission include sexual, perinatal, and parental/percutaneous transmission (Hou *et al*., 2005). Percutaneous HBV transmission includes injection drug use, transfusions and dialysis, acupuncture, working in a health-care setting, tattooing, circumcision and scarification, and household contact. Although HBV surface antigen (HBsAg) has been detected in variety of body fluids besides blood, including saliva, sweat, semen, breast milk, urine and faeces, only

blood serum, semen and saliva have been shown to transmit the virus efficiently (Bancroft *et al*., 1977, Gray *et al*., 1989). Sexual transmission of HBV occurs in all areas of the world; however it is more common in areas of low HBV endemicity such as North America, with homosexual men being considered to be at the highest risk (Alter, 2003).

The horizontal mode of transmission is the most common route in Africa, whereas perinatal route is very common in the far east countries, including China, and Japan (Hou *et al*., 2005). In Africa, transmission occurs predominantly in early childhood, by horizontal transmission of the virus via contact with siblings and unrelated playmates as well as other family members (Kew, 1996).

1.1.3 Epidemiology

As a result of different socioeconomic conditions, the proportion of the population with risky lifestyles, the current prevalence of HBV, the availability of vaccination programs and compliance to hygienic measures and transmission routes, HBV infection occurs at markedly different prevalence rates geographically. As measured by the presence of HBsAg, the world can be divided into areas of low (<2%), intermediate (2-8%) and high (>8) endemicity (Hou *et al*., 2005).

In Africa very high endemicity of HBV is seen in developing regions with large populations and most infections in these highly endemic areas occur during infancy or early childhood. More than 8 % of the population in these areas is chronically infected with HBV and 70-95 % of the population bears serological evidence of past or present HBV infection. Sub-Saharan Africa, most of the West, East, Central and sub-Saharan Africa with chronic infection rates

of 7-26%, nonetheless areas like Kenya, Cote d'Ivoire, Liberia, Sierra Leone, Senegal and Zambia are moderately affected with intermediate endimicity rates between 2 and 8 % (Kramvis and Kew, 2007a), this shows mixed patterns of transmission, including infant, early childhood and adult transmission (Hou *et al*., 2005, André, 2000). Low endemicity is found in the northern African countries of Egypt, Tunisia, Algeria and Morocco with prevalence rates of less than 2 % (*Figure 1.1*) (Kramvis and Kew, 2007a).

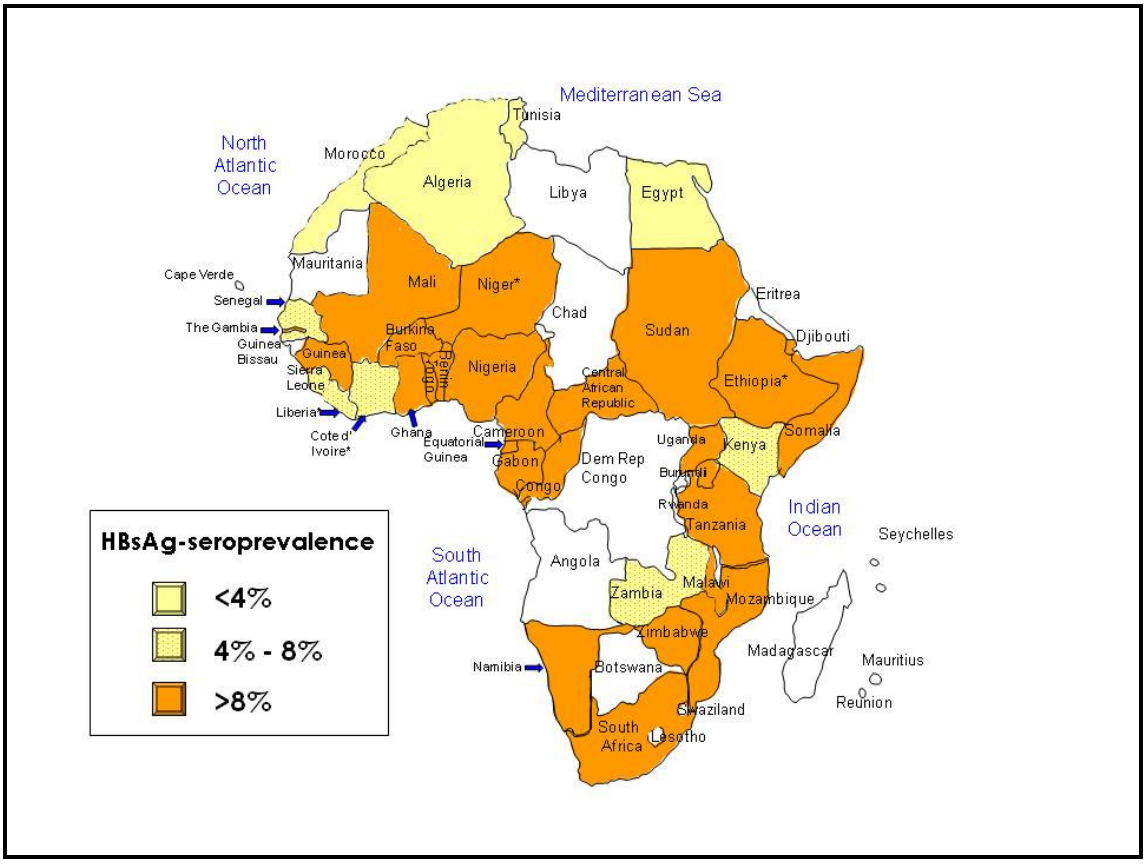


Figure 1.1: Geographic distribution of chronic HBV infection showing the high, intermediate and low prevalence areas in Africa (Kramvis and Kew, 2007a). Reproduced with permission from Prof. A. Kramvis.

Sub Saharan Africa remains the region most heavily affected by HIV. In 2008, sub-Saharan Africa accounted for 67% of the world's HIV infections, with Swaziland, South Africa, Lesotho, and Botswana being the African countries with the highest rates of HIV infection (Fix *et al*., 2007, Selabe *et al*., 2007). Of the 34 million people infected with HIV, an estimated 2-4 million are chronically infected with HBV.

The prevalence of HBV in HIV-infected individuals varies with the population studied. Several non-African studies support an increased prevalence of HBV in HIV-infected populations of sub-Saharan Africa with more than 80% of HIV-positive individuals in some of the countries carrying serum markers for HBV. African studies have found no increased prevalence of exposure to HBV in HIV-positive individuals, but have still raised questions about the impact that HIV may be having on the epidemiology of HBV. (Burnett *et al*., 2005, Selabe *et al*., 2007).

1.1.4 HBV Biology and viral structure

The Dane particle or complete virion of HBV has a diameter of 42- 47 nm and consists of a spherical outer shell, comprising of envelope proteins and a small amount of lipid, and an inner nucleocapsid enclosing the genome (*figure 1.2*) (Seeger and Mason, 2000, Locarnini *et al*., 2003). The envelope is composed of the large (LHBs), the middle (MHBs) and the small (SHBs) surface proteins (*figure 1.3 B*). The inner icosahedral nucleocapsid encloses a partially double stranded relaxed circular DNA particle, which is covalently bound to the viral polymerase (Kann, 2002). The negative strand of the HBV genome is approximately 3.2 kb in length and consists of four overlapping open reading frames and *cis*-acting elements

required for regulating HBV gene expression and replication(Su and Yee, 1992) The positive strand, which varies in length maintains the circular structure of the genome by cohesively binding to the 5' and 3' ends of the negative strand (Mahoney, 1999).

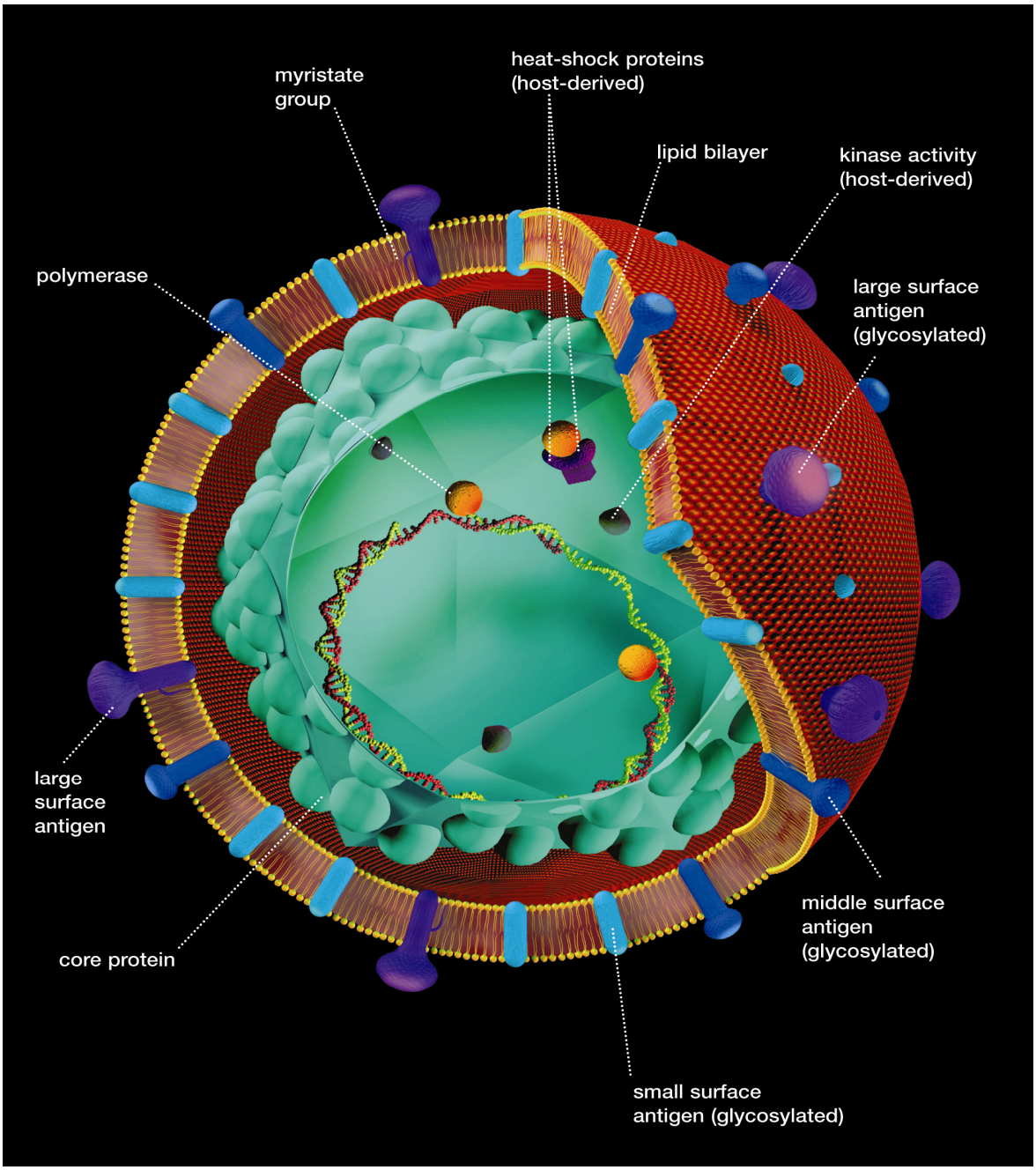
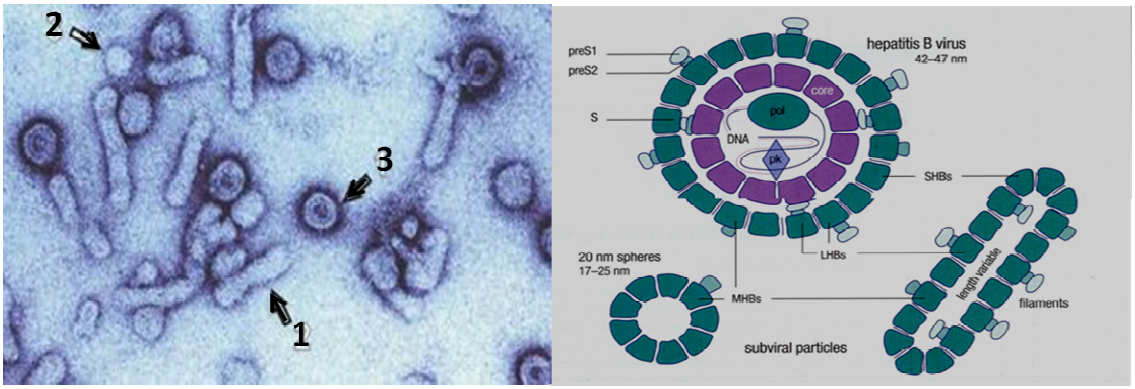


Figure 1.2 The hepatitis B virion structure (Dane particle) consisting of the small, middle and large glycosylated surface proteins as well as the lipid bilayer; the core; the polymerase protein; the enzymatic proteins and the partially double stranded DNA genome. (Kann, 2002). Reproduced with permission of International Medical Press, © 2008



A B

Figure 1.3 HBV virions and subviral particles structure: (**A**) An electron micrograph (x210,000) of hepatitis B virus in serum indicating the three distinct subviral particles; (1) represents the small empty spherical particles, (2) represent infectious virions (Dane particle) and (3) represent the filamentous particles (Zentgraf, 2002). (**B**): schematic drawing of the 20 nm sphere and filament virion particles (Kann, 2002). Reproduced with permission of International Medical Press, © 2008.

In the serum of HBV infected individuals, two different subviral particles can be found: the spherical 20 nm particles and the filamentous particles, which vary in length (*figure 1.3A*). The spherical and filamentous particles consist of viral surface proteins and host-derived lipid components (Kann, 2002), different from the complete Dane particle, these subviral particles lack nucleic acids and therefore are non-infectious. During infection, the subviral particles constitute over 90 % of the total HBV particles with titres ranging from between 10^6 and 10^{14}

particles per millimetre of infected serum (Scaglioni *et al*., 1996). The reason for the occurrence of these particles in higher titres is unknown, however, it is thought to the assist in shielding the infectious complete virions from host defences because these subviral particles are highly immunogenic and can induce anti-HBs. These subviral particles have been used as the basis of many vaccines against hepatitis B (Patient *et al*., 2007, Patient *et al*., 2009)

1.1.5 Natural history of HBV

HBV infection is a dynamic process characterised by replicative and non-replicative phases based on virus-host interaction. The hepatocytes are principal site of hepatitis viral infection. The liver plays an essential role in energy storage and conversion, blood homeostasis, chemical detoxification, and immunity to microbial infections (Seeger and Mason, 2000, Masson and Littwin, 2002), many of the functional activities of the liver reside in the hepatocytes, which constitutes 70 % of the liver. Therefore this makes them a target of hepatotropic viruses such as HBV. Any disturbance to the liver leads to a wide spectrum of clinical presentations ranging from an asymptomatic carrier state to self-limited acute or fulminant hepatitis to chronic hepatitis with progression to cirrhosis and hepatocellular carcinoma. Viral factors together with the host immune response have been implicated in the pathogenesis and clinical outcome of HBV infection (Baumert *et al*., 2007). The incubation period of HBV ranges from six weeks to six months, with clinical symptoms are highly dependent on the host (McMahon *et al*., 1985)

1.1.5.1 Acute HBV infection

Acute HBV infection can manifest be asymptomatic but can also manifest as acute hepatitis and rarely fulminant liver failure (Sulkowski, 2008). The most common presentation of asymptomatic infections begins with active HBV replication within the hepatocytes, HBsAg and HBeAg become detectable in blood within two weeks of infection before anti-HBcAg can be detected in the liver and may persist up to 8 weeks. Anti-HBc occurs early and decreases after 6 months. Anti-HBs appear after the decrease of HBV replication. Acute hepatitis varies in severity from mild to fulminant, which occurs rarely, in 1-2 % of acute hepatitis patients. The clinical manifestations of HBV infection are age dependant. In adults HBV is generally acute and self-limiting whilst it is mild in children and neonates (Fattovich, 2003). In neonates, clinical manifestation generally do not occur, in children aged 1-5, only 5% to 15 % develop clinical symptoms, whereas 33% to 50 % of older children and adults, develop symptomatic infections (Mahoney, 1999). Manifestations of acute hepatitis include high HBV DNA and serum transaminases (ALT) levels, which can persist. Acute infections usually resolve within 6 months (McMahon *et al.*, 1985). Clinical symptoms of acute hepatitis include fever, anorexia, nausea, malaise, vomiting, jaundice, dark urine, clay-coloured or pale stools, and abdominal pain (Mahoney, 1999, Sulkowski, 2008).

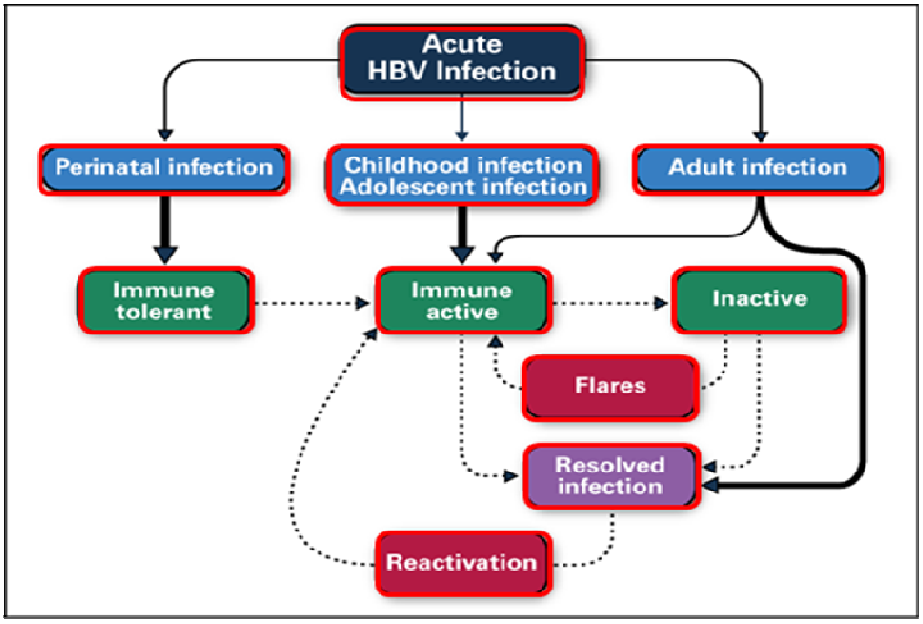


Figure 1.4: Natural history of acute Hepatitis B virus infection (modified from (McMahon and Spach, 2005).

1.1.5.2 Chronic HBV infection

Chronic HBV infection is defined as the persistent presence of HBsAg in the serum of an individual for 6 months or longer and the absence of anti-HBc immunoglobulin (IgM) (Mahoney, 1999). Persons with chronic HBV infection are at substantially increased risk of developing chronic liver diseases, including cirrhosis and primary HCC. The risk of developing chronic HBV infection ranges inversely with age: up to 90% in infants infected in perinatal period, between 25 to 50 % of children between the ages of 1 and 5 and 10 % in older children and adults (Mahoney, 1999). Chronic disease factors such as renal failure, HIV infection, and diabetes are associated with of developing chronic infection (Mahoney, 1999)

Three phases of chronic HBV infections are described: the immune tolerant phase has almost normal liver histology, high HBV DNA levels and HBeAg positivity, this phase is usually recognised in children and adolescents; the immune clearance phase is characterised by seroconversion from HBeAg to anti-HBe, followed by active hepatic inflammatory response and fibrosis, with fluctuating ALT levels and lastly, the residual phase, which is characterised by low HBV DNA and normal ALT levels (Sulkowski, 2008). Spontaneous reactivation is often seen during the course of chronic HBV infection and involves the reappearance of HBeAg and/or HBV DNA and a rise in serum ALT levels, after a period in which they are low or negative.(Baumert *et al*., 2007)

Persons with chronic HBV infection are generally classified as having one of three histologic patterns on liver biopsy: chronic persistent hepatitis, chronic active hepatitis, and cirrhosis. Cirrhosis is an irreversible condition in which regenerative nodules and fibrosis cause liver injury and may lead to development of HCC (Mahoney, 1999). The degree of histologic injury is hardly reflected by the symptoms, and persons with severe chronic liver disease are often asymptomatic appearing to be healthy with normal ALT levels until later in the course of their illness

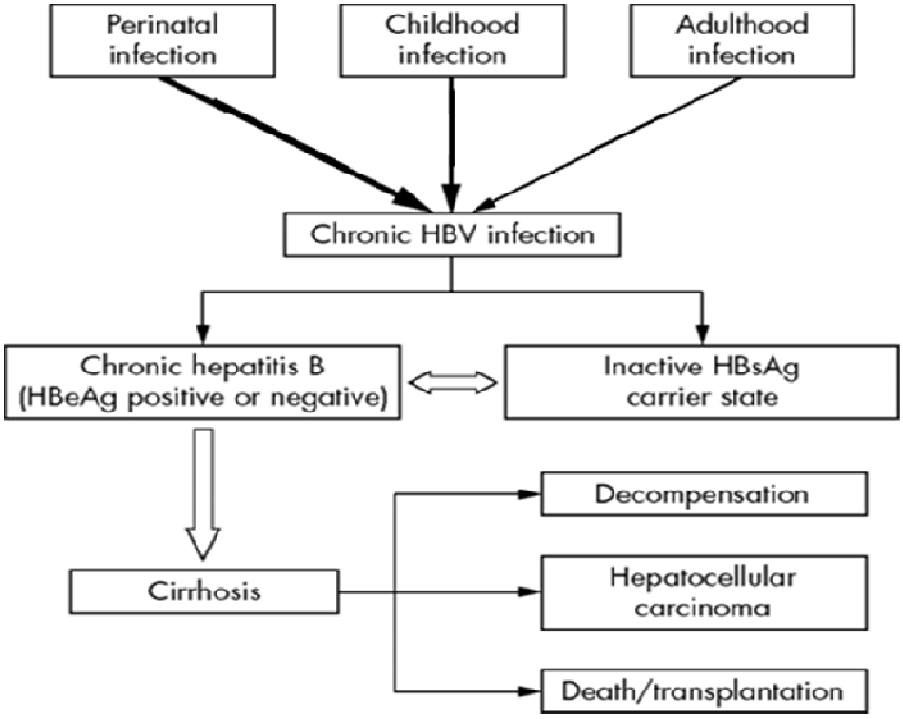


Figure 1.5: Natural history of HBV chronic infection (modified from Calabrese, *et al*, 2006)

1.1.6 The HBV genome Organization

The HBV viral genome is the smallest known fully replication competent genome among animal viruses. It has a genome size that is genotype dependent, varying from 3182 to 3248 nt (Kramvis *et al*., 2005a) (Jilbert *et al*., 2002). The HBV genome of an infectious virion is a relaxed circular double stranded DNA (rcDNA) consisting of an almost complete negative DNA strand of 3.2 kb and the shorter positive strand which varies in length (~1.6-2.8 kb) (Mahoney, 1999). The circular structure of the genome is maintained by the terminal redundancy, r, of 8 nt of the negative DNA strand and the incomplete positive strand with a variable 3'. The 5' end of the negative DNA strand is covalently linked to the terminal protein of the viral DNA polymerase, while the 5' of the positive DNA strand is capped by an

18 nt RNA oligonucleotide which is derived from the pre-genomic RNA obtained during replication (Jilbert *et al*., 2002, Kann, 2002).

The HBV genome encodes four overlapping open reading frames that encode for seven viral proteins: the core (C), polymerase (P), surface (S), and the X ORF's (figure 1.4) coded by the negative DNA strands whilst the positive strand is non-coding (Kann, 2002). All HBV regulatory elements are encoded within the protein coding sequences. Transcription results in formation of preC/pregenomic, preS1, preS2/S and X mRNA transcripts, which are modified by 3' polyadenylation and 5' cap (Moolla *et al*., 2002). A single polyadenylation signal terminates transcription at a common at 3' end for all four transcripts, however, the 5'ends vary depending on the location of each of the four ORF promoters (preC/pregenomic, preS1, preS2/S and X promoters) initiating transcription by binding of the RNA polymerase II (Zentgraf, 2002, Jilbert *et al*., 2002). In addition, two enhancers: enhancer I and enhancer II and the *cis* –acting negative regulatory elements regulate transcription (Moolla *et al*., 2002). The C ORF encodes for the core protein (HBcAg) and the e antigen (HBeAg), P encodes for the viral polymerase (reverse transcriptase), the S encodes for 3 surface proteins: SHBs, MHBs and LHBs. X encodes for the HBx *trans*-activator (*figure 1.4*) (Kann, 2002, Jilbert *et al*., 2002, Zentgraf, 2002)

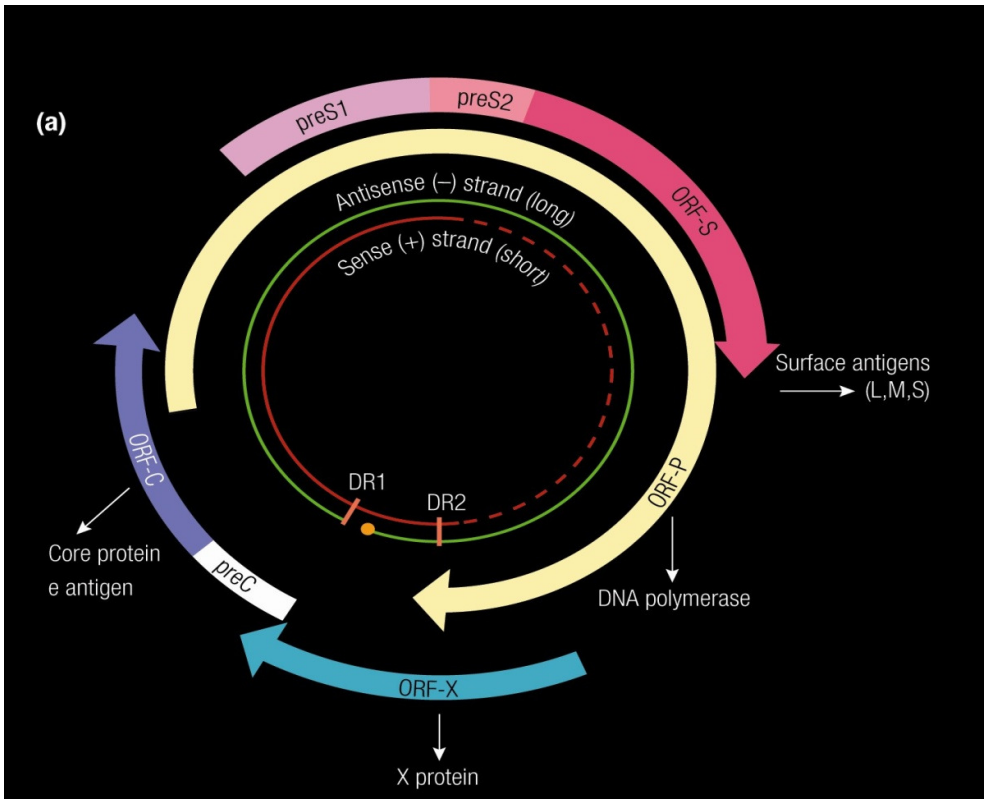


Figure 1.6: Structure and organization of the HBV genome presenting the plus (+) and minus (-) sense, showing four main mRNA transcripts indicated by the arrow bars and their corresponding protein products , DR1 and DR2 also illustrated on the 5' and 3' ends (Lai and Locarnini, 2008). Reproduced with permission of International Medical Press, © 2008

1.1.7 The viral gene products

Transcription of the overlapping HBV ORF's generates four mRNA transcripts (*figure 1.4 and 1.5*): the 3.5 kb pre-genomic, 2.4 kb S1, 2.1 kb S2 and 0.7 kb X mRNA transcripts. The 3.5 kb pregenomic mRNA is a template for translation of the core protein (HBeAg and HBcAg) and the viral polymerase (reverse transcriptase). The 2.4 kb and 2.1 kb mRNA transcripts encode the preS1 (large), preS2 (middle) and major surface proteins (HBsAg)

respectively which are essential for envelopment of the nucleocapsid. The 0.7 kb serves a template for the additional gene products during natural infection, the HBV X protein and the hepatitis e antigen (HBeAg), their function is unknown (Moolla *et al*., 2002, Mahoney, 1999, Seeger and Mason, 2000).

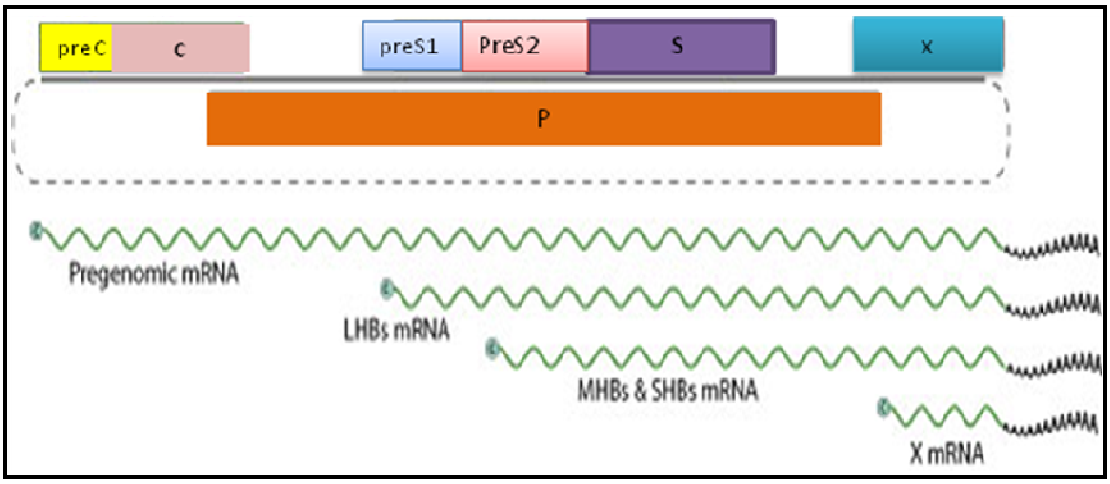


Figure 1.7: Four HBV primary transcripts that translates into seven HBV proteins

1.1.7.1 *Precore/core ORF*

The precore/core (C) open reading frame contains two start codons coding for two overlapping proteins, the HBeAg and the HBcAg (Mahoney, 1999), depending on the start (AUG) codon used. A 21.5 kDa core protein is synthesized when a translation start at the second start codon is used. This can be either 183, 185 or 195 amino acids in length depending on viral genotype (Kann, 2002, Locarnini *et al*., 2003). When translated, the HBcAg is targeted to the endoplasmic reticulum where it is cleaved and assembles into the nucleocapsid or viral capsid. The core protein has two functional distinct domains, the amino acid terminal (1-144) of the core protein are needed for assembly of the nucleocapsid and the arginine-rich C-terminal domain (amino acids 150-164) at end of the protein mediates nucleic

acid binding, encapsidation of the pgRNA and stabilization of the capsid protein (Locarnini *et al*., 2003). Assembly of the HBcAg into the icosahedral subviral particles requires formation of dimers or subunits, these are stabilized through two disulfide bonds, followed by assembly of 120 dimers into a single shell with a diameter of 32 nm and a T3 symmetry or a diameter of 36 nm consisting of T4 symmetry with 240 core proteins dimers (Seeger and Mason, 2000, Kann, 2002).

The first translation start codon of the precore/core ORF 87 nucleotides upstream of the core initiation site results in the HBeAg This is a 25 kDa protein consisting of a 19 amino acid (aa) pre-C domain and a core polypeptide chain of 183 or 185 aa. The pre-C region serves as a signal peptide that targets the precursor to the ER, where the N-terminus is cleaved to yield a 22-23 kDa protein. 34 aa of the protein is cleaved further from the carboxyl terminus in the Golgi apparatus into a mature product of 15-18 kDa HBeAg, which is secreted into the blood (Harrison, 2006, Kann, 2002). The HBe protein is essential for the establishment of persistent infection (Locarnini *et al*., 2003) and is an indicator of high viral replication and infectivity (François *et al*., 2001). It is also thought to be a tolerogen (Chen *et al*., 2004).

1.1.7.2 *Polymerase ORF*

The polymerase is the second protein to be translated from the pgRNA and longest ORF. It is 845 aa in length. Its translation start codon is 600 nt down stream of the 5' end of the RNA encoding a 90 kDa protein (Kann, 2002); (Seeger and Mason, 2000). The P gene, with 834–845 codons, has at least four domains: N-terminal domain, spacer, polymerase, and carboxyl terminal domain. The N-terminal domain (1-183 aa at the N-terminus of the protein) codes

for the terminal protein (TP), which is involved in minus-strand DNA synthesis and is required for the pregenomic RNA encapsidation.

The spacer covers aa 184 -354 and serves as an intervening domain of the N-terminus and the reverse transcriptase (RT) with no specific recognized function (Locarnini *et al*., 2003). The polymerase domain encodes for the reverse transcriptase (RT) extends through aa 355-694. It incorporates the YMDD motif; this is the catalytic domain essential for reverse transcriptase activity (Seeger and Mason, 2000, Yokosuka and Arai, 2006). The carboxyl terminal domain encompasses aa 695-844. It encodes for a ribonuclease (RNase H) that serves as a reverse transcriptase RNase H activity endowed domain that cleaves RNA in the RNA-DNA hybrids during reverse transcription (Seeger and Mason, 2000). The RNase H domains also plays a role in viral RNA packaging, in optimizing priming of minus-strand DNA synthesis, and in elongation of the minus-strand viral DNA. The polymerase domain also encodes at least two T cell epitopes within its catalytic domains at amino acid residues 107 to 115 and 227 to 235 (Locarnini *et al*., 2003).

1.1.7.3 *X ORF*

The X ORF is a highly conserved region and encodes a 17 kDa polypeptide of 154 amino acids (HBx) translated from the 0.7 kb mRNA (Locarnini *et al*., 2003, Moolla *et al*., 2002). Its function during natural viral infection is unknown. It is an essential component of the infectivity process *in vivo* (Seeger and Mason, 2000). The HBx is known to activate various signalling pathways (AP-1, NF-κB)
in vitro and to bind p53.

1.1.7.4 *Envelope ORF*

The envelope ORF encodes the hepatitis surface antigen (HBsAg). HBV has three distinct surface/envelope proteins, LHBs, MHBs and SHBs which can be distinguished by three domains (*figure 1.6).* The envelope ORF posseses three separate translational start sites but has the same stop codon (UAA). The longer preS1 mRNA of 2.4 kb encodes the LHBs, the first start codon of the shorter S mRNA (2.1 kb) encodes the MHBs and the SHBs is transcribed using the second start codon of the 2.1 kb S mRNA and is the most abundant (Locarnini *et al*., 2003).

Synthesis of the surface proteins occurs in the ER. Relative distribution of the three surface proteins varies between virions, filaments and spheres. More LHBs is found in virions and filaments than in spheres. Over expression of LHBs results in retention within the ER which prevents particle formation (Kann, 2002). The envelope proteins exist in two forms, the glycosylated and unglycosylated form and are covalently linked to the other viral proteins by disulphide bridges formed by cysteine residues (Seeger and Mason, 2000).

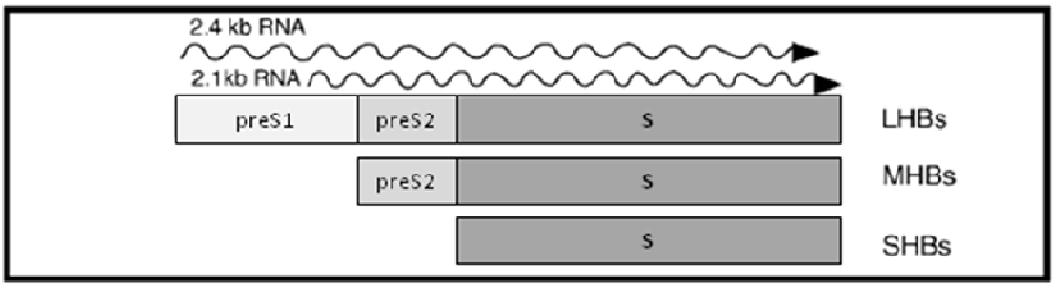


Figure 1.8: The three domains of HBsAg, The single 2.4-kb RNA makes LHBs while the various 2.1- kb mRNAs translate MHBs and SHBs (modified from Locarnini *et al*., 2003)

1.1.7.4.1 *Small envelope protein*

SHBs protein is referred to as the major surface (HBsAg) protein is composed of 226 amino acids and the most abundant protein in all three HBV-associated particles. It is 24 and 27 kDa in its unglycosylated and glycosylated form respectively. 50%-60% of the small envelope protein is folded into α-helices numbered I – V, by 4-5 lengths of hydrophobic amino acids (*Figure 1.7a*). The first helix (I) is located between amino acids 11-29, crosses the ER membrane and translocates the upstream sequence into the ER lumen (Kann, 2002). The next amino acids 30 to 79 comprise the first polar hydrophilic domain, which is the major cytoplasmic loop and afterwards localised in the virus interior. The next helix II , signal II covers aa residues 80-98 and is also inserted in to the ER membrane to mediate translocation of downstream sequences and directed to the core particle (Kann, 2002).

Second hydrophilic domain of HBsAg is after helix II; it consists of 70 aa and covers residues 99 to 168. This region contains a high number of cysteine residues, which are cross-linked with each other by disulfide bonds to form a loop configuration, the major antigenic determinant of HBsAg, known as the 'a' determinant (Locarnini *et al*., 2003). This region is exposed on the surface of the virus and subviral particles containing the antigenic determinants, the epitope ("a" determinant) are able to elicit virus-neutralising antibodies (anti-HBs) following immunization (Seeger and Mason, 2000, Locarnini *et al*., 2003)

The 'a' determinant is common for all nine serological subtypes and is defined by a threonine or isoleucine at position 126 of HBsAg. The serological subtypes are differentiated by amino acid substitutions at positions 122 and 160 of HBsAg and are used to distinguish between serotypes *d/y* and *w/r (Kramvis et al., 2005b)*. During an infection, antibodies against all

determinants will be produced, those against the 'a' determinant entirely will be able to protect against other HBV serological subtypes, and hence the 'a' determinant is clinically the most crucial. This has lead to use of the HBsAg as the basis of many vaccines against HBV (Torresi, 2002). Downstream of helix II is helix III, passing through the ER, following helices IV and V spanning the ER membrane (Kann, 2002).

1.1.7.4.2 Medium envelope protein

The MHBs pre-S2 domain consists of the S and a 55-amino-acid N-terminal extension (*figure 1.7 B*) and is 281 aa in length. It is a minor component of the virion or HBs particle. PreS2 is N-glycosylated with a glycan at amino acid 4 and may additionally have an O-glycan at position 37 in HBV genotypes B-F. Two glycosylated forms can be distinguished: gp33 and gp36. Ninety percent of preS2 molecules are modified further by the acetylation of their N-terminal methionines (Kann, 2002). The role of MHBs is not well understood, however, the central part of the Pre-S2 domain carries the major antigenic epitope and the region between amino acids 3 and 16 has the ability to bind polymerized human serum albumin but the significance of this binding is unknown (Locarnini *et al*., 2003) .

1.1.7.4.3 Large envelope protein

The LHBs is the largest surface protein with 389-400 aa residues and it consist of three domains, pre-S1, pre-S2, and S (*figure 1.6)*, in addition to an extra 108-119 amino acids on its N-terminus, depending on subtype/genotype and is glycosylated (Locarnini *et al*., 2003). It shares carboxyl terminal sequence with the MHBS and SHBs surface proteins (figure *1.7 A and C).*

LHBs constitute only 10-30 % of the total surface proteins which is an important ratio for virion assembly and secretion (Kann, 2002). When the LHBs is localised in the ER and exposed on the virion surface (Schädler and Hildt, 2009) (*figure 1.7 C),* it is accessible to antibodies, potential receptors and proteases, or in the hepatocyte cytosol (Kann, 2002), followed by localisation in the virion interior (*figure 1.7 D*) where it is involved in envelopment of the mature core particles,essential for viral assembly (Kann, 2002). The N-terminal end of the pre-S domain is myristylated, this is not required for virion formation and release but is essential for virion infectivity. The LHBs has important antigenic sites for both B and T cells that appear to play critical roles in recovery from viral infection. The coding regions at amino acids 27 to 35, 72 to 78, and 95 to 107 are very crucial immunogenic epitopes within the LHBs protein (Locarnini *et al*., 2003).

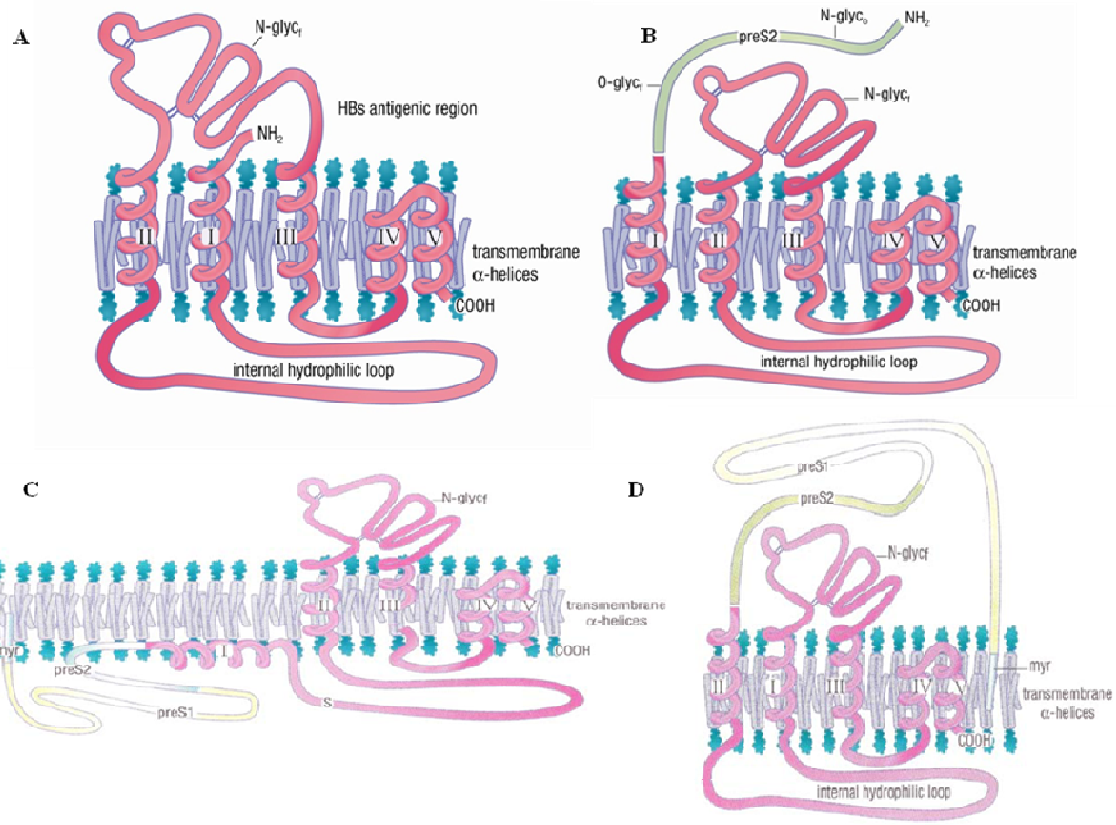


Figure 1.9: **Topological models of the three HBV surface proteins:** (A) Small surface protein (SHBs), (B) middle surface protein (MHBs). (C) Large surface protein (LHBs) with ER localized preS1 domain, (D) large surface protein with cytoplasmically localized preS. The α-helices are numbered I-V. N- or O-glycosylations are marked N- or O-glyc. The top of each picture represents the lumen of the ER/pre Golgi compartment, whilst the bottom corresponds to the hepatocytes cytosol or interior of the virus. (Kann, 2002). Reproduced with permission of International Medical Press, © 2008

1.1.8 HBV life cycle and genome replication

1.1.8.1 *Entry into hepatocytes*

The hepatocytes are the primary site for HBV viral DNA replication. It is generally believed that HBV interacts with a specific, yet unidentified, cell-surface receptor. The virus enters the cell via receptor-mediated endocytosis of virus into the hepatocytes (Scaglioni *et al*., 1996). Following entry into the host cell, the viral envelope is thought to fuse with the endosome, resulting in the release of the viral nucleocapsid into the cell cytoplasm (Jilbert *et al*., 2002). Before entering the nucleus, the nucleocapsid is disassembled on the cytoplasmic side of the nuclear membrane, thus allowing the viral DNA to enter the nucleus and initiate replication where the conversion into covalently closed circular DNA (cccDNA) occurs (figure 1.7) (Scaglioni *et al*., 1996, Schädler and Hildt, 2009).

1.1.8.2 *Conversion of rcDNA to cccDNA*

At the end of viral entry process, the rcDNA is delivered into the nucleus and converted into a nuclear, episomal cccDNA by an endogenous DNA polymerase activity. This involves

completion of positive plus (+) strand DNA synthesis, removal of the bond that links the polymerase to the 5' end of the negative (-) strand DNA, removal of the RNA primer from the 5' end of (+) strand DNA and ligation of the DNA ends (Scaglioni *et al*., 1996). The cccDNA represents the central intracellular intermediate in viral replication and serves as a template for all transcripts involved in protein production and replication (Jilbert *et al*., 2002).

1.1.8.3 *pgRNA transcription from cccDNA*

By utilizing the host cell RNA polymerase II, the cccDNA is used as a transcriptional template for the pre-genomic RNA (pgRNA) of 3.5 kb in length, which encodes the core and polymerase proteins (reverse transcriptase), the 2.4 kb and 2.1 kb transcripts, which encode the surface envelope proteins, whilst a 0.7 kb transcript encodes the X-protein (Mahoney, 1999). Transcription of these four proteins is initiated by different gene promoters; the enhancer II/basal core, large surface antigen, major surface antigen and enhancer I/X, respectively (Scaglioni *et al*., 1996).

1.1.8.4 *Reverse Transcription*

HBV replicates its genome by reverse transcription of the pgRNA, which is characterised by terminal repeats at the 5' and 3' ends which include the direct repeats: DR1 at both ends, DR2 at the 3' terminal and the stem loop RNA packaging signal (ε) (Moolla *et al*., 2002).. First, the pgRNA-polymerase complex is packed into subviral packages composed of HBcAg subunits, whereas the viral polymerase binds to the encapsidation signal ε, which is a cis-element on the pgRNA. This is aided by three host chaperone proteins: p23 and heat shock

protein 70 (Hsp70) aid the binding of the polymerase to ε, and Hsp60 is required in the activation of the viral polymerase (Kramvis and Kew, 1998, Seeger and Mason, 2000).

The ε-polymerase interaction, also activates reverse transcription, where the binding of the polymerase acts as a primer at the 5'UUC3' motif within the bulge of the 5' ε, initiating the synthesis of the first 3 nt of the negative DNA strand 5'GAA3'. The formed Pol-oligonucleotide complex is then translocated into DR1 at the 3' end of the pgRNA and continues with the rest of the negative DNA strand synthesis till the 5' end of the pgRNA (figure 1.6)(Siegel, 2007). The elongation of the negative DNA strand occurs simultaneously with degradation of the pgRNA by RNAse H activity, which leaves only the last 18 bases including the DR1 motif. This translocates s to the DR2 motif and serves as a primer for the positive DNA strand synthesis. Elongation of the positive strands continues to the 5' end of the negative DNA strand, this includes a 9 base terminal redundancy sequence complementary to the 3' end of the negative strand DNA. This results in the elongating 5' end of the positive DNA strand being transferred to the 3'end of the negative DNA strand and therefore circularising the genome, and finally leading to rcDNA synthesis (Jilbert *et al*., 2002, Seeger and Mason, 2000, Scaglioni *et al*., 1996, Schädler and Hildt, 2009).

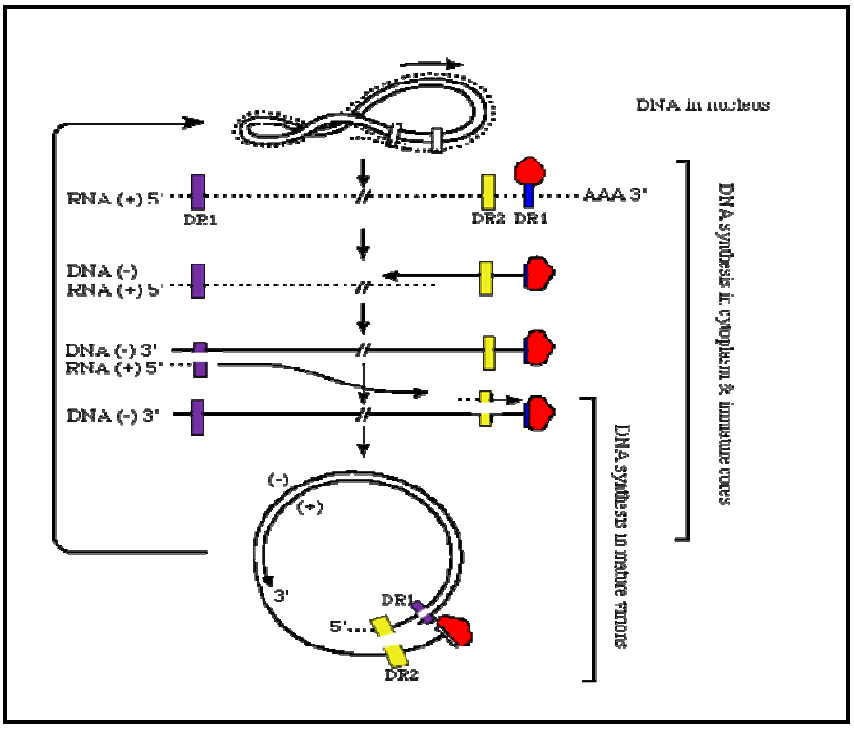


Figure 1.10: Viral DNA replication by reverse transcriptase. DR1 and 2: Direct repeat sequences (modified from http://www.stanford.edu/group/virus/1999/tchang/replication.htmm, Accessed November 2010) .

1.1.8.5 *Capsid maturation, Envelopment and budding*

The nucleocapsid containing the newly the synthesised rcDNA is either recycled back to the nucleus for the conversion into cccDNA for synthesis of more viral particles or enveloped selectively, before budding off the cell. The envelopment occurs in the Golgi apparatus, where the nucleocapsid are translocated from the cytoplasm Golgi complex through the ER to acquire an ER membrane containing lipids and the three viral surface proteins (SHBs, MHBs and LHBs). In the Golgi complex, the surface proteins are glycosylated, this is followed by

release of mature virions into the bloodstream for infection of more cells (figure l.7) (Seeger and Mason, 2000).

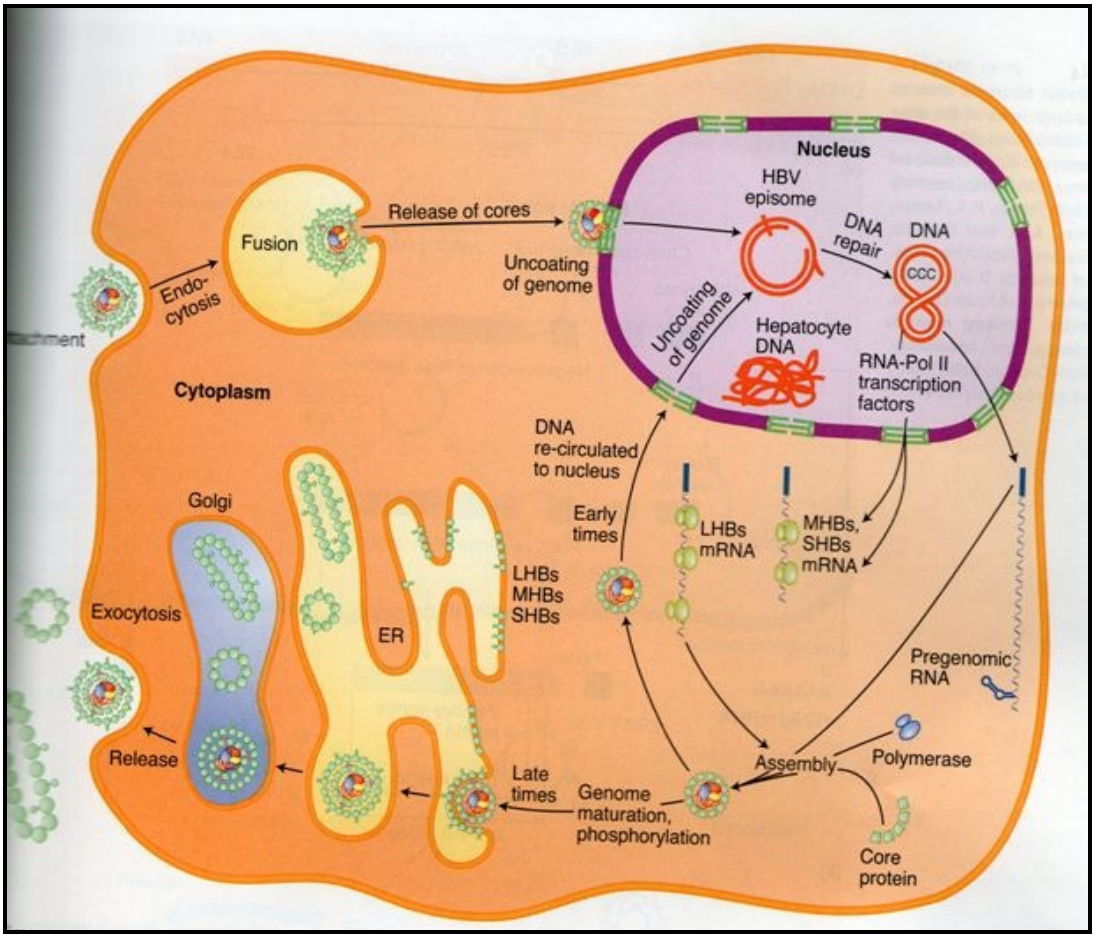


Figure 1.11 HBV life cycle. The cycle commences with attachment of the infections virion on to the target cell receptor via the preS1 domain of LHBs protein. Uncoating is followed by relocation of the nucleocapsid into the nucleus where the rcDNA is converted into cccDNA. The cccDNA serves as template for transcription of mRNA and the pgRNA. The mRNAs proceed to the cytoplasm and translated into viral proteins. The pgRNA is reverse transcribed into rcDNA which is either converted into cccDNA or translocated across the endoplasmic reticulum and coated surface proteins and ends with release into the bloodstream. (modified from http://prolacmalaysia.com/page.php?10)

1.2 HBV GENOTYPES

A genotype is generally defined as the genetic constitution of an organism. In viruses, it is a form in which the genomic sequence of a virus that is replication competent has stabilized after a prolonged period of time (Kramvis *et al*., 2005b, François *et al*., 2001). HBV replicates by reverse transcription of the pregenomic RNA using a viral-encoded polymerase that lacks proof-reading activity. The genome may evolve at an estimated error rate of 1.4 - 5 x 10^{-5} nucleotide substitutions/site/year (Fares and Holmes, 2002, Orito *et al*., 1989, Gunther *et al*., 1998), which results in a wide virus heterogeneity (Ramos *et al*., 2007). As a result of this genetic variability, genotypes of HBV have been identified (Kramvis *et al*., 2005). defined by the inter-genotypic differences of more than 7.5% in the complete nucleotide sequence (Okamoto *et al*., 1988, Norder *et al*., 1992b). To date, phylogenetic analysis of the HBV genome has lead to recognition of nine genotypes of HBV: A to D (Norder *et al*., 1994, Okamoto *et al*., 1988), genotype E to F (Norder *et al*., 1994, Naumann *et al*., 1993, Norder *et al*., 1992b), genotype G (Stuyver *et al*., 2000), genotype H (Arauz-Ruiz *et al*., 2002) genotype I(Olinger *et al*., 2008) and more recently a tenth genotype J has been proposed (Tatematsu *et al*., 2009).

Before identification of HBV genotypes, HBV was classified according to serotypes. four major serological subtypes were identified based: *adw, adr, ayw* and *ayr* (Norder *et al*., 1992a, Okamoto *et al*., 1987) and further into 10 serological subtypes (*ayw1*, *ayw2*, *ayw3*, *ayw4*, *ayr*, *adw2*, *adwq*, *adr*, *adrq−* and *adrq*$^{+}$), depending on the amino acid substitutions on the antigenic determinant at positions 122, 127 and 160 (Kramvis *et al*., 2005b) . There is a limited relationship between the HBV genotypes and the serological subtypes and their geographical distribution (Table 1).

Table 1.1 Relationship between genotypes and serotypes and their geographical distribution [§]

Genotype	Serotype	Geographical distribution
A	*adw2, ayw1, ayw2, adw4*	North western Europe, USA, sub-Saharan Africa, East Africa and central Africa , Japan, and the Philippines
B	*adw2, ayw1, adr*	Indonesia, South and East Asia, Vietnam, Brazil, Indonesia, the Philippines and Venezuela
C	*adr*, *adrq*$^{+}$, *adrq*$^{-}$ *ayr, adw, adw2, ayw2, ayw3, adwr*	Venezuela, Korea, China, Japan Polynesia, Vietnam, East Asia, Japan, the Philippines, Tibet Australia [Aborigines] and Japan
D	*ayw, ayw3, ayw4*, *adw3*	West, Central and, East Africa, India Mediterranean area, Middle East, USA and Central America
E	*ayw4*	West Africa, Nigeria, Western and Central Africa
F	*adw4, adw4q−*, *adw2*, *ayw4*	South and Central American Hispanics, France, Alaska,
G	*adw2*	France, Germany, USA and the United Kingdom
H	*adw4*	Northern part of Latin America, Central America and Mexico

[§]adapted from (Kramvis *et al*., 2005b), *adw2* occur in many parts of the world

Subgenotypes of five genotypes (A, B, C, D, and F) have been identified (Kramvis *et al*., 2005b), based on more than 4% divergence in the complete genome (Norder *et al*, 2004) The HBV genotypes and subgenotypes show a distinctive geographical distribution (figure 4.). This may change over time as a result of human migration patterns or due to specific risk behaviour such as injection drug use and men having sex with men (Ramos *et al*., 2007, Günther, 2006). Genotype A predominates in the USA and South America, North and Middle Europe, Southern Africa, Asia (Kramvis et al., 2002, Kramvis and Kew, 2007a). Genotype B and C are dominant in mainland and South East Asia, Japan, Australia and the Pacific

Islands. Genotypes B and C have been subdivided into six and five subgenotypes, respectively (Tanaka *et al*., 2005, Kramvis *et al*., 2008, Sakamoto *et al*., 2006, Sugauchi *et al*., 2002).

Genotype D occurs in many places in the world, including France, Germany, India, Alaska , USA, Canada, West and South Africa (figure 4) where the transmission route is mainly horizontal (Owiredu *et al*., 2001, De Maddalena *et al*., 2007, Sugauchi *et al*., 2002). Genotype E only occurs in Africa and is closely related to genotype D (Takahashi *et al*., 2000, Mulders *et al*., 2004, Shibayama *et al*., 2005). Genotype F is indigenous in Central and South America and Polynesia; it has only four subgenotypes (Huy *et al*., 2006, Arauz-Ruiz *et al*., 1997, Devesa *et al*., 2008). Genotype G has been found in Germany, North America, France and the UK (Kato *et al*., 2002, Günther, 2006). Genotype H occurs in Central America, Mexico and Canada (Arauz-Ruiz *et al*., 2002). Recently genotype I have been identified in Vietnam, South East Asia and North East India (Huy *et al*., 2008, Arankalle *et al*., 2010, Hannoun *et al*., 2000, Yu *et al*., 2010). The proposed Genotype J was isolated from a Japanese HCC patient from Borneo. It is most genetically related to a non-human primate (Tatematsu *et al*., 2009).

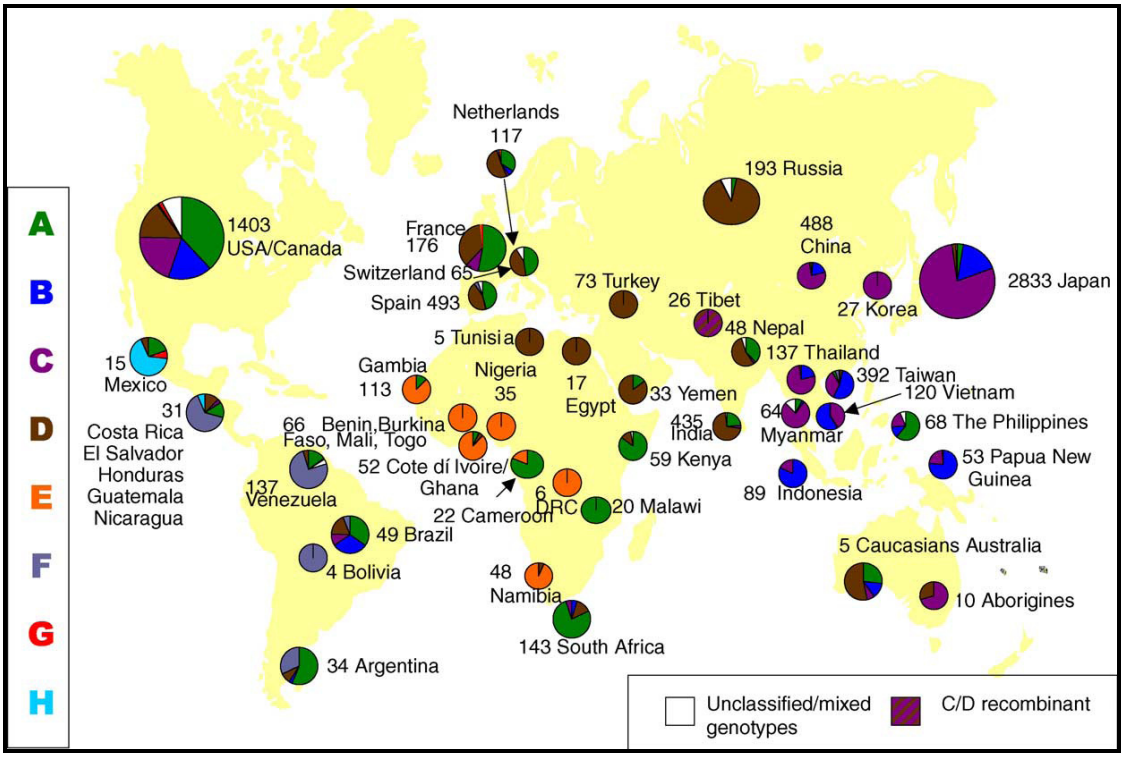


Figure 1.12. Geographical distributions of the eight hepatitis B virus genotypes, the numbers next to the pie charts are the number of isolates genotyped. Adapted from Kramvis *et al*, 2005. Reproduced with permission of Prof. A. Kramvis

1.2.1 African HBV genotypes and subgenotypes

Africa is an area of very high endemicity for HBV, with three genotypes A, D and E predominating (Kramvis and Kew, 2007b), with genotypes B and C sporadically detected (Zekri *et al*., 2007). Genotypes A is found mainly in southern and eastern Africa, genotype D in northern Africa and genotype E Central and West Africa (*figure 1.5*) (Sbai *et al*., 2007, Borchani-Chabchoub *et al*., 2000). Despite the importance of HBV infection in Africa up to five years ago there was a limited number of HBV genotyping studies in Africa

1.2.1.1 *Genotype A*

HBV genotype A genomes have a characteristic 6 nucleotide (nt) insert at the carboxy-terminal end of the core gene, which is not found in the other genotypes. It has distinctive sequence characteristics in all ORFs and in the transcriptional regulatory elements. Genotype A was the first genotype in which subgenotypes were recognised (Bowyer et al., 1997b). Subgenotype A1 was first identified in HBV isolates from South Africa using phylogenetic analysis of preS2/S sequences (Bowyer et al., 1997b). However, later studies then identified subgenotype A1 in Malawi, Mexico, Brazil, Philippines, India and Nepal (Sugauchi *et al*., 2003, Sánchez *et al*., 2002, Estacio *et al*., 1988, Sugauchi *et al*., 2004)

Subgenotype A1 has distinct sequence characteristics in all ORF's and transcription regulatory elements that may affect both replication of the virus and the expression of its proteins, which differentiates it from subgenotype A2 (Kimbi *et al.*, 2004). Compared to subgenotype A2, subgenotype A1 has a greater mean nucleotide divergence, indicating that subgenotype A1 has been endemic to the South African Black population and has a long natural history within the population (Kimbi *et al*., 2004). Subgenotype A2 has been reported to be widely distributed in European countries and the USA (Olinger *et al*., 2006). Characterization of additional strains has lead to the identification of subgenotype A3 in the Cameroon, Democratic Republic of Congo and Gabon, Central African Republic. Subgenotype A3 characteristic feature include a recombination of a non-overlapping short fragment of the polymerase RT domain with genotype E (Kurbanov *et al*., 2005), found in one of the Cameroonian strains. The complete genome analysis of subgenotype A3 suggests a long natural history within the native population of Cameroon (Kurbanov *et al*., 2005, Makuwa *et al*., 2006). Recently, subgenotype A4 and A5 have been isolated in Mali and

Nigeria respectively (Olinger *et al*., 2006). More recently, Subgenotypes A6 and A7 have been proposed (Hübschen *et al*., 2010, Pourkarim *et al*., 2010). Subgenotype A6 strains have been isolated from African–Belgian patients originating from Congo and Rwanda (Pourkarim *et al*., 2010) and subgenotype A7 strains have so far only been found in Rwanda and Western Cameroon (Hübschen *et al*., 2010).

1.2.1.2 *Genotype D*

Genotype D is found in many places around the world. In Africa, it has been described in Egypt, Morocco, Tunisia, West and South Africa (Borchani-Chabchoub *et al*., 2000, Zekri *et al*., 2007, Sbai *et al*., 2007) . Genotype D is 3 182 nucleotides in length and is characterized by a 33-nucleotide deletion at the N terminus of the preS1 region. Genotype D also has been divided into five subgenotypes: D1 to D5 (De Maddalena *et al*., 2007, Chandra *et al*., 2009) isolates of subgenotype D1 are mainly from the Middle East. Subgenotype D2 were mostly from Germany and India. Subgenotype D3 was from Alaska, Sweden France and South Africa. And lastly subgenotype D4 isolates were from Somalia, and Oceania (Sugauchi *et al*., 2002). Subgenotype D5 has been described in East India (Chandra *et al*., 2009). Recombination has been reported between genotype A and D over the non-overlapping regions of the S and P open reading frames (Owiredu *et al*., 2001, De Maddalena *et al*., 2007).

1.2.1.3 *Genotype E*

Genotype E is restricted to Africa and mainly found in central and West Africa (Mulders *et al*., 2004, Kao, 2002) (figure 5). A feature of genotype E genome isolates is that they have a

3-nucleotide deletion at the amino terminus, However, Genotype E does not separate from genotype D in the X and C ORFs (Kramvis *et al*., 2005b, Kidd-Ljunggren *et al*., 2002), and its existence has been questioned (Kidd-Ljunggren *et al*., 2002, Bowyer and Sim, 2000) . Genotype E has been isolated form chimpanzees; this has lead to speculations that genotype E may have once been a chimpanzee hepadnavirus, however, no direct transmission could be established (Takahashi *et al*., 2000).

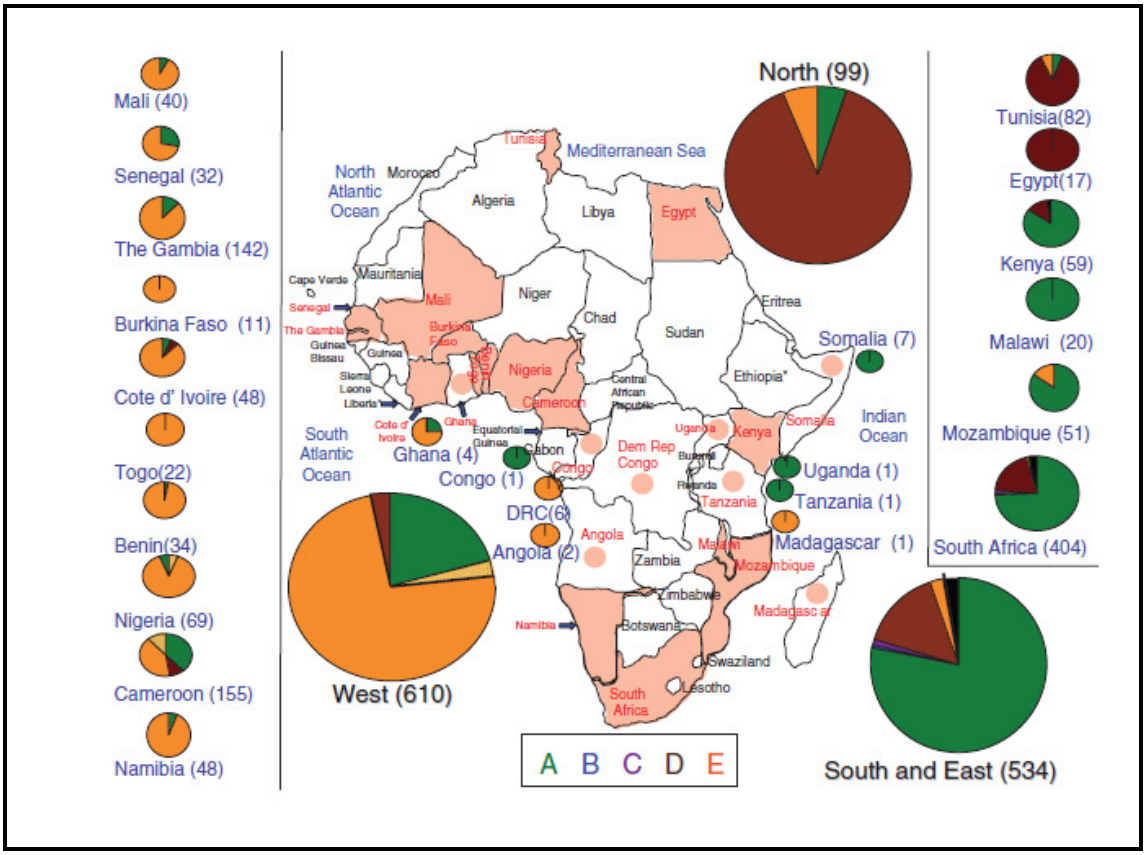


Figure 1.13. Hepatitis B virus (HBV) genotypes A, B, C, C and D distribution across the African continent. (Kramvis & Kew, 2007). Reproduced with permission of Prof. A. Kramvis

1.2.2 Clinical relevance of the HBV genotypes

Studies have suggested possible pathogenic and therapeutic differences among HBV genotypes. Many of the studies have been done comparing and contrasting genotypes molecular and clinical similarities and differences. In Southern Africa genotypes A, D, and E of HBV circulate, with genotype A dominating (Kew *et al*., 2005). From earlier studies, a higher prevalence of genotype A in patients with HCC compared with asymptomatic carriers was deduced (Zeng *et al*., 2005, Thakur *et al*., 2002). A South African study showed a 4.5-fold increased risk for HCC and an earlier age of cancer diagnosis (on average, 6.5 years younger) in HBV carriers infected with genotype A compared with non-A genotypes (Kew *et al*., 2005). Subgenotype A1 was found to have a lower frequency of HBeAg positivity compared to genotype D (Kramvis and Kew, 2007a) .Genotype A is known to induce chronic hepatitis more often and hold much higher response to interferon treatment than genotype D (Thakur *et al*., 2002, Kao, 2002).

An Indian study suggested that HBV genotype D is associated with more severe diseases and may predict the occurrence of HCC in young patients (Thakur *et al*., 2002).In a European study, genotype D was found to be more associated with earlier HBeAg sero-conversion, with much higher incidence of HCC when compared to genotype A. In addition, genotype D had a stronger resistance to anti-viral drugs. (Schaefer, 2005). Genotype E, which predominantly occurs in Africa is reported to be associated with progression to chronic HBV carrier during the childhood in West Africa (Schaefer, 2005)

1.2.3 HBV genotypes in HIV co-infected individual

There are very few studies documenting HBV genotypes in HIV infected individuals. In a number of European studies, genotype A has been found to be the most predominant followed by genotype D and genotype E (Pérez-Olmeda *et al*., 2003, Soriano *et al*., 2010, Ramos *et al*., 2007, Cooley *et al*., 2003). Despite the high prevalence of both HBV and HIV in Africa, the genotypes prevailing in African HIV infected individuals are unknown. As far as we can ascertain, this is the first study to examine the HBV genotypes in South Africa.

1.3 HBV MUTANTS: DISTRIBUTION AND DISEASE PROGRESSION

Considering the compact and overlapping arrangement of the HBV genome together with the lack of proof reading activity by the viral reverse transcriptase, the host and anti-viral pressure the mutation rate occurring in the HBV genome is relatively high. The nucleotide substitution rate, per site per year, has been estimated to be 1.4×10^{-5} to 5×10^{-5} (Fares and Holmes, 2002, Kramvis and Kew, 2005, Sugauchi *et al*., 2000, Okamoto *et al*., 1987). The mutation errors in genomic replication are of great importance in viral evolution, they allow the virus mutants to escape host immune responses (immune/vaccine escape mutants) and be able to resist antiviral treatment pressures (drug resistant mutants) (Bonhoeffer and Sniegowski, 2002). Therefore this results in changes which bear on the progression of the disease, virus replication and expression of viral proteins.

1.3.1 Precore/core mutants

The core promoter directs transcription of the 3.5 kb mRNA: the pgRNA and the precore mRNA (pre-C mRNA). The pgRNA is translated into the core protein (HBcAg) and the viral polymerase and serves as a template for reverse transcription after being packaged into the virus core particles (figure 1.7). Therefore the precore/core core promoter is very important because it plays a central role in viral DNA replication and morphogenesis (Tiollais *et al*., 1985, Mayerat *et al*., 1999). The pre-C mRNA is translated into the pre-C/C fusion protein that is post-translationally modified into HBeAg, which is not required for viral replication, and may be involved in induction of immune tolerance (Milich and Liang, 2003). The core promoter (CP) consists of the Basic core promoter (BCP) and the upper regulatory region URR.

The PC gene contain important elements that are very crucial for viral replication: the start site for pgRNA synthesis, DR1 and the ε signal, hence its sequence variation within this region is limited. The CP encompasses nt 1814 to 1901. One of the most important mutations occurring in this region is the G1896A stop codon mutation, which occurs within the ε signal bulge within the priming region. This mutation truncates the precore/core protein into a 28 aa peptide and completely abolishes HBeAg expression (Carman *et al*., 1989). The presence of A1896 depends on C1858 or T1858, which is genotype specific. Genotypes A and H have C1858, genotype B, D and E have T1858 and the other genotypes can have either of the variants (Rodriguez-Frias *et al*., 1995). In genomes with T1858, the A1896 mutation stabilises the ε signal, resulting in a an enhanced stability of the, pgRNA promoting the initiation of DNA synthesis (Lok *et al*., 1994). The C1858 with the mutation A1896 results in a wobble pair, which destabilises the ε signal and is therefore detrimental to reverse

transcription initiation (Lok *et al*). Therefore the A1896 mutant is not common in genotypes with C1858. The G1896A variant is less prevalent in chronic carriers from South Africa because genotype A, which T1858 , is prevalent.

In a South African study, when the precore region of HBV isolated from sera and HCC tumour tissue was analysed, the most common missense mutation was G1862T (Kramvis et al., 1997a, Kramvis et al., 1998b). This mutation was more prevalent in HBeAg-negative than HBeAg positive asymptomatic carriers and HCC patients (Kramvis et al., 1997a, Kramvis et al., 1998b). This mutation results in a valine to phenylalanine mutation in the precore region of HBV causing intracellular retention and impaired secretion of HBe-antigen (Chen *et al*., 2008b).

1.3.2 Basic core promoter mutants

The BCP region encompasses nt 1724 to 1849 (Kramvis and Kew, 1999). The region contains DR1 and *cis*-acting elements that independently direct transcription of pre-C mRNA and pgRNA: The A-T rich regions control transcription of pre-C mRNA and pgRNA. Mutations within this region may severely reduce pgRNA and may dysregulate viral gene expression contributing to disease progression. The most frequently described mutations within this region are the A1762T together with G1764A. This double mutant is accompanied by a reduced level of hepatitis B e antigen (HBeAg) expression (Takahashi *et al*., 1995, Laskus *et al*., 1995). These mutations are often seen in chronic patients or fulminant hepatitis than asymptomatic carriers (Kramvis and Kew, 1999, Baptista *et al*., 1999). They have been isolated from sera, liver and tissue samples from patients with HBV-related HCC. The double mutation has been often seen in genotypes that have C1858 which prelude favours

development of pre-C stop codon mutation G1896A. The A1762T G1764A double mutation together with 1753 mutation may be a marker for advanced liver disease (Kramvis and Kew, 1999). Mutations in the region 1753 -1757 together with the A1762T G1764A have been detected in isolated with HCC and fulminant hepatitis (Kramvis and Kew, 1999).

Other mutaions that occur within the BCP regions include the G1809T C1812T. In the Kozak region, which is upstream of the precore start codon (UAG) , 80 % of Sounth African blacks hobour a double or triple mutation on this region (1809-1812 (Baptista *et al*., 1999). The translational product of the core gene HBeAg antigen requires initation of translation from the UAG upstream of the core gene on the precore messenger RNA. The triple mutaion on this region was found to reduce translational initiation and therefore the reducing HBeAg expresssion, while theh double mutation was found to slightly enhance HBeAg expression (Ahn *et al*., 2003).

1.3.3 Complete Surface gene mutants

The surface gene has a vital role in the life cycle of the hepatitis B virus and infectivity of the virus. The HBsAg protein contains the cysteine-rich "a" determinant (nt 99 to 169) it is this region that is recognised by antibodies that are elicited by active hepatitis B vaccination and by anti-HBs antibody present in hepatitis B immune globulin (Torresi *et al*., 2002). Mutations of the surface gene antigen lead to problems in the antigenicity and immunogenicity of the HBsAg. This results in immune escape mutants and false HBsAg negatives (Iacomi *et al*., 2009).

The most described mutations in the S gene are the immune/ vaccine escape mutants. These are the mutants on the 'a' determinant These vaccine escape mutants can infect vaccinated individuals and are prevalent in countries with high endemic rates of HBV infection and with established HBV vaccination programs (Torresi *et al*., 2002). The most common mutants described is the G145R (Gly→Arg) and Q129R (Gln →Arg) (Szomor *et al*., 2008, Carman *et al*., 1990),Other 'a' determinant mutations that have been reported include P120L (Pro→ Leu), T123N (Thr →Asn), T126 N (Thr→Asn), M133L (Met→Leu), and D144 A/G (Asp→Ala/Glu) (Sheldon *et al*., 2006, Torresi, 2002) .

1.3.4 Polymerase mutants

The polymerase gene is the longest gene of HBV, spanning almost 80% of the genome and overlapping the three other genes (surface, core and x) (Tiollais *et al*., 1985), with the polymerase gene overlapping the envelope gene. Mutations in the catalytic domain of the polymerase gene can affect the amino-acid sequence of the envelope protein (HBsAg) and *vice versa*. since the 'a' determinant overlaps the major catalytic regions of the viral polymerase protein from amino acid 454 to 524 and known as domains A and B (Locarnini, 1998).

Mutations in the polymerase are lethal to the virus because this region encodes for the reverse transcriptase DNA polymerase and RNase H activity, packaging of the pgRNA, which are important activities in the viral life cycle. Mutations in the polymerase region mostly result following the use of nucleoside analogues during therapy.

The most reported analogue resistance is to lamuvudine (LMV), which is a member of a class of antiviral nucleoside analogues that inhibit hepadnavirus replication specifically by inhibiting viral reverse transcriptase by terminating viral DNA synthesis (Allen *et al*., 1998, Iacomi *et al*., 2009). LMV induces resistance mutations in the major catalytic region of the reverse transcriptase known as the YMDD motif (tyrosine-methionine-aspartate-aspartate motif) (Torresi, 2002, Ramos *et al*., 2007). Common LMV resistance mutations in this region include the triple mutation, rtV173L+ rtL180M + rtM204V (*figure 1.13*) (Zoulim and Locarnini, 2009). These mutant viruses are functional and pathogenic but less efficient than wild-type viruses. Because of the overlapping orientation between the polymerase and the HBsAg, the amino acid changes in the HBsAg selected by LMV alter the antigenicity of the HBsAg and therefore the ability of anti-HBs antibody to recognise and bind to the protein (Torresi *et al*., 2002). M204I and L180M/M204I produce I195M and W196S in the HBsAg. Other HBsAg overlapping polymerase region mutations selected by LMV include the mutants E164D and M198I (figure 1.12) (Torresi, 2002) .

1.4 HBV AND HIV CO-INFECTION

1.4.1 Characteristics of co-infection

The co-infection of HBV with HIV is very common due to shared mode of blood-borne transmission (Audsley *et al*., 2010, Thio *et al*., 2002, Alter, 2006). An estimated 4 million out of the 40 million HIV-infected people worldwide are co-infected with HBV (Fix *et al*., 2007) Hepatitis B virus is an important cause of mortality and morbidity among HIV infected persons (Thio, 2003, Bloquel *et al*., 2010). A number of studies from around the world have found that the prevalence of HBV infection among HIV-positive individuals is

significantly higher than in HIV-negative individuals (Burnett *et al*., 2005, Bloquel *et al*., 2010). Higher frequencies of liver disease have been reported amongst the co-infection and exceedingly high incidence rates of serological markers of prior or active HBV infection have been reported in HIV-infected populations and have been estimated to be between 10- 80 % depending on the population being studied and geographic region, (Bloquel *et al*., 2010, Fix *et al*., 2007, Burnett *et al*., 2005), For example, the prevalence of co-infection in injection drug user population is 5 % to 10 %, in South African region, 20 % prevalence has been reported (Soriano *et al*., 2008), 9%, 16.9 %, 25.9 %, and 73 % has been reported in small cohorts the geographical regions: Tanzania, Malawi, Nigeria and Uganda respectively (Peters, 2007).

HIV induced immune suppression have been reported to have serious and harmful effects on the natural history, physiology, diagnosis and therapeutic responses to hepatitis B virus (Kottilil, *et al*, 2005). These include a lower response rate to HBV vaccination, higher chances of developing chronic diseases after HBV exposure, higher HBV DNA levels and more profound liver disease experienced as a result of the chronic HBV infection and the presence of the HBV pre-core mutants tends to present a more aggressive and more prolonged liver disease and more hepatoxicity under antiretroviral treatment regimens. HBV has been associated with increased risk of progression of immunodeficiency progress of HIV infection into AIDS and a premature death (Firnhaber *et al*, 2005, 4).

1.4.2 HBV-HIV co-infection impact on disease progression and treatment

HIV has a significant impact on the natural history of HBV infection. Patients co-infected with HIV and HBV are particularly at risk of hepatitis B reactivation (Peters, 2007, Burnett *et*

al., 2005). In these patients, the mortality rate due to liver related disease is 19-fold higher than those with HIV mono infection and are up to six-fold more likely to develop chronic hepatitis B than are HIV-negative individuals (Thio, 2009).

Cirrhosis is more common in HIV-HBV co-infection in spite of lower ALT levels than in HBV mono-infection. There is some evidence that lower CD4_ T cell counts are associated with increased risk for HCC in HBV-HIV coinfected individuals, (Thio, 2009). HIV infection also decreases the rate of HBeAg clearance up to five-fold and increases the level of HBV replication as manifested by higher HBV DNA levels (Sulkowski, 2008) . In a study done by Colin al, it was found that HIV infection is associated with a higher level of HBV replication and a higher risk of cirrhosis in homosexual men with chronic hepatitis B (Colin *et al*., 1999). Even HIV-infected persons who acquire protective antibody to hepatitis B surface antigen (anti-HBs) remain at risk for loss of anti-HBs and subsequent reactivation latent HBV infection (reverse seroconversion) (Firnhaber and Ive, 2009)

Occult hepatitis B infection is also seen more commonly in HIV-positive individuals, in this case, co-infection may escape diagnosis, especially in resource-limited countries where it is not possible to measure the HBV DNA viral load or use nucleic acid tests, which are more expensive. This may lead to an increased risk of hepatic cirrhosis caused by hepatitis B and of hepatic-related deaths in HIV patient if not treated (Firnhaber and Ive, 2009, Mphahlele *et al*., 2006).

1.4.3 Management and treatment of HBV-HIV co-infection

Co-infection with hepatitis B and HIV can influence antiretroviral treatment and prognosis of both diseases. HIV infection can complicate the diagnosis of hepatitis B because spontaneous reverse seroconversion marked by the disappearance of anti-HBs and reappearance of HBsAg can occur, especially if CD4T cell counts are less than 200 cells/mm, therefore allowing reactivation of HBV infection (Thio, 2009). In a South African study occult HBV infection was found in 22.1% of HIV positive patients. This increased prevalence of occult hepatitis B in HIV-positive patients clearly supports that HIV infection is one of the risk factors for false negatives in serological HBsAg tests (Mphahlele *et al*., 2006).

The guidelines for the South African HIV Comprehensive Care, Management and Treatment (CCMT) programme do not include viral hepatitis studies. Therefore, hepatitis B serological tests are done only if serum amino transferases are elevated in the absence of other known causes (Firnhaber *et al*., 2008). In South Africa, LMV is currently one of the three drugs in the highly active anti-retroviral therapy (HAART) and is active against both the HIV and HBV. LMV has been reported to cause high rate LMV-selected resistant variants and more predominantly in patients infected with HBV genotype A (Iacomi *et al*., 2009).

1.4.4 HBV-HIV co-infection in the Sub-Saharan Africa

HBV infection has long been recognised as highly endemic in Southern Africa including Botswana, South Africa, Zambia and Zimbabwe with many of the studies conducted among black Africans (Botha *et al*., 1984, Dibisceglie *et al*., 1986). Majority of the people have been exposed to HBV horizontally as children and by the time they are exposed to HIV infection for the first time are already protected from HBV infection, or are chronic carriers

of HBV (Burnett *et al*., 2005). HIV in South Africa is a major public health problem with HIV seroprevalence as high as 65% depending on population, geographical location and ethnicity (Kleinschmidt *et al*., 2007).

Most of the research from around the globe conducted in areas of low HBV endemicity found that the prevalence of HBV infection among HIV-positive individuals is significantly higher than in HIV-negative individuals (Horvath and Raffanti, 1994, Bodsworth *et al*., 1991, Hess *et al*., 1989), mainly as a result of shared transmission routes. In South Africa few studies have been conducted and the generated is slightly divergent from that of the world. In study done in an urban government clinic, an HBV-HIV prevalence rate of 5 % was found, determined by the surface antigen serology. A 50 % prevalence of markers for previous exposure to HBV the HIV sero-positive patients was also reported (Firnhaber *et al*., 2008, Firnhaber *et al*., 2009). The alanine aminotransferase (ALT) and aspartate aminotransferase were within the normal range in 84% and 57% of these HBsAg-positive patients and most of the patients had low HBV DNA viral load (Bisceglie A. M. Di *et al*., 2010, Firnhaber *et al*., 2008, Firnhaber *et al*., 2009).

In another study by Mphahlele *et al*, HBsAg antigen was more prevalent in HIV-positive that HIV-negative individuals, with 22.1 % HBsAg negativity in HIV-positive compared to 2.4% of HIV-negative individuals and there indicating that in a South African setting, HIV infection might be a risk factor for occult hepatitis B (Mphahlele *et al*., 2006). Lukhwareni *et al*, investigated the prevalence of HBV in sera from HIV positive South African patients initiating antiretroviral therapy (ART). Sixty three percent of the study patients had evidence of past of HBV infection, with 40.6 % of the patients having detectable HBV DNA, 22.9 % were HBsAg positive and were significantly associated with high HBV Viral loads. Twenty

three percent of the HBsAg negatives had occult infections. This study demonstrated that HIV/AIDS patients initiating ART in South Africa have chronic or acute HBV infections and that HIV is a factor for occult HBV infection in a South African setting (Lukhwareni *et al*., 2009).

1.5 RATIONALE, AIMS AND OBJECTIVES OF THE STUDY

It is evident that the epidemiology, the global distribution of the different genotypes of the HBV and the modes of transmission vary around the world (Kramvis *et al*., 2005b, Shibayama *et al*., 2005, Orito *et al*., 2001) and that data obtained outside Africa cannot be extrapolated to the Southern African context. Many of the areas where HBV is highly endemic overlap with areas having high rates of HIV infection (Idoko *et al*., 2009). Many of the HBV/HIV co-infection studies have been conducted in areas of low endemicity, with very few studies being conducted in regions where both HBV and HIV are endemic.

Therefore it is important that HBV and HIV co-infection is studied locally in order to generate data that are relevant to South Africa, so that clear insight and understanding on the effect of the two viruses on the disease/s can be obtained. Although the genotypes in HIV-negative individuals have been studied extensively in South Africa (Kew et al., 2005, Kramvis et al., 2005b, Kramvis and Kew, 2007a), there is great paucity of data of the genotypes prevailing in HIV-infected individuals.

The main objective of the study was to molecularly characterise HBV isolates from HIV infected South Africans from two distinct locations, namely, Helen Joseph Hospital, an urban

hospital in Johannesburg and Shongwe Hospital, a rural hospital in Mpumlanga Province. The aims of the study include the following:

1. Amplification and molecular characterization of the basic core promoter/precore and complete surface region of HBV
2. Genotyping of HBV
3. Identification of mutations

Chapter 2: MATERIALS AND METHODS

2.1 0VERVIEW AND ORGANISATION OF THE STUDY

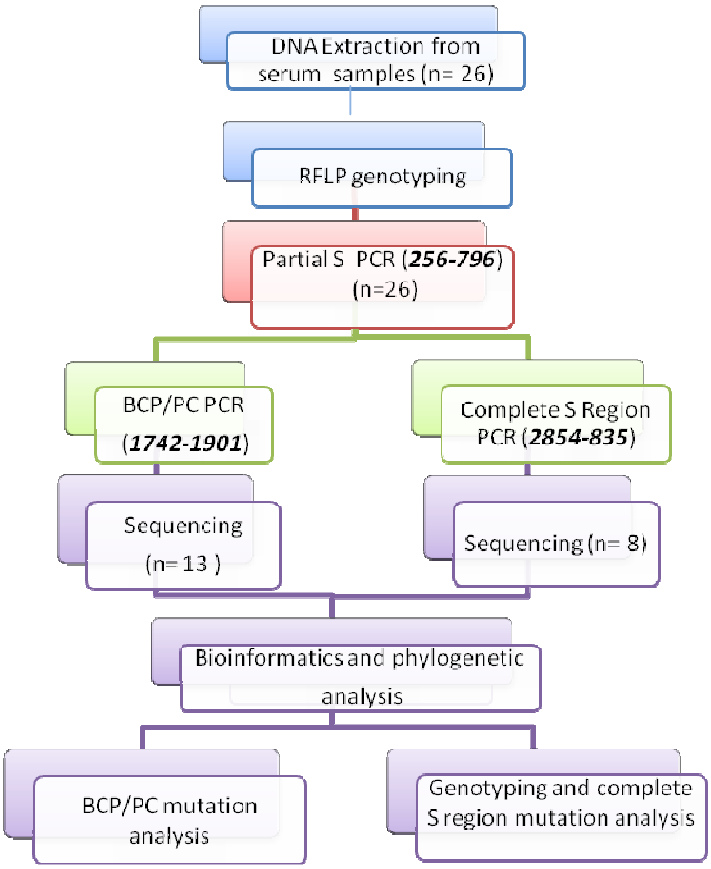


Figure 2.1. An overview of the study participants recruited and the methods used in the Helen Joseph Hospital study. Numbers in bold refer to the region in HBV genome from the *EcoR*1 site.

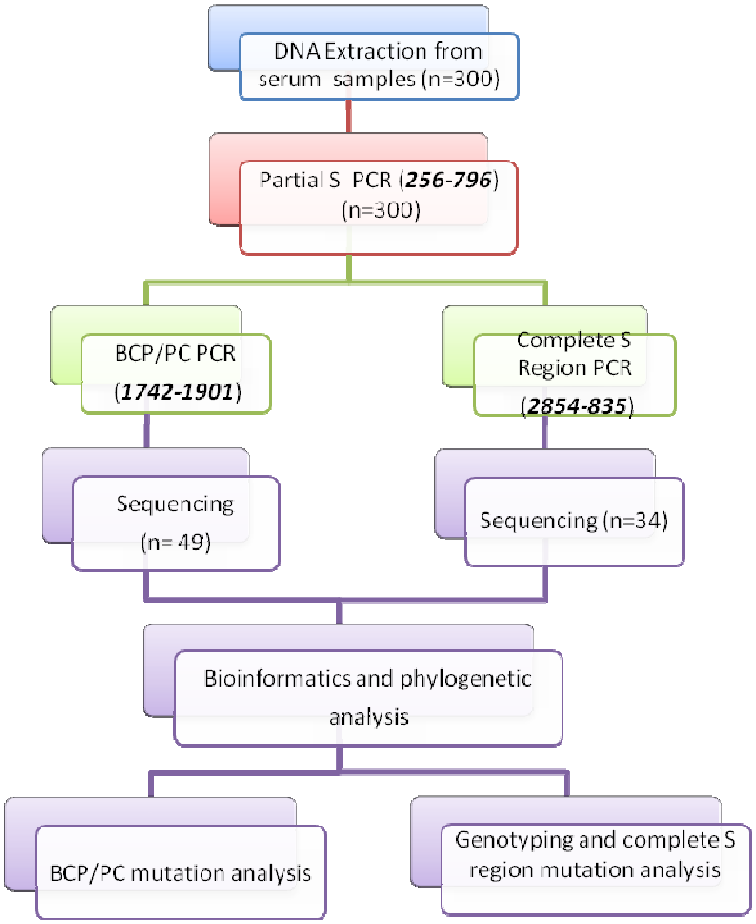


Figure 2.2: Flow diagram of methodology followed in the Shongwe Hospital study. Numbers in bold refer to the region in HBV genome from the *EcoR*1 site.

2.2 STUDY PARTICIPANTS AND SERUM SAMPLES

2.2.1 The Helen Joseph Hospital Urban Cohort

For the urban cohort, serum samples from participants recruited from Helen Joseph Hospital in Johannesburg, South Africa were utilised. Blood samples were obtained with informed

consent from 17 individuals who have been confirmed to be HBV and HIV positive from a previous study (firnhaber *et al*, 2008). Blood was drawn before initiation of HAART (baseline) and at six months after initiation of HAART.

2.2.2 The Shongwe Hospital Rural cohort

For the rural cohort, the samples were collected from participants attending a rural hospital, Shongwe Hospital in Malelane in Mpumalanga Province, East of South Africa. This area is located between the Mozambique and Swaziland Borders. Three hundred HIV positive patients were recruited before initiation of HAART. Of these patients, 114(38 %) were male and 186 (62 %) were female patients with CD4 cell counts less than 200 cells/mm^3. Only patients older than 18 years of age, with signed informed consent were allowed to participate in the study. The study participants were from the surrounding area, as well as from Swaziland and Mozambique.

2.3 ETHICS CLEARANCE

For the Helen Joseph Study, Ethics Clearance certificate was obtained by Dr. C. Firnhaber (Medical: M050620). An umbrella Ethics clearance certificate for the Shongwe Hospital study was obtained By Prof. A Kramvis from the Witwatersrand Human Research Ethics Committee (Medical: M080450). Ethics clearance for this research project was also obtained

from Witwatersrand Human Research Ethics Committee (Medical: M090414) and from the Mpumalanga Provincial Health Research and Ethics Committee. (*appendix C*).

2.4 DNA EXTRACTION

Serum obtained from patients was used to extract HBV DNA using the QIAamp® DNA Blood Mini Kit (QIAGEN, GmbH, Hilden, Germany), as per manufacturer's instructions. This kit allows for the purification of total DNA, free of protein, nucleases and other contaminants or inhibitors, from blood, plasma, serum or other body fluids.

Before starting, the samples and the buffers were equilibrated to room temperature, and a heating block was pre-heated to 56ºC. An aliquot of 200 µl of the serum sample was added to a 1.5 ml Eppendorff tube, together with 20 µl QIAGEN Protease (Proteinase K). 200 µl Buffer AL was then added to the sample and the mixture pulse-vortexed for 15 sec to mix, followed by 10 min incubation at 56ºC. A volume of 200 µl of absolute ethanol was then added to the sample and again mixed by pulse-vortexing for 15 sec. The mixture was then carefully applied to a QIAamp Spin Column and centrifuged at 8 000 rpm for 1 min. The column was placed in a clean collection tube and 500 µl Buffer AW1 added. Centrifugation was again carried out at 8 000 rpm for 1 min and the column placed in a clean collection tube. After addition of 500 µl Buffer AW2, the QIAamp Spin Column was centrifuged at 14 000 rpm for 3 min. To eliminate any chance of Buffer AW2 carryover, an optional centrifugation step was carried out for an extra min at 14 000 rpm. The QIAamp Spin Column was then placed in a sterile 1.5ml Eppendorff tube, 75 µl best quality water (BQW) added directly to

the column and incubated for 5 min at room temperature. The DNA was eluted by centrifugation for 1 min at 8 000 rpm and stored at -20ºC until needed. In addition to the serum sample, a negative control consisting of BQW was included in the extraction procedure to check for contamination.

2.5 POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION

PCR is a highly specific and sensitive method designed to amplify large quantities of a DNA sequence without the use of cloning. The basic three steps of PCR involve a denaturation step in which double stranded DNA is denatured to single stranded DNA by heating to 94ºC, an annealing step in which the primers anneal to either side of the target sequence via their complementary bases and lastly, the extension of the target sequence by a DNA polymerase. Each of the aforementioned steps occurs at different temperatures in a thermal-cycler, resulting in an exponential increase in the target DNA.

Table 2.1: Primer sequences used for the PCR.

Region Amplified	Primer Name	Primer Sequence *	Nucleotide Position in HBV Genome*
Complete S-region	S1F	5'TCA ATC GCC GCG TCG CAG AAG ATC TCA ATC 3'	nt 2410-2439
	S1R	5'TCC AGA CCX GCT GCG AGC AAA ACA 3'	nt 1314-1291
	S2F	5'AAT GTT AGT ATT CCT TGG ACT CAT AAG GTG GG 3'	nt 245–2482
	S2R	5'AGT TCC GCA GTA TGG ATC GGC AGA GGA 3'	nt 1254 -1286
P7/p8	230F	5'TCA CAA TAC CGC AGA GTC T 3'	nt 230 -249
	800R	5'AAC AGC GGT ATA AAG GGA CT 3'	nt 800-820
	P7	5' GTG GTG GAC TTC TCT CAA TTT TC 3'	nt 25 -278
	P8	5' CGG TAT AAA GGG ACT CAC GAT 3'	nt 796-776
BCP	BCP1F	5' GCA TGG AGA CCA CCG TGA AC 3'	nt 1606-1625
	BCP1R	5' GGA AAG AAG TCA GAA GGC CAA 3'	nt 1955-1974
	BCP2F	5' CTA AAG AGG ACT CTT GGA CT 3'	nt 1653-1672
	BCP2R	5' GGC AAA AAA AGA GTA ACT A 3'	nt 1940-1959

* Nucleotide position of HBV *adw* genome (GenBank accession number (AY233276), where position 1 is the *Eco*RI cleavage site.

2.5.1 Partial S region PCR

A nested PCR was carried out to amplify a small region of the HBsAg of HBV(256 to 796 from the *EcoR*1 site). This PCR was carried out on the MyCycler thermal cycler™ PCR machine (BioRad, USA). The first round PCR consisted of 22.5 µl of reaction mixture containing 15.4 µl BQW, 2.5 µl 10X NH_4 Buffer, 1.5 µl 25 mM MgCl2, 0.5 µl 40mM dNTP's (10 mM each dNTP), 1.25 µl each of the outer primers, 230F and 800R, respectively (*Table 2.1*), and 0.1 µl Promega Taq DNA Polymerase (Promega, Madison, WI) in a 0.2 ml PCR tube. To this mixture, 2.5 µl of DNA was added to bring the total reaction mixture to 25 µl.

PCR cycling for the first round involved 40 cycles of denaturation at 94ºC for 60 sec, annealing at 55 ºC for 1 min and extension at 72ºC for 2 min. An aliquot of 5 µl of the above reaction was then added to a second round mixture containing; 32.3 µl BQW, 5 µl 5.0 NH_4 µl, 1.5 µl, 25 mM MgCl2, 1 µl 40 mM dNTP's (10 mM each dNTP), 2.5 µl each of the inner primers P7 and P8, respectively (*Table 2.1*), and 0.2 µl Promega Taq DNA Polymerase (Promega, Madison, WI) in a 0.2 ml PCR tube. The second round cycling profile consisted of 40 cycles of the same cycling conditions as the first round.

2.5.2 Basic Core Promoter (BCP)/precore PCR

A nested PCR was carried out to amplify the basic core promoter/ precore region of HBV. This PCR was carried out on the MyCycler thermal cycler™ PCR machine (BioRad,

USA)First round PCR consisted of 22.5 µl of a reaction mixture containing 15.4 µl BQW, 2.5 µl 10X NH_4 buffer, 1.5 µl 25 mM MgCl2, 0.5 µl 40mM dNTP's (10 mM each dNTP), 1.25 µl each of the outer primers, BCP1F and BCP1R, respectively (*Table 2.1*), and 0.1 µl Promega *Taq* DNA Polymerase (Promega, Madison, WI) in a 0.2 ml Eppendorff tube. To this mixture, 2.5 µl of DNA was added to bring the total reaction mixture to 25 µl.

PCR cycling for the first round involved 40 cycles of denaturation at 94ºC for 60 sec, annealing at 55 ºC for 1 min and extension at 72ºC for 2 min. An aliquot of 5 µl of the above reaction was then added to a second round mixture containing; 32.3 µl BQW, 5 µl 5.0 NH_4 buffer µl, 1.5 µl, 25 mM MgCl2, 1 µl 40 mM dNTP's (10 mM each dNTP), 2.5 µl each of the inner primers BCP2F and BCP2R, respectively (*Table 2.1*), and 0.2 µl Promega Taq DNA Polymerase (Promega, Madison, WI) in a 0.2 ml PCR tube. The second round cycling profile consisted of 40 cycles of the same cycling conditions as the first round.

2.5.3 Complete S-region PCR

A nested PCR was carried out to amplify the entire S region of HBV (preS1/S2/S region). First round PCR consisted of 22.5 µl of a reaction mixture containing 9.25 µl BQW, 12.5 µl Qiagen Hot Start master mix, outer primers, S1F and S1R, respectively (*Table 2.1*), in a 0.2 ml Eppendorff tube. To this mixture, 2.5 µl of the DNA was added to bring the total reaction mixture to 25 µl

PCR cycling for the first round involved 40 cycles of denaturation at 94ºC for 60 sec, annealing at 65ºC for 1 mins and extension at 72ºC for 3 min. 5 µl of the above reaction was

then added to a second round mixture containing; 18.5 µl BQW, 25 µl Qiagen master mix 0.75 µl each of the inner primers S2F and 1S2R, respectively (*Table 2.1*), in a 0.2 ml Eppendorff tube. The second round cycling profile consisted of 40 cycles of denaturation at 94ºC for 60 sec, annealing at 66ºC for 1 min and extension at 72ºC for 3 mins.

2.6 DETECTION AND CONFIRMATION OF AMPLIFIED PCR PRODUCT

Following PCR, the resulting DNA products were separated by size via electrophoresis on a 1% agarose gel containing ethidium bromide (*Appendix A*). Sizes of PCR products were estimated relative to the migration patterns of a 100 bp DNA ladder (Promega, Madison, WI, USA) for the BCP/ PC and P7P8 PCR product and a 1 kb DNA ladder (Promega, Madison, WI, USA) for the complete surface gene PCR products. Products were visualized by placing the gel under ultraviolet light. Images were captured using the GelDoc system (BioRad, US

2.7 GEL PURIFICATION OF PCR PRODUCT

For some of the complete surface gene amplifications, the PCR products were gel purified before being sent for sequencing. The PCR amplicons were electrophoresed on a 0.8% agarose gel without ethidium bromide (*Appendix A*) together with a 1 kb molecular weight marker (Promega, Madison, WI, USA). An aliquot of 40 µl of the PCR product was loaded onto the gel with 10 µl Loading Buffer containing GelRed^{TM} nucleic acid gel stain (Biotium,

Hayward, CA, USA) and allowed to run at ~60 volts for 1½ to 2 hours, or once the crystal violet in the gel had run about a quarter of the way down the gel.

To excise the PCR product, the buffer was poured off and the gel placed on a fluorescent light box to help visualize the fragments. Using a new sterile surgical blade, the band was carefully excised from the gel and placed into a sterile 1.5 ml Eppendorff tube. To purify the DNA from the agarose gel, the Zymoclean Gel Recovery Kit™ (Zymoresearch, CA, U.S.A.).was used The volume of the agarose pieces was estimated by weighing the gel (1mg ~ 1µl), and 3 times this volume of ADB buffer was added. By periodically shaking the tube vigorously and incubating at 55ºC on a hot block, the agarose was completely dissolved in 5-10 mins. The melted agarose solution was then added to into a Zymo-Spin Column and placed onto a collection tube. This was centrifuged for 30 sec and 200 ul of wash buffer was added of wash buffer was added to the column then spun for 30 sec. After repeating the wash step once, the Zymo-Spin column was placed on to a clean 1.5 ml eppindorf tube and 10 µl of water was added on the column matrix and spun down to elute the DNA.

2.8 LINDH GENOTYPING

Lindh genotyping is a method for genotyping HBV, making use of restriction fragment length polymorphism (RFLP), using the restriction enzymes *Tsp509*I which recognizes AATT and *Hinf*I which recognizes GANTC. Following amplification of the HBV DNA with a nested PCR using primers 230F and 800R for the first round PCR and second round primers P7 and P8 were used to amplify nucleotides 256 to 796 of the S region, The amplicons were then restricted using restriction enzymes *Hinf*I and *Tsp*509I, in separate reactions, to give the

characteristic restriction fragment length polymorphism (RFLP) patterns for the different genotypes (Lindh *et al*, 1997). This genotyping method can be used to discriminate genotypes A to H.

2.8.1 RFLP assay for Genotyping

A master mix was prepared for each of the enzymes, *Hinf* I (Promega, Madison, WI) and *Tsp* 509I (New England Biolabs, Beverly, MA), used for genotyping (*Table 2.2)*

Table 2.2: Master mixes for genotyping HBV p7/p8 PCR products

Stock Reagent	***HinfI***	***TSP509*I**
BQW	2.5 µl	2.5 µl
10X Buffer	2 µl	2 µl
Restriction Enzyme	0.5 µl	0.5 µl
Final volume	5 µl	5 µl

A volume of 5 µl of the each of the master mixes was added to two separate 0.5 ml Eppendorff tubes, followed by 15 µl of the PCR product. A drop of sterile mineral oil was added to each tube to prevent evaporation of the product. Both reactions were incubated for 3 hours; *Hinf* I at 37ºC and *Tsp509*I at 65ºC. After incubation, the reaction was terminated by

the addition of 4 µl loading dye. 10 µl of the sample was then run overnight on a 3% ethidium bromide-containing composite agarose gel (*Appendix A*) at ~30V, alongside 100 bp and 1 kb molecular weight markers (Promega, Madison, WI, USA). The resulting restricted fragments were visualized under UV light and images captured using the GelDoc system (BioRad, USA)

2.9 SEQUENCING

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 performed by the Central Analytical Facility (Marrone *et al.*), Stellenbosch University, South Africa, using the ABI 3130XL Genetic analyser (Applied Biosystems, Foster City, CA). Specific sequencing primers were used. BCP sequences were analysed in both the forward and reverse directions, whilst the complete S sequences were analysed in 3 overlapping fragments in forward direction only.

Table 2.3. Primers used for sequencing of the BCP/PC region and the complete S region.

Region	Primer Name	Sequence	Nucleotide Position in HBV Genome*
BCP/PC	BCP1	5'-GAG GCA TAC TTC AAA GAC TG-3'	nt 1698-1717
	BCP2	5'-AGT AAC TCC ACA GTA GCT CC-3'	nt 1928-1947
Complete S	2497F	5'-TTC CTT GGA CTC ATA AGG TG-3'	nt 2461 – 2480
	3188F	5'-AG TCA GGA AGG CAG CCT AC-3'	nt 3152 – 3170
	591F	5'-ATT GCA CCT GTA TTC CCA TCC-3'	nt 591 – 611

*From the *EcoR*I site

2.10 BIOINFORMATICS AND PHYLOGENETIC ANALYSIS

Both complete surface and the precore/core DNA sequences were assembled and aligned manually using GeneDoc (http://evolution.genetics.Washington.edu, 1995) The sequences were compared with corresponding African Genotype A sequences of HBV from GenBank. Multiple sequence alignments and nucleotide divergence calculations were carried out using Dambe (Xia, 2000). The alignments were fed into PHYLIP (Phylogeny inference package) version 3.5c (Page, 1996) DNAML (maximum likelihood) alone and DNADIST consecutively with NEIGHBOR (neighbour-joining) were used to generate dendograms. SEQBOOT, DNADIST and NEIGBOR were be used for bootstrapping of 1 000 data sets. CONSENSE were used to compute a consensus tree. TreeView Win 32 version 1.6.1 software program were to visualize the trees (Page, 1996).

Chapter 3: RESULTS

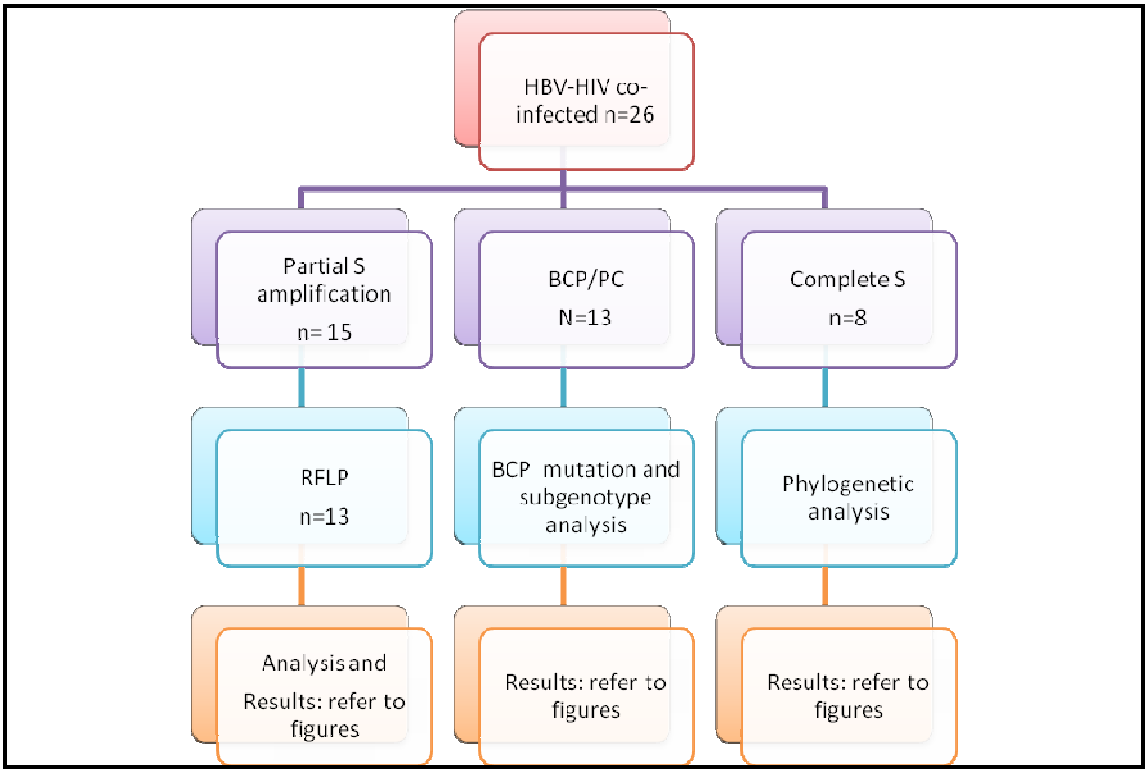


Figure 3.1A: Summary of the analysis of BCP/PC and complete surface open reading frame from Helen Joseph Hospital (HJH) pilot study.

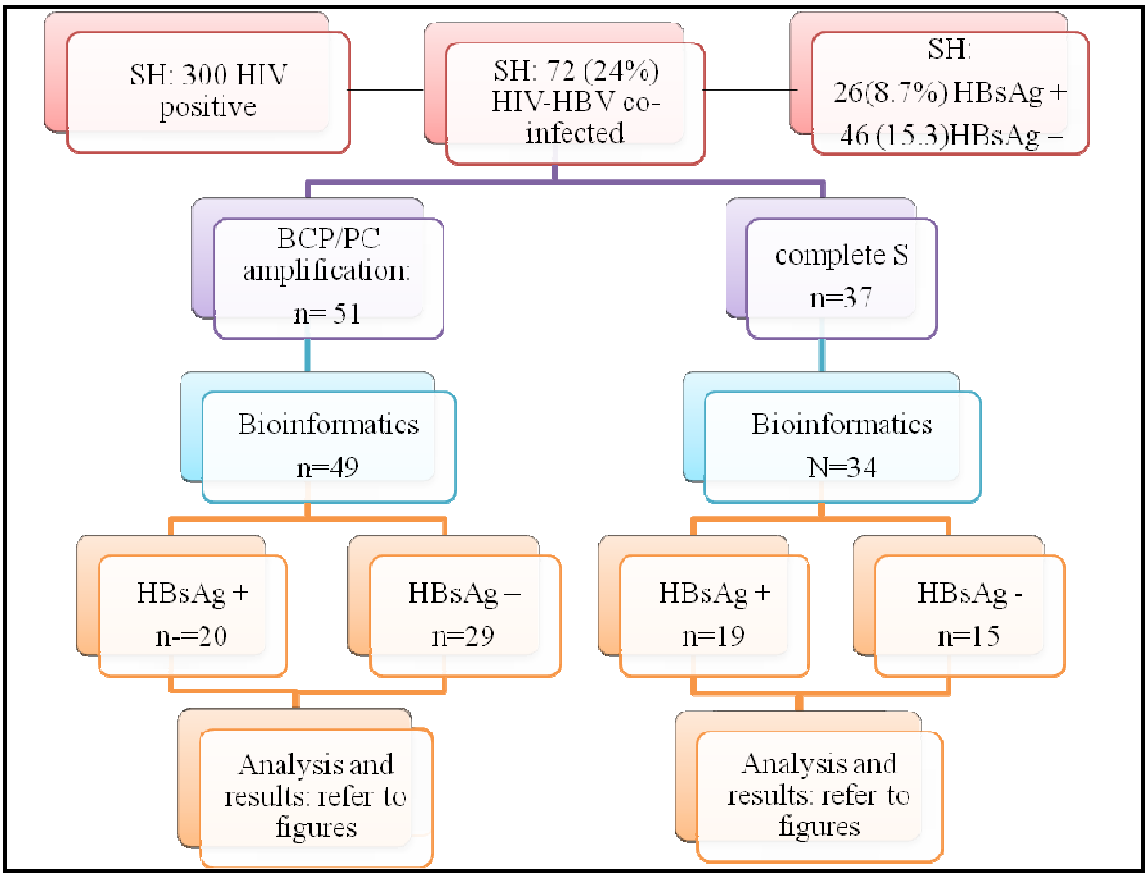


Figure 3.1B: Summary of the analysis of BCP/PC and complete surface open reading frame from Shongwe Hospital (SH) isolates.

3.1 DETECTION OF AMPLIFIED PRODUCTS

3.1.1 Partial S region PCR

The partial surface gene (256 to 796 from the *Eco*RI site) was successfully amplified by nested PCR. After amplifying the HBV DNA, a PCR product of 541 bp was visualized (*figure 3.2).*

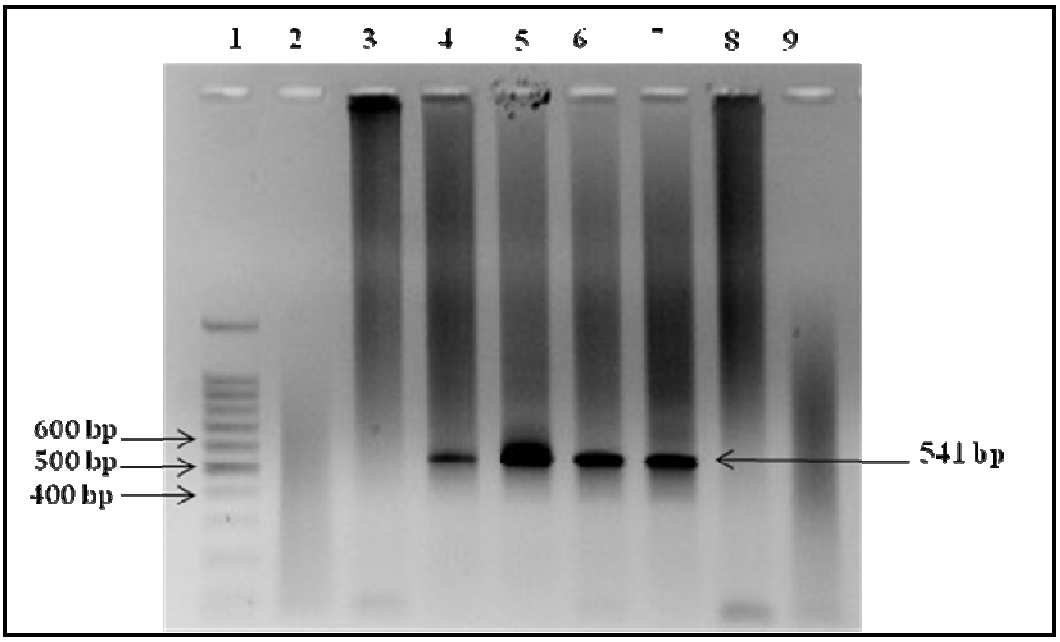


Figure 3.2: Detection of the amplified partial surface gene, (256 to 796 from the *EcoR*I site), visualised as 541 bp PCR product on an ethidium bromides-stained 1 % agarose gel (inverted image).

Lane 1 = 100 bp molecular weight (Promega)

Lanes 2 and 9 = second round PCR water controls

Lanes 3 and 8= first round PCR water controls

Lanes 4-7= amplified partial surface gene.

3.1.2 BCP/PC amplicons

The basic core promoter/precore region of HBV was successfully amplified from DNA extracted from HIV co-infected patients, shown below as 307 bp bands (*figure 3.3*).

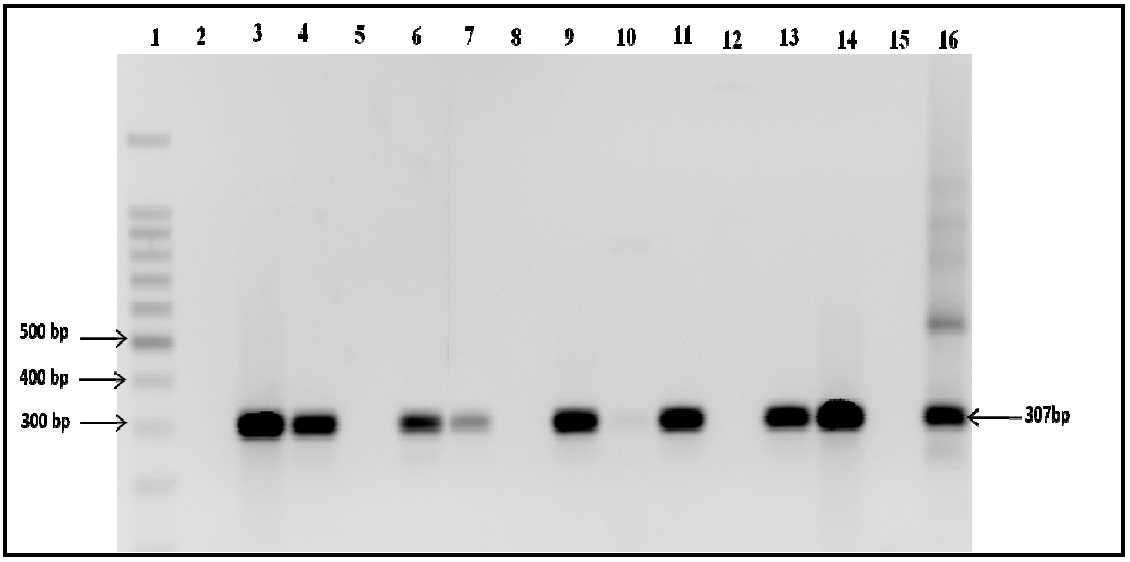


Figure 3.3: Detection of the amplified partial BCP/PC region of the HBV genome (1742-1901 from the *EcoR*I site) visualised as 307 bp PCR product on an ethidium bromides-stained 1 % agarose gel (inverted image).

Lanes = 100 bp molecular weight marker (Promega)

Lanes 2 and 15 = PCR water control

Lanes 5, 8, 10 and 12 = unsuccessful amplification of the BCP/PC region (no DNA bands)

Lanes 3-4, 6-7,9, 11, 13-14 = amplified PCR product

Lane 16 = PCR positive control

3.1.3 Complete surface gene amplicons

The complete surface gene amplification was successful for most serum samples with high HBV viral loads. Successful amplification was shown by detection of a ~2.1 kb DNA band (*figure 3.4*).

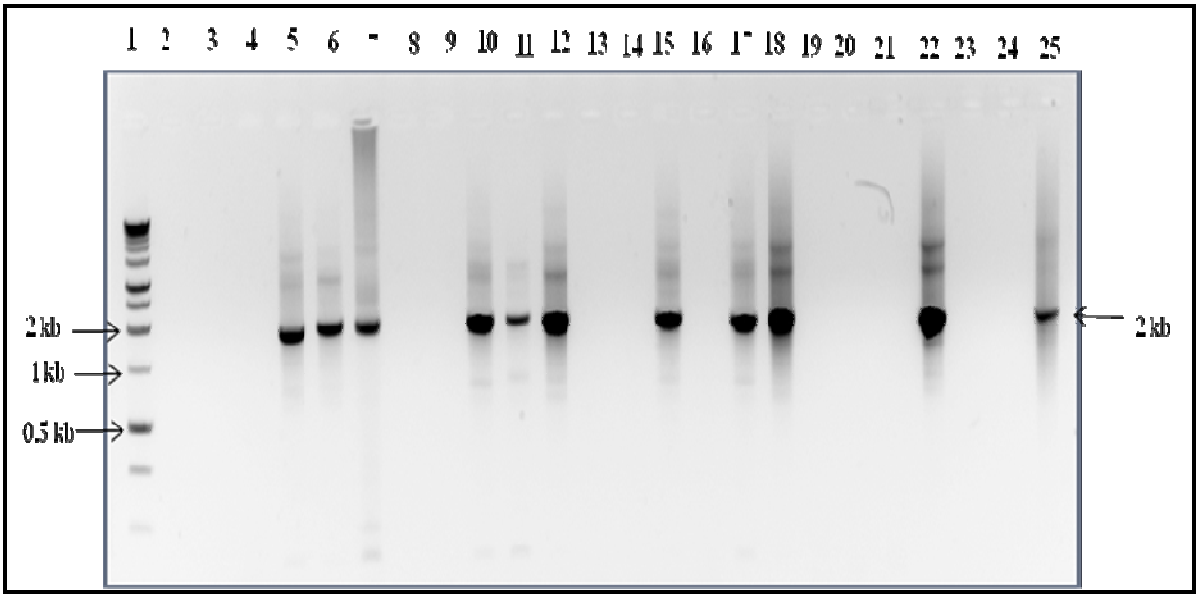


Figure 3.4: Visualisation of HBV complete surface gene amplicon (2854-835 from the *EcoR*I site) (inverted image).

Lane 1 = 1 kb molecular weight

Lanes 2, 24 = BQW control from second round PCR

Lanes 3, 23 = BQW control from first round PCR round PCR

Lanes 4, 8-9, 13-14, 16, 19-21= unsuccessful amplification of the complete surface antigen

Lanes 5-7, 10-12, 15, 17-18, 22 = successfully amplified complete surface gene from HIV co-infected samples.

Lane 25 = PCR Positive control.

3.2 GENOTYPING BY A RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) ASSAY

Genotyping of HBV PCR amplicons from section 3.1.1 (*figure 3.2*) was performed on the Helen Joseph Hospital samples only. HBV isolated from baseline (prior to HAART

initiation) and at six months follow up (after initiation of HAART) were genotyped. This method of genotyping relies on the amplification of a region of the partial S gene of HBV, followed by the digestion of the amplicons by restriction enzymes and RFLP analysis. Nucleotides 256 to 796 of the surface gene of HBV were successfully amplified from DNA extracted from serum samples and detected by the presence of a 541 bp band on a 1% agarose gel (*figure 3.2*).

The PCR products were then digested using restriction enzymes *Hinf* I and *Tsp* 509I, in separate reactions. The RFLP pattern for six amplicons at baseline and five at six months follow up corresponded to that for genotype A: 274 bp, 252 bp and 15 bp fragments for *Hinf* I and 207 bp, 126 bp, 109 bp, 47 bp and 16 bp fragments for *Tsp 509*I (Lindh *et al.*, 1997) (*Figure 3.5*), Fragments smaller than 40 bp could not be visualized properly on the agarose gel because of their small size. Six serum samples at baseline and five out of six followed up six months after initiation of HAART were infected with genotype A. One isolate could not be amplified at six months follow up because of the decrease in HBV DNA viral load. According to the Lindh genotyping method , one isolate (HJH20B) at six month follow up had RLFP pattern corresponding to that of genotype D: 526 bp and 15 bp for *Hinf*I restriction and 173 bp, 164 bp and 109 bp for the *Tsp 509*I restriction (Lindh *et al*., 1997) (*figure 3.5,* lanes H4 T4). Isolate (HJH01A) had an unusual RLFR pattern and therefore the genotype could not be determined using this method.

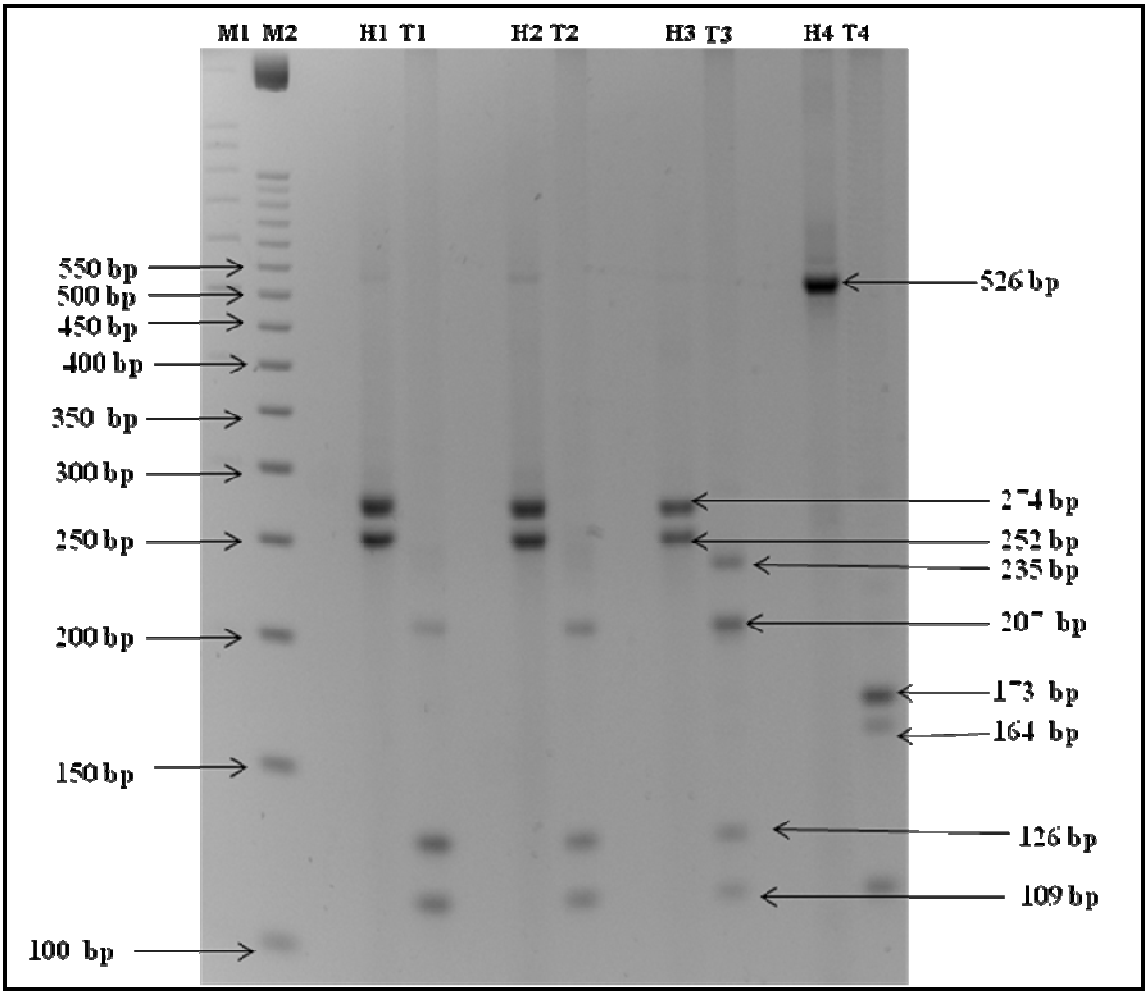


Figure 3.5: Visualisation of RFLP analysis of partial S-region amplified HBV clones on a 3% composite gel,(inverted image).

Lane M1 = 100 bp molecular weight marker

Lane M2 = 50 bp molecular weight marker

Lanes H1, H2, H3, H4 = *Hinf* I restricted PCR product

Lanes T1, T2, T3, T4 = *Tsp 509*I restricted PCR products

3.3 DNA SEQUENCING ANALYSIS

The PCR products of both the BCP/PC and the complete surface gene were sent for direct sequencing at the Central Analytical Facility (Marrone *et al.*), Stellenbosch University. The generated chromas (*figure 3.6)* and fasta format DNA sequences were manually edited and analysed for mutations and used for phylogenetic analysis. Two isolates were found to have mixed genotype sequences. One showed double peaks on the chromatograms (*figure 3.7)* and the other had the BCP/PC sequence with characteristics of genotype A whilst the complete S region had characteristics of genotype D and double peaks on part of the S region sequence.

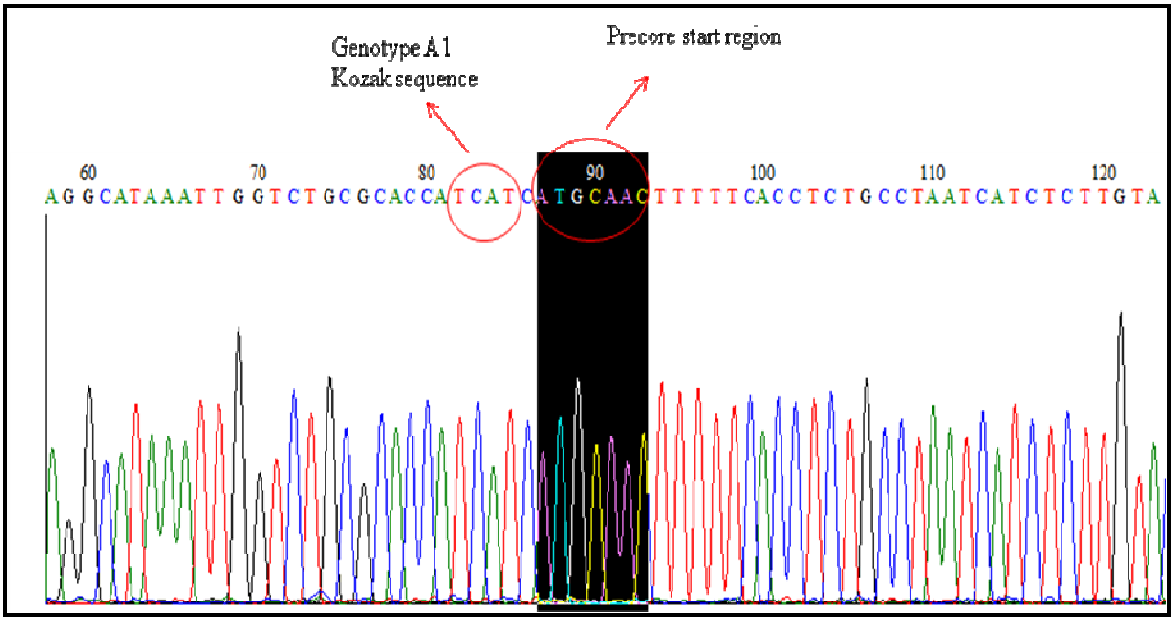


Figure 3.6: Chromatogram acquired after automated sequencing of the BCP/PC gene of the HBV genome from an HBV-HIV co-infected patient, showing the Kozak sequence (1809-1812 from the *EcoR1* cleavage site) preceding the precore start codon of an HBV isolate belonging to subgenotype A1.

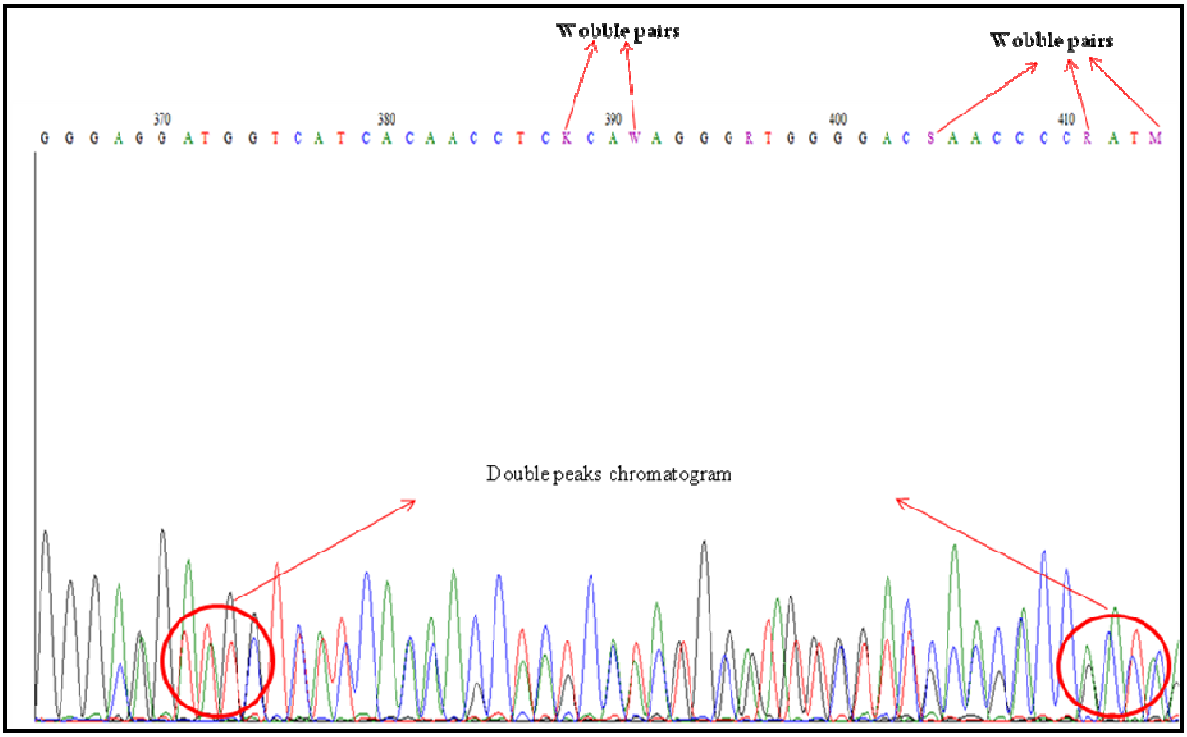


Figure 3.7: Chromatogram generated following direct sequencing of the complete surface gene of isolate number S027A. This shows double peaks indicating detection of mixed genotypes/ quasispecies of HBV isolated from a single serum sample.

3.4 BCP/PC MUTATION ANALYSIS

3.4.1 Helen Joseph Hospital cohort

HBV DNA from confirmed co-infected patients from the Helen Joseph cohort was extracted from 26 samples from baseline and at six month after initiation of HAART. Thirteen BCP/PC DNA sequences were analysed for mutations at 10 loci in the region 1750 to 1900 from the *Eco*RI site as described by Kramvis *et al*, 2008. Eight sequences prior to HAART initiation and five sequences at six months follow-up were analysed (*figure 3.8)*. Five sequences of the 13 had wild type BCP sequences. Four isolates: HJH01A, HJH03A, HJH10A and HJH01B

had the A1762T G1764A mutation, with HJH10A having in addition T1753C, known to down regulate HBeAg expression and a HCC marker (Kramvis and Kew, 1999). Isolate HJH05A had the A1762T mutation alone with G1888T. Two isolates (HJH11B and HJH20B had the GCAC at the Kozak sequence (1809-1812) preceding the precore start codon together with C1858T and A1888G, characteristic of isolates belonging to genotype D/E (HJH11B and HJH20B, highlighted in pink in *figure 3.8*). All the sequences showed wild type sequences at 1802-1803, 1814-1816, 1817-1819, 1862, 1896 and 1899.

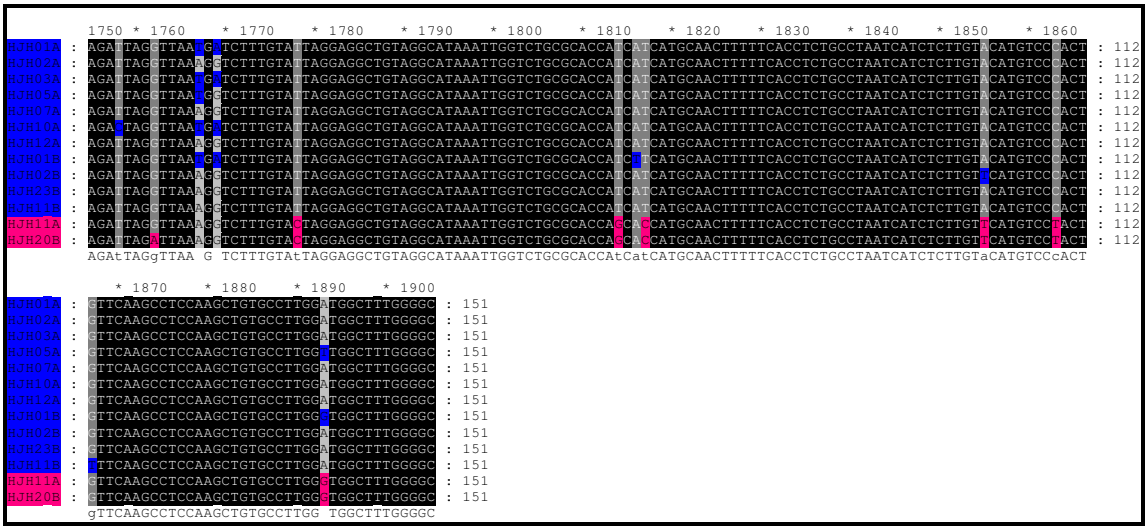


Figure 3.8: BCP/PC region analysis of the Helen Joseph Hospital cohort. A baseline isolates and **B** represents isolates from six month follow up after initiation. Highlighted regions indicate regions with mutations. Blue shows genotype A isolates and pink indicates genotype D or E isolates. Position 1 corresponds to *Eco*RI cleavage site of HBV genome.

3.4.1 The Shongwe Hospital Rural cohort

Table 3.1. BCP/PC region analysis of the Shongwe Hospital Rural cohort

Samples	subgenotype	1753	1762	1764	1802-1803	1809-1812	1814-1816	1817-1819	1858	1862	1888	1896	1899
S121A	A1	T	A	G	CG	TCAT	ATG	CAA	C	G	A	G	G
S159A	A1	T	A	G	CG	TCAT	ATG	CAA	C	G	A	G	G
S253A	A1	T	A	G	CG	TCCT	ATG	CAA	C	G	A	G	G
S255A	A1	T	A	G	CG	TCAT	ATG	CAA	C	G	A	G	G
S274A	A1	T	T	A	CG	TCAT	ACG	CAA	C	G	G	G	G
S001A	A1	T	A	G	CG	TCAT	CTG	CAA	C	G	G	G	G
S009A	A1	T	T	A	CG	TCAT	ATG	CAA	C	G	A	G	G
S016A	D1/E	T	A	G	CG	GCAC	ATG	CAA	T	G	G	A	G
S022A	A1	T	A	G	CG	TCAT	ATG	CAA	C	G	A	G	G
S042A	D1/E	T	A	G	CG	GCAC	ATG	CAA	T	G	G	A	G
S048A	A1	T	A	G	CG	TCAT	TTG	CAA	C	G	A	G	G
S070A	A1	T	A	G	CG	TCAT	CTG	CAA	C	G	T	G	G
S100A	A1	T	A	G	CG	TCAT	ATG	CAA	C	G	A	G	G
S109A	A1	T	A	G	CG	TCAT	ATG	CAA	C	G	A	G	G
S126A	A1	T	A	G	CG	TCAT	ATG	CAA	C	G	A	G	G
S148A	A1	C	T	A	CG	TCAC	AAG	CAA	C	G	G	G	G
S167A	A1	T	A	G	CG	TACT	ATG	CAA	T	G	A	A	A
S180A	A1	T	T	A	CG	TTCT	ATG	CAA	C	G	G	G	A
S256A	A1	T	A	G	CG	TCAT	ATG	CAA	C	G	A	G	G
S300A	A1	T	A	G	CG	ACAT	ACG	CAA	C	G	G	G	A
S027A	A2	T	A	G	CG	TCAC	ATG	CAA	T	G	G	A	G
S029A	A1	T	A	G	CG	TCAT	ATG	CAA	C	G	A	G	G
S032A	A2	T	A	G	CG	GCAC	ATG	CAA	C	G	G	A	G
S039A	A1	T	A	G	CG	TCAT	ATG	CAA	C	G	A	G	G
S044A	A1	T	A	G	CG	TCAT	ATG	CAA	C	T	A	G	G
S053A	D1/E	T	A	G	CG	GCAC	ATG	CAA	T	T	G	G	G
S060A	A2	T	A	G	CG	p	ATG	CAA	C	G	G	G	G
S061A	A1	C	T	A	CG	TCTT	ATG	CAA	C	G	T	G	G
S083A	A1	T	A	G	CG	TCAT	ATG	CAA	C	G	A	G	G
S094A	A2	C	A	G	CG	ACAC	ATG	CAA	C	G	G	G	G
S107A	A1	T	A	G	CG	TCAT	ATG	CAA	C	G	A	G	G
S110A	A1	T	A	G	CG	TCAT	CTG	CAA	C	G	A	G	G
S123A	A1	T	A	G	CG	TCAT	ATG	CAA	C	G	A	G	G
S128A	A1	T	A	G	CG	TCAT	ATG	CAA	C	G	A	G	G
S131A	A1	T	A	G	CG	TCAT	ATG	CAA	C	G	A	G	G
S132A	A1	T	A	G	CG	TCAT	ATG	CAA	C	G	A	G	G
S130A	A1	T	A	G	CG	TCAT	CTG	CAA	C	G	C	G	G
S162A	A1	T	A	G	CG	TCAT	ATG	CAA	C	G	G	G	G
S173A	A1	T	A	G	CG	TCAT	ATG	CAA	C	T	A	G	G
S184A	A1	C	T	A	CG	TCTT	ATG	CAA	C	G	T	G	G
S187A	A1	T	A	G	CG	TCAT	ATG	CAA	C	T	A	G	G
S193A	A1	T	A	G	CG	TCTT	ATG	CAA	C	G	A	G	G
S217A	A2	T	A	G	CG	GCAC	ATG	CAA	C	G	G	G	G
S219A	A1	T	A	G	CG	TCAT	ATG	CAA	C	T	A	G	G
S221A	A2	C	T	A	CG	GCAC	CTG	CGA	C	T	A	G	G
S240A	A1	T	A	A	CG	TCCT	ATG	CAA	C	G	A	G	G
S246A	A1	T	A	G	CG	TCAT	ATG	CAA	C	T	A	G	G
S249A	A2	T	A	G	CG	GCAC	ATG	CAA	C	G	G	G	G
S264A	A1	C	T	A	CG	TCAT	ATG	CAA	C	G	G	G	G

HBeAg positie
HBV active infection
HBV occult infection

Out of 300 HIV infected individuals 72 were found to be co-infected with HBV using detection of HBV DNA in at least two regions of the HBV genome. The BCP/precore region of 51 of these samples was successfully amplified by nested PCR and sent for sequencing. Forty nine DNA sequences were generated, assembled, aligned and analysed by GeneDoc software. From the sequence analysis of the 49 BCP/PC sequences it subgenotypes could be deduced according to Kramivis *et al*, 2008. Three isolates (SH16A, SH42A and SH53A) had HBV subgenotype D/E with Kozak sequence GCAC at nt 1809-1812, 1888G and 1858T. Seven isolates (SH27A, SH32A, SH60A, SG94A, SH217A, SH221A and SH249A) were subgenotype A2 with BCP/PC characteristic Kozak sequence GCAC at 1809-1812, 1888G and 1858C. Thirty nine sequences found to be subgenotype A1 with Kozak sequence TCAT (or mutant) at nt 1809-1812, 1888A and 1858C/T (*figure 3.9*) .

Of the 49 cases, 5 were HBeAg-positive and the remaining 41 HBeAg-negative. Four (S121A, S159A, S253A, S255A) of the five isolates from HBeAg positive cases did not show any mutations in the BCP/precore region, which can abolish or down regulate the expression of the HBeAg. They had wild type sequences at positions 1762A/ 1764G, 1809-1812 (TCAT Kozak), 1862G and 1888A relative to the consensus for subgenotype A1 of HBV.

The 44 BCP/precore sequences derived from HBeAg negative individuals were analysed for mutations, which are known to abolish the synthesis of HBeAg or down regulate its expression. This may be at transcriptional, translational, or at post translational level. mutations A1762T G1764A known to down regulate HBeAg expression at transcriptional level occurred in eight isolates (S274A, S009A, S148A, S180A, S061A, S184A, S221A and S264A), with five cases occurring together with the T1753C and two belonging to

subgenotype A2. The Kozak mutations affecting the expression of HBeAg at translational level were found in nine isolates, with two occurring together with the precore initiation codon (1814-1816). Four (S027A, S94A, S148A and S300A) had double mutation and five isolates (S061A, S180A, S184A, S194A and S240A) had triple mutation. In addition, seven isolates had the precore initiation codon, which completely abolishes HBeAg expression at translational level. The G1896A mutation occurred in five isolates and in four cases it occurred together with the C1858T mutation.

The G1862T, which interferes with post-translational modification HBeAg-precursor and abolishes HBeAg expression occurred in seven HBsAg negative isolates and none in the HBsAg positive isolates ($p<0.05$) (*figure 3.10).* Position 1888 had a single nucleotide substitution mutation in 20 cases, with 3 having the unusual G to T mutation and two cases having in addition the G1899A mutation. All sequences had wild type sequences at positions 1802-1803 and 1817-1819, Only 13 isolates out of the 49 BCP/PC sequences had wild type sequences at region 1750-1900.

Of the 39 samples belonging to subgenotype A1, for which the BCP/precore region was successfully sequenced, 20 were from HBsAg-positive and from 21 HBsAg-negative sera. The frequency of the various BCP/precore mutations was compared between the two groups. There was no difference in the frequency of the mutations except for G1862T, which occurred more frequently in the HBsAg-negative (figure 3.9, Table.3.1).

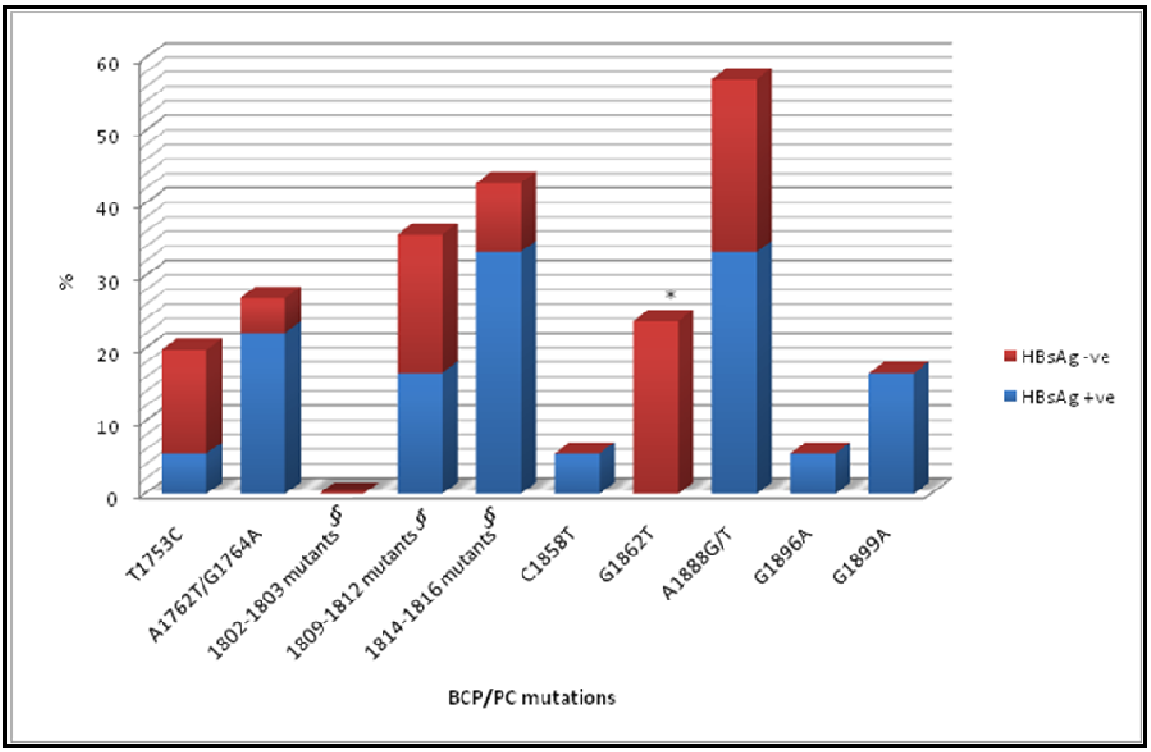


Figure 3.9: Mutations present in the BCP/PC region of the HBV subgenotype A1.

***** G1862T statistically significant (P< 0.05).

§ Wild type: 1802-1803 (GC); 1809-1912 (TCAT); 1814-1816 (ATG).

3.5 PHYLOGENETIC ANALYSIS

DNADIST was used consecutively with NEIGHBOR (neighbour-joining) to determine the genotypes of all the isolates from both Helen Joseph and Shongwe Hospital, dendograms generated using all complete surface gene sequences together with representative sequences derived from Genbank belonging to genotypes A-I. The trees were then visualized using Tree View Win 32 software program (*figure 3.10*). All the isolates clustered with subgenotype A1, except for isolate S055A which clustered with genotype D (*figure 3.10).* Nine isolates were serological subtype of *ayw2* (blue) and the remaining 33 were serological subtype *adw2* (pink)

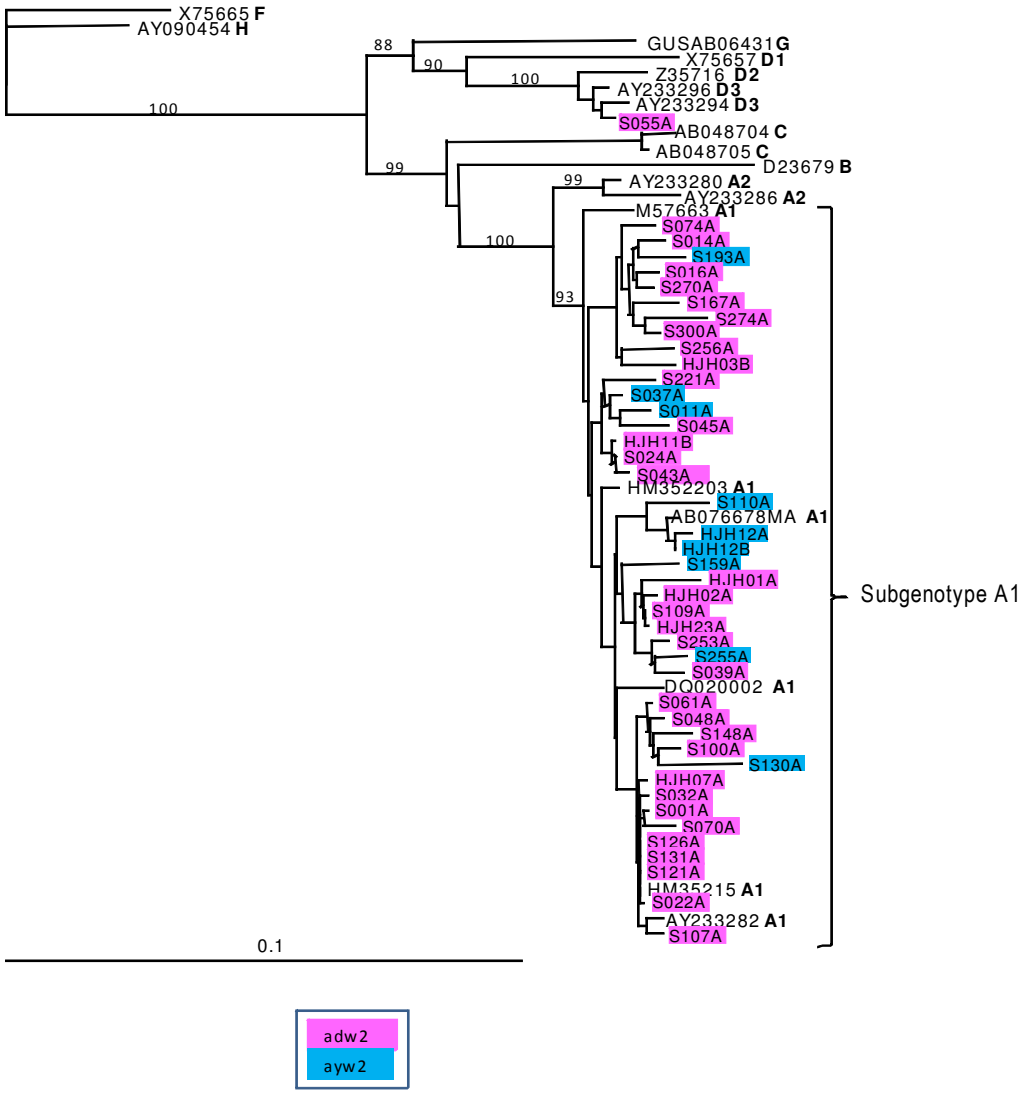


Figure 3.10. Phylogenetic relationship of the hepatitis B virus S open reading frame (position 2854-835 from the *EcoR1* site) of Shongwe Hospital (number with "S" prefix) and Helen Joseph Hospital (numbered with "HJH" prefix). Isolates from HBV-HIV co-infected isolates prior to ARV treatment are marked with suffix "A" and those at six months following treatment with "B". Trees established using neighbour-joining method. Bootstrap statistical analysis was performed using 1 000 data sets and the numbers on the nodes indicate the percentage of occurrences.

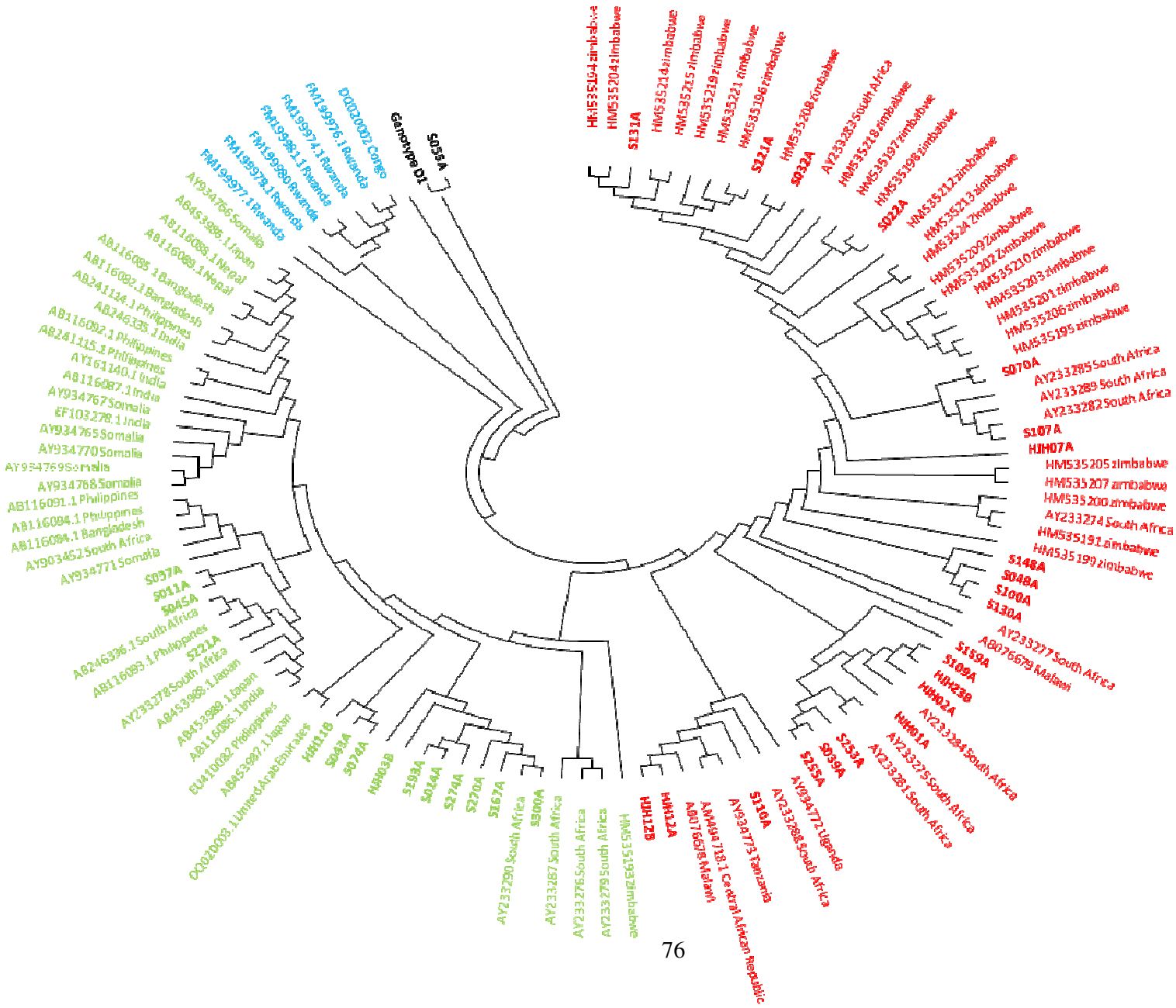

Genotype D1
S055A
DQ020007 Congo
FM199976.1 Rwanda
FM199974.1 Rwanda
FM199980 Rwanda
FM199981.1 Rwanda
FM199978.1 Rwanda
FM199977.1 Rwanda
AY934766 Somalia
AB453988.1 Japan
AB116089.1 Nepal
AB116085.1 Bangladesh
AB116092.1 Bangladesh
AB241114.1 Philippines
AB346335.1 India
AB241115.1 Philippines
AB116087.1 India
AY161140.1 India
AY934767 Somalia
EF103278.1 India
AY934765 Somalia
AY934770 Somalia
AY934769 Somalia
AY934768 Somalia
AB116091.1 Philippines
AB116094.1 Philippines
AB116094.1 Bangladesh
AY903452 South Africa
AY934771 Somalia
S037A
S011A
S045A
AB246396.1 South Africa
AB116093.1 Philippines
S221A
AY233278 South Africa
AB453989.1 Japan
AB453987.1 Japan
AB116086.1 India
EU410082 Philippines
AB453987.1 Japan
OQ020003.1 United Arab Emirates
HM611B
S043A
S074A
HM863B
S193A
S014A
S274A
S270A
S167A
AY233290 South Africa
S300A
AY233287 South Africa
AY233276 South Africa
AY233279 South Africa
HM535193 Zimbabwe
HM612B
HM612A
AM949718.1 Central African Republic
AB076678 Malawi
AY934773 Tanzania
S116A
AY233288 South Africa
S255A
S039A
S153A
AY233284 South Africa
AY233285 South Africa
HM601A
HM602A
HM239
S109A
S159A
AB076679 Malawi
AY233277 South Africa
S130A
S100A
S048A
S148A
HM535191 Zimbabwe
AY233274 South Africa
HM535199 Zimbabwe
HM535207 Zimbabwe
HM535200 Zimbabwe
HM535205 Zimbabwe
HIM07A
S107A
AY233282 South Africa
AY233289 South Africa
AY233285 South Africa
S070A
HM535195 Zimbabwe
HM535206 Zimbabwe
HM535201 Zimbabwe
HM535203 Zimbabwe
HM535210 Zimbabwe
HM535207 Zimbabwe
HM535209 Zimbabwe
HM535214 Zimbabwe
HM535213 Zimbabwe
HM535212 Zimbabwe
S022A
HM535196 Zimbabwe
HM535197 Zimbabwe
HM535218 Zimbabwe
AY233283 South Africa
S032A
HM535208 Zimbabwe
S121A
HM535221 Zimbabwe
HM535219 Zimbabwe
HM535215 Zimbabwe
HM535214 Zimbabwe
HM535204 Zimbabwe
S131A
HM535194 Zimbabwe

Figure 3.11. A circular phylogenetic tree comparing the South African subgenotype A1 complete S region with Asian and African subgenotype A1 obtained from GeneBank (designated by accession numbers), established using neighbor-joining. Red: African cluster, Blue: Central African cluster and Green: South eastern African/Asian cluster ('Asian" cluster).

Subgenotype A1 isolates from co-infected individuals were compared to subgenotype A1 isolates from Asian and African countries using a circular unrooted phylogenetic tree (*figure 3.11*). Three clusters could be discerned: containing strains from Central Africa (blue), one from southern eastern Africa (red) and an African/Asian cluster ("Asian"). The details of these clusters are shown in figure 3.12A and 3.12B. Fifteen isolates (HJH011B, HJ03B, S011A, S014A, S037A, S043A, S045A, S074A, S159A, S167A, S193A, S221A, S270A, S274A and S300A) clustered with the "Asian" subgenotype A1 (*figure 3.12 A*) cluster and the remainder of the isolates clustered with the African subgenotype A1 isolates (*figure 3.12B).*

Upon translation of the preS1/preS2/HBsAg and overlapping polymerase region, majority of the isolates from both African, Central African and "Asian" clusters isolates showed distinct subgenotype A1 amino acids 54Q, 74V 86A and 91V in the pre-S1 region and 32L in the preS2 region (Kimbi *et al*., 2004). The following mutations distinguished the isolates in the "Asian" cluster from the southern eastern African ones: F25L and V88L in the preS1 region, R48T/K, A53V and L54P in the preS2 region. Eleven isolates from the "Asian" cluster had 194A whereas the remaining four and all isolates from the southern eastern African clusters had 194V.

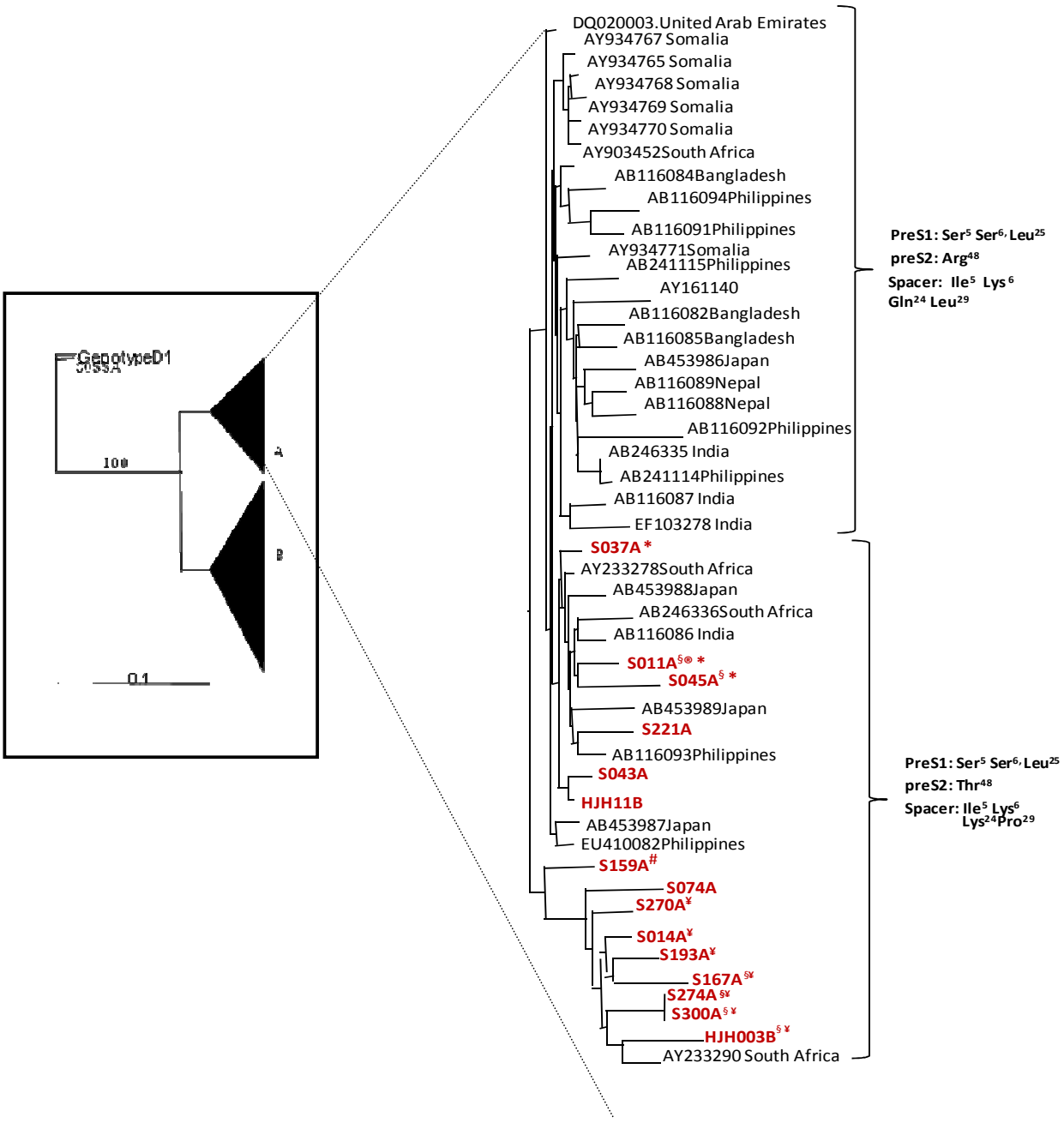


Figure 3.12A. Phylogenetic tree comparing the South African subgenotype A1 in the "Asian" cluster to Asian subgenotype A1 obtained from GenBank (designated by accession numbers), established using neighbor-joining, rooted using genotype D. Bootstrap statistical analysis was performed using 1 000 data set and the numbers on the nodes indicate the percentage of occurrences, **®PreS1 deletion , § preS2 deletions. * Spacer: 32Q 28L , #Spacer: 5L 6K, Reverse transcriptase: ¥ 1D**

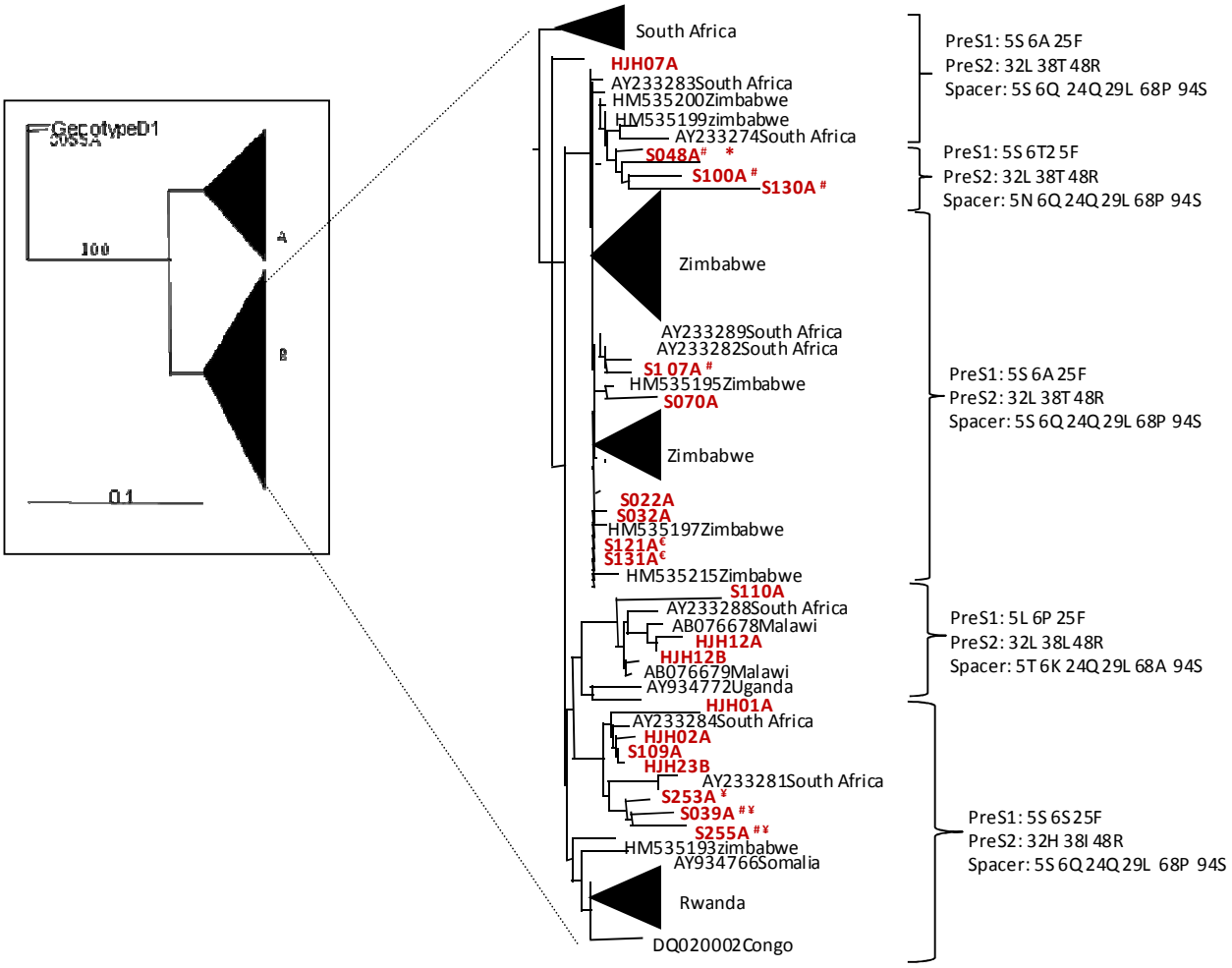


Figure 3.12B. Phylogenetic tree comparing the South African subgenotype A1 in the southern eastern African cluster with African subgenotype A1 obtained from GeneBank (designated by accession numbers), established using neighbor-joining, rooted with genotype D. Bootstrap statistical analysis was performed using 1000 data set and the numbers on the nodes indicate the percentage of occurrences. ***PreS1: 5T 6S, # Spacer: 94N, € Spacer 5S 6Q , ¥ 1D** . GenBank accession numbers: South Africa: AY233287, AY233279, AY233277, AY233276; Zimbabwe: HM535209, HM535221, HM535219, HM535208, HM535214, HM535205, HM535207, HM535194, HM535204, HM535215, HM535210, HM535206, HM535203, HM535201, HM535202, HM535121; Rwanda: FM199977, FM199981, FM19997, FM199976, FM199979, FM199980.

The southern eastern African cluster consisting of twenty four of isolates had distinct amino acid motifs in the preS1/preS2/S region compared to the "Asian" cluster isolates (*figures 3.12A* and *3.12B)*. The African isolates had greater variation in the preS1 compared to the "Asian" cluster (*figures 3.12A* and *3.12B)*. In the preS2 region, seven isolates had mutation L32H, while the remainder of the African isolates and "Asian" isolates had 32L.

In the spacer region of the overlapping polymerase region, 13 of 15 isolates from the Asian cluster had 5I 6K and the remaining two had 5LK 6K. The strains in the southern eastern African cluster had different amino acid motifs at position 5 and 6 for the five different clades: two isolates (S107A and S148A) had 5I 6Q, isolates S100A and S130A had 5S 6Q, 5T 6K together with 64A was found in three isolates (S110A, HJH12A A and HJH12B), seven isolates had 5S 6Q and 11 isolates had 5I 6 (*figure 3.12B*).

Ten of the 15 isolates had 24L together with P29 whilst the remaining 5 together with the African cluster isolates had 24Q and 29L. Ten "Asian" isolates had 59S while the remainder of the isolates including the isolates clustering with African cluster had 59P. All "Asian" isolates had 68A compared to the southern eastern African isolates, which had 68P.

The first amino acid of the reverse transcriptase domain of the overlapping polymerase was Asp in 10 of the 15 isolates instead of Glu . To check for validity of this mutation, 457 sequences from the public databases were checked. This mutation was found in the following: all genotype G sequences, some Japanese subgenotype B1 (AB010291,

AB073854, AB010290, AB106884, AB073856, AB073849, AB0733838 and AB033554), Taiwanese subgenotype B2 (AY596105, AY167098, AY167093), Swedish subgenotype B2 isolate (A7121250) and one South African subgenotype A1 isolate (AY233290) (results not shown).

3.6 Complete S region analysis

3.6.1 Helen Joseph Hospital cohort

Eight complete surface gene sequences of HBV isolated from the Helen Joseph Hospital cohort were analysed for mutations in the preS1, preS2, HBsAg regions and the polymerase region overlapping the HBsAg. In the preS1 region, F25L substitution mutation in the major pres1 antigenic epitope occurred in one isolate. The V90A occurred together with the S6A/P mutation in four isolates. Other mutations found in this region were S5L, I48V, H51Q, N40I and W77G.

The preS2 region showed two deletion mutants previously described in HBV-HIV co-infected patients (Audsley *et al*, 2010). Deletion mutants of 16 and 18 codons, one from a baseline isolate and from a 6 month follow up isolate, respectively, were found. Subgenotype A1 determinant mutation L32H occurred in 3 isolates, twice with S47A mutation. R48T/K occurred in two isolates from six months follow up patients only. The A53V mutation together with L54P was found in one isolate which had the 18 codon deletion mutation.

In the HBsAg region, four isolates showed the following mutations in the "a" determinant epitope (aa 99 to 150): I110M, K122R, T125M, M133T and F134I. One baseline patient was infected with immune/vaccine escape mutant E164V. R24K, L49R, S155L, W156G, V194A and L209V mutations were also found in this region. The polymerase region overlapping the HBsAg region of the HBV genome showed no drug resistant mutations in isolates derived from either the baseline or 6 month follow up patients. Substitution mutations rtQ125E and rtP109S occurred in 3 and 2 six months follow up isolates, respectively. The following mutations only occurred in a single isolate: rtH9Q + rtS119A+ rtV142N, rtY126H, rtI163T, rtL164W and rtE1D+ rtT128A

3.6.2 The Shongwe Hospital Rural cohort

The complete surface gene DNA sequences were obtained for 34 isolates. Of the 34 isolates 19 were HBsAg negative and 15 were HBsAg positive. (*Figure 3.1*). Mutation analysis was done for all 34 isolates. The preS1, preS2, HBsAg and the overlapping polymerase regions of the complete surface gene were analysed for the following clinically significant mutations: the deletion mutants, vaccine/immune escape mutants, HBsAg "a" determinant mutations, HBV reactivation maker mutations, the overlapping polymerase drug resistant mutations and other significant mutations. These are summarized in table 3.1.

Table1 3.1. Mutations observed in the complete surface open reading and their statistical significance

HBV region	HBV mutation	Percentage of mutations in HBsAg positive % (n = 19)	Percentage of mutations in HBsAg negative % (n= 15)	Statistical significance P value
PreS1	S5T	5.26	6.67	0.6952
	F25L	55.55	20.0	0.4941
	I48V	0	46.67	**0.0008***
	V88L	55.55	33.33	0.4709
	L85M	10.53	0	0.3048
	T90V	36.84	0	**0.0094***
	P94T	0	20.0	0.0760
PreS2	M1I	10.53	13.33	0.4150
	A7T	0	26.67	**0.0294***
	Q10R	10.53	20.0	0.3836
	A11T	5.26	6.67	0.6952
	F22L	21.05	13.33	0.6952
	L32H	21.05	6.67	0.4525
	R48K/T	36.84	20.0	0.2507
	A53AV	31.58	20.0	0.2466
	L54P/S	31.58	40.0	0.4394
HBsAg	V14G	10.53	0	0.3048
	68 (M)	5.26	0	0.5588
	Q101H/K	10.53	0	0.3048
	M103L	5.26	0	0.5588
	T115R	0	6.67	0.4412
	S117N	0	6.67	0.4412
	T118K	0	6.67	0.4412
	Y100C	5.26	6.67	0.6952
	P120T	10.53	0	0.3048
	Q129R/H	5.26	20.0	0.2158
	N131K	5.26	13.33	0.4150
	D144E	5.26	0	0.5588
	E164A/G	10.53	13.33	0.4150
	V168A[§]	10.53	26.67	0.2197
	S174N[§]	0	20.0	0.0760
	I195M	0	6.67	0.4545
	Y206H/C	10.53	0	0.3048
Overlapping Polymerase	rtE1D	36.84	13.33	0.1242
	rtS105T	10.53	13.33	0.4150
	rtH122N	15.79	13.33	0.6164
	rtQ125E	21.05	6.67	0.2507
	rtT128N/S	10.53	0	0.3048
	rtL129M	0	6.67	0.4412
	rtV173L	0	6.67	0.4412
	rtL180M	0	6.67	0.4412
	rtM204V	0	6.67	0.4412
	rtV214A	5.26	0	0.5588

*Statistically significant[§]

V168A together with S174N are reactivation markers and were found to be significantly higher in HBsAg-negative individuals (Henke-Gendo *et al*., 2008)**.**

In the preS1 region, the F25L amino acid mutation in the major preS1 binding occurred more frequently together with the V88L in three isolates (S70A, S193A and S270A) HBsAg negative isolates and in four HBsAg positive isolates (S014A, S016A S159A, and S167A). The T90V mutation occurred in 6 HBsAg positive isolates and 3 times with the V88L and none in the HBsAg negative isolates. Other mutations found in this region were I48V, L85M, P94T, S5T and one 30 nt deletion mutant at nucleotide position 2900-2929, commonly found in HCC patients (Kew *et al*., 2005).

In the preS2 region, the R48T/K occurred in 12 isolates: 8 times together with the A53V and 4 times with L32H. The Q10R mutation in the major preS2 antigenic epitope region was found together with the V53A and R48K frequently. PreS1/preS2 deletion mutants ranging from 11 to 33 codons previously associated with HIV co-infection (*figure 3.13)* (Audsley *et al*, 2010), were found in five isolates (S011A, S045A, S167A, S274A and S300A), Other mutations in the preS2 region include the amino acid substitution mutations M1I which occurred together with F22L, A7T, A11T and L54P/S *(table 3.1*)

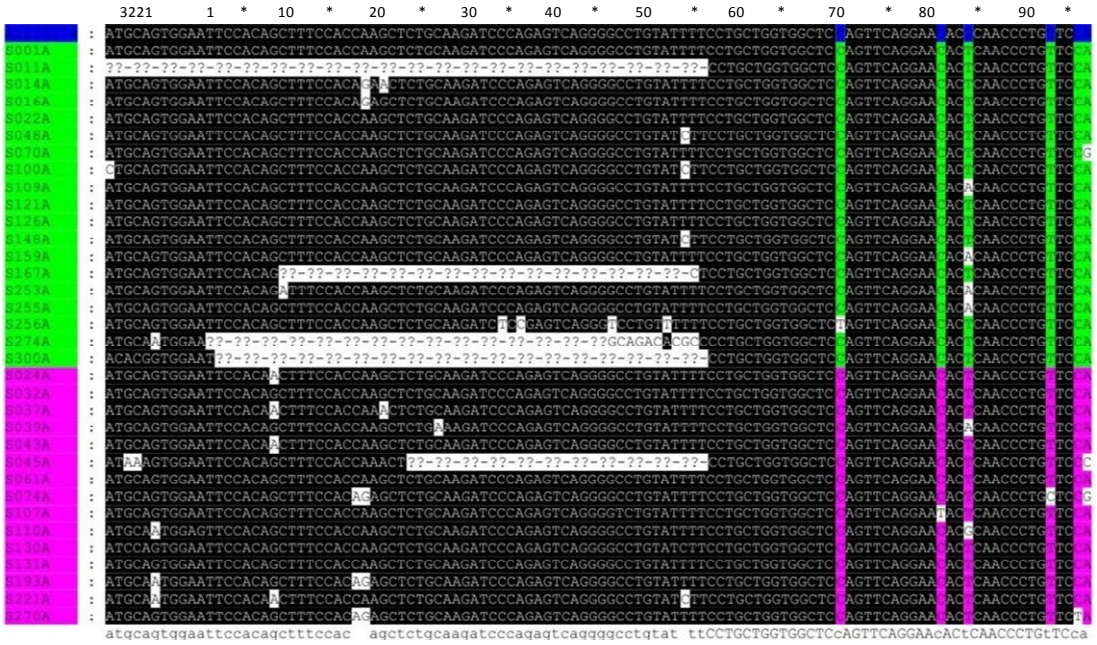


Figure 3. 13: **Deletions observed in the PreS1/PreS2 region of the HBV subgenotype A1 genome from HIV co-infected.** Position 1 corresponds to the *Eco*RI cleavage site of HBV genome.

The HBsAg region was analysed for vaccine/immune escape mutants. Immune escape mutants P120T/A, Q129R and E164D/G were found in 7 isolates (*figure 3.14*). The following mutations were found in the vicinity of the 'a' determinant epitope of the HBsAg: Y100C, Q101H/K, M103L, T115R, S117n, T118K and N131K (*figure 3.14*). The HBV reactivation markers V168A + S174N (Henke-Gendo *et al*., 2008) were found in 23.81 % of the HBsAg negative and none in the HBsAg positive infections (*figure 3.15*). The following mutations: D144E, E164D, I195M and Y206C also resulted in substitutions occurring in the overlapping polymerase region.

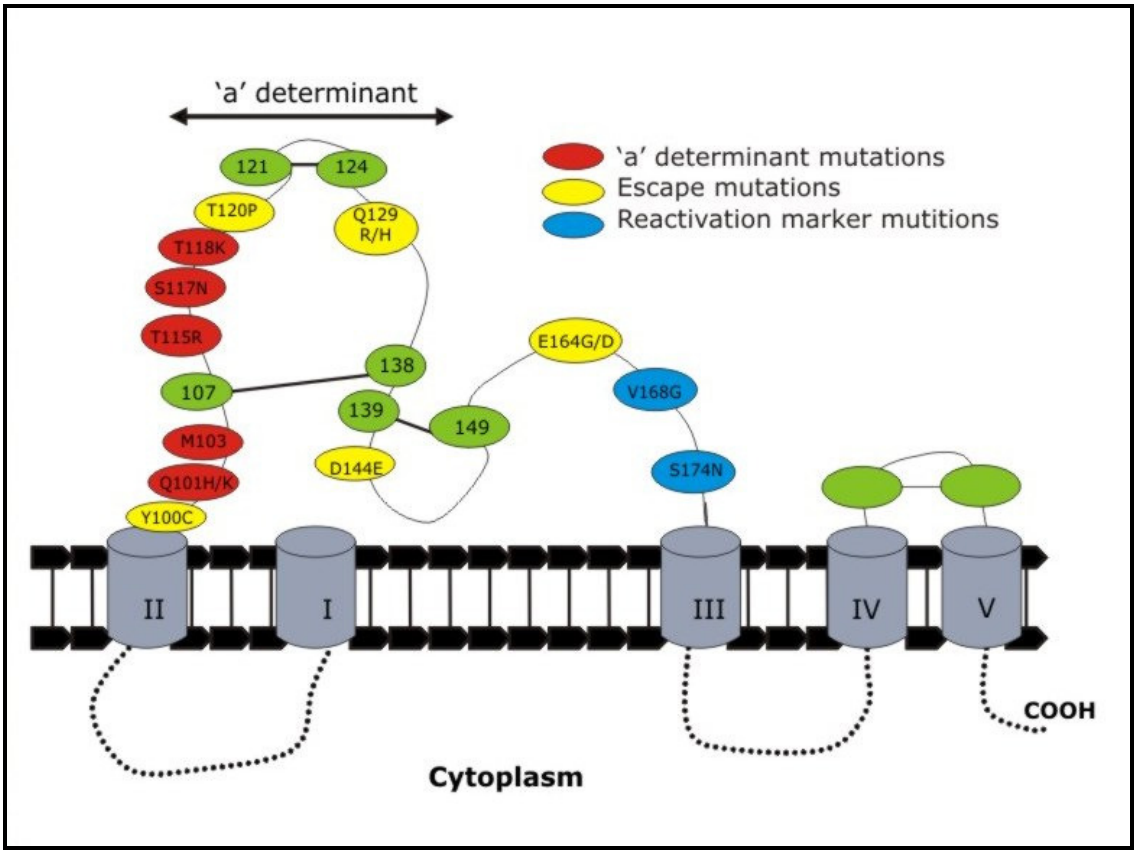


Figure 3.14: Graphical Representation of the mutations found on the HBsAg small envelope protein from the HBsAg-negatives. The red circles show the mutations found in the HBsAg "a" determinant epitope, yellow circles show the immune/vaccine escape mutants and the blue circles represents the HBV reactivation mutation markers.

In the polymerase region, overlapping the HBsAg region, the following clinically significant drug resistant mutations were found in 3 isolates (S011A, S074A and S130A): rtL180M +rtM204V, rt V173L in HBsAg negative isolates and rtV214A in one HBsAg positive isolate (*figure 3.15).* The substitution mutation on rtT128N/S occurred in 2 isolates (S255A and S274A) as a result of the HBsAg overlapping mutation P120T. Other

mutations that were found in this region include rtS105T, which frequently occurred together with rtH122N, rtQ125E and rtL129M (*table 3.1*).

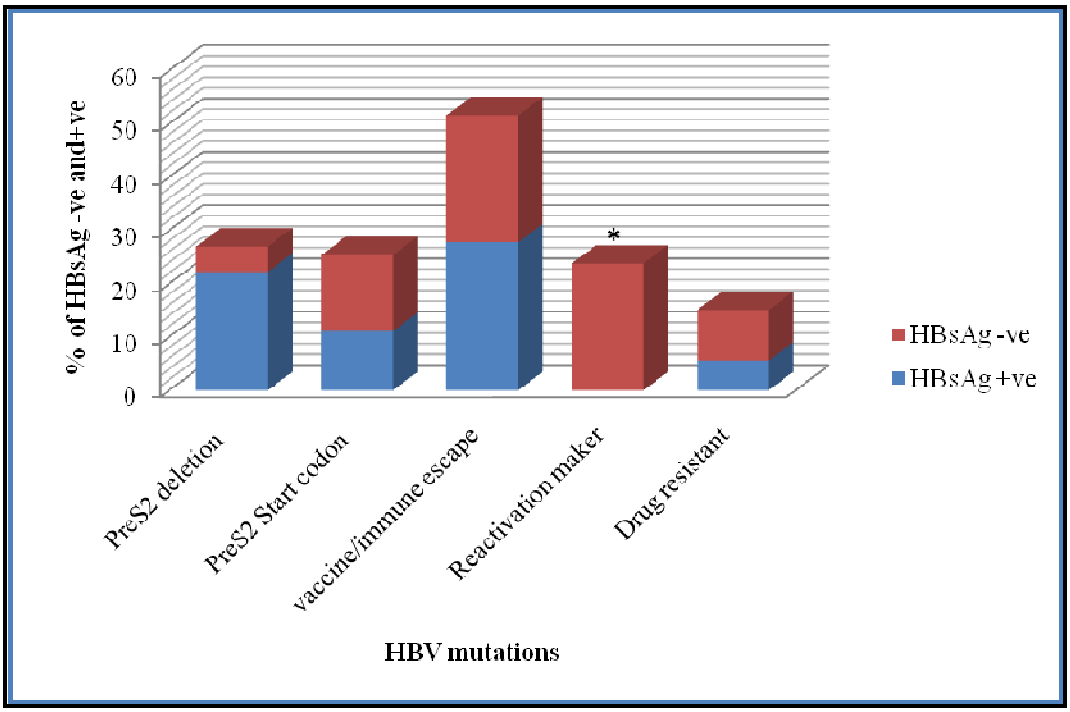


Figure 3. 15. Clinically significant mutations of the complete surface open reading frame in the HBsAg positive and negative HBV infections.* statistically significant P<0.05. PreS2 deletions depicted in figure 3.10; PreS2 start codon mutation: M1I; vaccine/immune escape mutants: P120T/A, Q129R and E164D/G; reactivation markers: V168A + S174N; Drug resistant mutations: rtV173L, rtL180M + rtM204V and rtV214A

Chapter 4: DISCUSSION

HBV and HIV are both endemic in sub-Saharan Africa. Most of the research on HBV and HIV coinfection has been conducted in areas of low HBV endemicity, where a number of studies have found a significantly higher prevalence of HBV infection in HIV-positive individuals (Colin *et al*., 1999, Thio *et al*., 2002, Shire *et al*., 2007) . Therefore it is of great importance to study HBV-HIV co-infection in a South African context. It is known that HBV genotype A is predominant in South African HBV-mono infected population (Bowyer and Sim, 2000, Kimbi *et al*., 2004); however genotypes prevailing in a HBV/HIV co-infected population were previously unknown.

Sequence variability is an important characteristic of the HBV because the viral polymerase (reverse transcriptase) lacks proofreading activity (Kramvis *et al*., 2005b). Host–virus interaction and selective pressure imposed internally by the immune system and externally by vaccination and antiviral treatment can also affect DNA sequence heterogeneity of the HBV genome (Kramvis and Kew, 2005). Compared to areas of low endemicity, where HIV and HBV are most likely transmitted at the same time during sexual maturity, in South Africa, the main route of HBV transmission is by horizontal transmission, early in infancy. Transmission occurs generally before the age of 5 years and these children then become chronic carriers of HBV in adulthood (Kew, 1996). Therefore majority of the South African population are naturally protected by antibodies to HBV by the time they acquire HIV at the age of sexual maturity (Burnett *et al*., 2005). However, immunosuppression as a result of HIV infection has been shown to result in HBV infection and/or reactivation (Henke-Gendo *et al*., 2008, Bloquel *et al*., 2010) , hence increasing HBV infection in HIV positive patients,

with previously resolved HBV infections. Reactivation of HBV infection can result in sequence heterogeneity leading to clinically significant mutations on the HBV genome (Henke-Gendo *et al*., 2008), which may have impact on liver disease progression. Moreover, the development of the mutations can be influenced by the HBV genotypes prevailing in a population. Therefore this study was conducted to determine the HBV genotypes and to identify mutations occurring on the BCP/PC, the complete surface and the polymerase region overlapping the HBsAg, of HBV isolated from South African individuals with HIV infection.

In order to determine the genotypes and to characterize the clinically significant mutations prevailing amongst the South African HIV positive population, blood serum samples were collected from HIV positive patients from both rural and urban hospitals. Following nucleic acid extraction, the partial surface gene, BCP/PC and the complete surface regions were amplified by PCR. The complete surface and the BCP/PC amplified products were sent for sequencing followed by bioinformatics analysis to determine the HBV genotypes and to identify mutations relative to the consensus sequences of the respective genotypes.

4.1 HBV GENOTYPING METHODS

Classical genotyping methods for HBV include PCR amplification and full genome sequencing followed by phylogenetic analysis. However, a number of other methods have been developed and used over the years. These include: restriction fragment length polymorphism (RFLP) (Lindh *et al*., 1997)**,** PCR with specific primers for single genotypes, multiplex-PCR for many HBV genotypes (McIver *et al*., 2005), hybridisation technologies

and serology based approaches (Laperche *et al*., 2001, Usuda *et al*., 1999). In this study PCR-RFLP method and PCR-sequencing followed by phylogenetic analysis was employed.

4.1.1 HBV genotyping by RFLP

The RFLP method for genotyping HBV is based on the amplification of the partial S gene (nt 256- 796), followed by restriction digestion of the amplicons. The partial surface region (nt 256- 796) of 26 HBV isolates from HIV-infected individuals attending the Helen Joseph hospital were successfully amplified using nested PCR. HBV could only be visualized following nested PCR (*figure 3.2),* indicating relatively low viral loads. Using a RFLP genotyping assay (Lindh *et al*., 1997) , 13 of the partial surface region amplified products were restricted using the enzymes *Hinf*I and *Tsp109*. Six isolates from baseline samples and five from the six months follow up were found to belong to genotype A. One isolate (HJH20B) yielded a restriction pattern found to belong to genotype D (*figure 3.5)* which was further confirmed by direct sequencing of the complete surface gene.

The RFLP method for HBV genotyping has an advantage over sequencing in that it does not require highly skilled personnel and may be more practical for large scale genotyping studies. This method can give an indication of the HBV genotypes prevailing in a population much quicker than sequencing and phylogenetic analysis because it does not require special sophisticated equipment. However, the RFLP restriction method for genotyping has shortcomings, which may result in poor results. Low viral load/ low viral DNA concentrations may cause poor PCR amplification and restriction, single nucleotide mutations

resulting in change within the restriction sites may affect the digestion and the quality of genotyping.

Since the method was described fourteen years ago, unusual patterns as a result of recombination, the presence of mixed genotypes and the increased number of genotypes and strains having been identified Thus this method may yield unreliable results, selecting only the most dominant genotype and missing mixed genotype populations. The high cost of restriction enzymes may also limit the method as number different enzymes are required depending on the number of genotypes being studied in a specific region. The method cannot differentiate subgenotypes or provide information about mutations in the HBV genome. Thereforethis RFLP genotyping method was not used in the subsequent analyses of the HBV isolates from the Shongwe cohort.

4.1.2 HBV genotyping by BCP/PC sequencing analysis

Sequence and phylogenetic analysis using individual gene or gene sequence can also be employed for genotyping of the HBV. The BCP/PC region is a small region of the HBV genome, which directs the transcription of the mRNAs which are translated into the precore HBeAg, the HBcAg and the polymerase (Gunther *et al*., 1996). Sequence analysis allows identification of specific nucleotides or nucleotide polymorphism distinct to certain genotype (Bartholomeusz and Schaefer, 2004), therefore the BCP/PC region sequence analysis may allow to distinguish the different HBV genotype by looking at specific nucleotides between nt 1750 to 1900 (from the *EcoR*1 site). Compared to RFLP method, by using the BCP/PC

sequence analysis method, certain subgenotypes, such as subgenotype A1 and A2 may also be distinguished.

The BCP/PC region was successfully amplified by a nested PCR, using standard DNA *Taq* polymerase, because it involved amplification of a relatively short fragment of 307 bp , which amplified even low viral loads. Because of high sensitivity, samples with very high viral load showed a very strong DNA band on the agarose gel when viewed under UV light following a single PCR and following a second round of PCR yielded smeared bands. Therefore samples with very high viral loads were sent for sequencing following only a single PCR or diluted 1:100 before running the second round PCR in order to get rid of all the non specificbanding and to obtain a clear specific single DNA band of 307 bp. Amplification of the BCP/PC region of HBV isolated from the Shongwe Hospital samples needed additional optimisation. Because of the sensitivity of the PCR, it was very difficult to avoid contamination and extra precautions were taken. Amplifications were repeated using one sample alternating with negative water control, dilution of the first round PCR products in 1:10, 1:50 and 1:100 to minimise carry over contamination and to reduce non-specificity. A new pair of sterile gloves was worn before handling of each sample to avoid carryover contamination of negative samples. To prevent cross contamination, different PCR hoods for first round and second round PCR were used. They were cleaned with bleach and 70 % ethanol and left under UV light for 20 to 30 min prior to each use. All the pipettes designated for each round of PCR were also cleaned with bleach and 70 % ethanol, and left under UV light prior to each use. The racks were soaked in bleach overnight and all the tubes used were autoclaved before use.

From a total of 51 Shongwe HBV isolates and 13 and Helen Joseph HBV isolates, the BCP/PC region was successfully amplified and sent for sequencing, A total of 49 isolates from Shongwe and 13 isolates from Helen Joseph generated DNA sequence data that were of good quality and could be properly assembled, aligned and analysed. Using the nucleotide region 1750 t0 1900 (from the *EcoR*1 site) in the BCP/PC region, sequence analysis was done and the subgenotypes were determined.

The following loci in this region were analysed to identify genotype specific nucleotides: 1809-1812, 1858, and 1888 (table 3.1). From the Helen Joseph study, BCP/PC region of 13 isolates were amplified, sequenced and analysed. Eleven isolates had the TCAT Kozak sequence at nt 1809-1812, 1858C together with 1888A (from the *EcoR1* site) (*figure 3.8*), therefore it could be deduced that belonged to subgenotype A1(Kramvis *et al*., 2008). The two other isolates (HJH11A and HJH 20B) had sequence characteristic of genotype D/E: Kozak sequence GCAC (1809-1812 from the *EcoR1* site) and 1858T.

Of the 49 BCP/PC DNA Shongwe Hospital sequences analysed, Thirty nine isolates were subgenotype A1 with the Kozak sequence TCAT at nt 1809-1812, T1858C and 1888A/G, which predominates in the HBV mono-infected population in South Africa. Three isolates had the Kozak sequence GCAC (1809-1812) with C1858T and 1888G, characteristics of genotype D or E. Seven isolates with Kozak sequence GCAC, 1858C and 1888G were deduced to belong subgenotype A2 (Kramvis *et al*., 2008).

Genotyping by BCP/PC sequence analysis advantages include easier PCR-amplification and PCR sensitivity due to the region's small size. The analysis allows differentiation between subgenotypes A1 and A2, which cannot be done using the RFLP method. However, the

method also has restrictions in that it cannot differentiate genotypes D and E. Although not predominant, both genotypes D and E may be found in South Africa and cannot be differentiated using the BCP/PC region. This is a limitation of genotyping using the BCP sequence analysis; however it can be overcame by phylogenetic analysis of the complete surface gene or full genome sequence.

4.1.3 HBV genotyping by Complete S sequencing analysis

The same procedure as the BCP/PC amplification was followed in amplification of the complete S region to avoid contamination. The complete surface gene PCR was less sensitive because of the longer length of the PCR product of 2 kb (*figure 3.4*). Different temperatures and primer concentrations were tried using the normal Bioline *Taq* polymerase but yielded no PCR product. Therefore the Qiagen Hotstar *Taq* master mix was used, this master mix has optimised conditions and ready mixed buffers and to give high PCR specificity and reduced non-specificity. HBV isolates with relatively high HBV DNA viral loads were amplified successfully. Those with much higher HBV viral loads had non-specific amplification and therefore required PCR gel purification prior to direct sequencing.

Following PCR amplification, the complete surface region of 8 HBV isolates from Helen Joseph Hospital and 34 isolates from Shongwe Hospital were directly sequenced. Following sequencing, the DNA sequences were analysed by phylogenetics and construction of phylogenetic tree against HBV genotypes A- H. The complete S analysis also allows to

determine HBV serological subtypes from translated HBsAg gene DNA sequences by looking at amino acids 122, 127 , 134, and 160 (Kramvis *et al*., 2008).

A total of 37 complete surface DNA sequences were generated from the Shongwe Hospital study and eight isolated from the Helen Joseph Hospital. Two isolates (HJH12A and HJH12B) from the Helen Joseph Hospital and eight isolates from Shongwe belonged to serological subtype *ayw*2 with amino acid sequence 122R, 127P, 134F, and 160K The remaining isolates from Shongwe and Helen Joseph had amino acid characteristics (122K, 127P , 134F, and 160K) depicting serological subtype *adw2.* Serological subtype *adw2* is the most common serological subtype in South African genotype A/subgenotype A1 isolates (Kramvis *et al*., 2005b).

Two isolates from Shongwe could not be accurately genotyped. In the first case, the genotype deduced from the BCP/PC region was genotype A. Using the S region it was deduced to belong to genotypes D or A, because the chromatogram yielded a mixed pattern. In the second case, both the BCP/PC and S region amplifications yielded mixed chromatograms. The discordant result between the BCP/PC and S region, as well as the mixed chromatograms can be either because the patient was infected with both genotypes A and D or with a genotype D/A recombinant. Genotype D/A recombinants have been shown to occur in South Africa (Owiredu *et al*). The only way that this could be differentiated would be by carrying out full genome amplification to identify the recombinant and/or cloning to identify the mixed population.

Upon construction of the phylogenetic tree of all 42 complete subgenotype A1 S DNA sequences (from both Shongwe and Helen Joseph) (*figure 3.10*), only one isolate (S055A) from Shongwe was found to cluster with genotype D. Phylogenetic comparison with

different subgenotypes of D (data not shown) this isolate was found to belong to subgenotype D3, previously found in HBV mono-infected individuals (Kimbi *et al*., 2004). Therefore as in the case of HBV mono-infection, the majority of HBV isolates from HIV co-infected individuals belonged to subgenotype A1. All HBV isolates had amino acids in the BCP/PC and preS1/preS2/HBsAg region characteristic of subgenotype A1 (Kimbi *et al*., 2004). Subgenotype A1 is the most prevalent subgenotype in South Africa. Genotyping by complete S analysis was found to be more accurate compared to the BCP/PC sequence analysis, since the S region amplified is longer than the BCP/PC and therefore more informative. Sequencing of these two regions is generally sufficient to accurately genotype HBV isolates except in the case of mixed infections and/or recombinants, where full genome amplification and cloning would be necessary.

4.1 .4 Phylogenetic analysis

Phylogenetic analysis is a method to assess the relative and evolutionary relatedness of sequences to each other and control sequences (McCormack and Clewley, 2002). Genotyping by phylogenetic analysis employs special algorithms and phylogenetic software packages to construct evolutionary relatedness tree using statistical analysis of DNA sequences of full genomes, or specific gene/ regions (Bartholomeusz and Schaefer, 2004).

In this study, a circular un rooted phylogenetic tree was constructed for the 39 subgenotype A1 isolates from the Helen Joseph and the Shongwe cohort (*figure 3.11*) together with HBV subgenotype A1 isolates from different African and Asian countries. The HBV subgenotype

A1 isolated from HIV infected individuals clustered together with African and Asian HBV isolates belonging to subgenotype A1 (*figure 3.11*), Even though these HBV subgenotype A1 isolates were obtained from one small region in South Africa, these isolates showed high nucleotide diversity when compared to others from other geographical regions of the world. Phylogenetic analysis showed three different clusters: the Central African cluster (blue), the southern eastern African cluster and the African/Asian ("Asian") cluster (*figure 3.10).*

Isolates from the southern eastern African cluster formed different clades: subgenotype A1 isolates from central eastern African countries (Malawi, Tanzania, Uganda and Central African Republic), Zimbabwe and South African (*figure 3.11* Red). The isolates from the "Asian" cluster formed five major clades where the isolates from the HBV-HIV co-infected individuals clustered together with some South African HBV mono-infected subgenotype A1 isolates, the United Arab Emirates clade, South Eastern Asia (Japan, Philippines and India). Somalian isolates clustered in this cluster and formed clades with HBV subgenotype A1 from Bangladesh, India, Japan, Nepal and Philippines (*figure 3.11* Green), whilst the Rwandan isolates formed an outlier slightly apart from the others (*figure 3.11* Blue).

Phylogenetic analysis does not only give genotype specific information but may also indicate relatedness of the transmission patterns, human behavioural patterns, and historical human migration patterns between the countries represented by the clusters or clades (McCormack and Clewley, 2002). It also gives evolutionary information and statistical reliability. However, because of the complexity of the method's expensive algorithms required to do the analysis, specially trained personnel are required in interpreting the results and it may be time consuming (Bartholomeusz and Schaefer, 2004).

In summary, by employing the HBV genotyping methods discussed above, subgenotype A1 was found to be most prevalent in both rural and urban South African HIV-infected individuals. This higher prevalence same as in HBV mono-infections although the ratio of genotype A to non-A is slightly higher in the HBV/HIV co-infected individuals. The previous study was carried out in 2004 (Kimbi *et al*) making direct comparison unfeasible. However, others have shown a predominance of genotype A in HIV infected individuals (Quarleri *et al*., 2007, Audsley *et al*., 2010)

The HBV isolates were found in both African and "Asian" clusters intimating a high diversity in the strains circulating in both the urban and rural cohorts. This high diversity may be indicative of the high mobility of populations to and from different northern countries in Africa to South Africa. Johannesburg being an urban centre attracts a lot of immigrants (Collinson *et al*., 2007). Moreover, Shongwe is close to the borders with Swaziland, Zimbabwe and Mozambique. Hence high levels of migration from these surrounding countries may lead to higher risk of sexually transmitted infections including HBV and further increased genetic variability of HBV (Collinson *et al*., 2007).

4.2 BCP/PC mutation analysis.

The BCP and PC/C regions of HBV encode for HBeAg. The BCP, which covers the distal X region and the proximal pre-core region, directs the transcription of pre-core mRNA, which is translated into the pre-core/core fusion protein that is the precursor of HBeAg. This region

possesses two start codons, HBeAg precursor protein, which is synthesized by translation from the first start codon and the second start codon translates into the of the core protein (HBcAg), when it is translated from the pgRNA (Byung Chul *et al*., 2003). A number of mutations that abolish and/or decrease HBeAg expression have previously been described (Kramvis et al., 1997b, Kramvis et al., 1998a, **Lim** and **Ton**, 2006). In this study the BCP/PC region of 49 HBV isolates from Shongwe and 13 from Helen Joseph Hospital were analysed.

In the Shongwe Hospital cohort, only five HBV isolates were HBeAg-positive and the remainder were HBeAg-negative. For the Helen Joseph Hospital cohort, HBeAg status was not determined because of the depletion of the serum. Variations or mutations that are characteristic of subgenotype A1 have been described, that can account for the HBeAg negativity. These mutations can affect expression of HBeAg at the transcriptional, translational and post-translational levels (Kramvis and Kew, 2007a, Chen et al., 2008a).

The BCP/PC region was analysed for mutations in region 1750 - 1900 (from the *EcoR*Isite) as described in Kramvis *et al*, 2008 In five Shongwe isolates (S016A, S027A, S032A, S042A and S167A), the classic precore G1896A mutation was detected (Carman, 1997) This mutation completely abolishes HBeAg expression and it occurred with other mutation. The G1896A converts the codon TGG for tryptophan to a stop codon leading to the truncation of the HBeAg precursor (Carman *et al*., 1989). The G1896A mutation occurred together with the C1858T in four of the five isolates *(table 3.1, Appendix D*), because in genotype A isolates the G1896A disrupts the G-C Watson Crick bond, destabilizing ε and compromising viral replication (Lok *et al*., 1994). Thus the C1858T mutation is frequently associated with the 1896 stop codon mutation (Kramvis *et al*., 2005b, Revill *et al*., 2010).

Another important mutation in the precore/core region is the first initiation codon mutation at region 1814-1816 (non-ATG). This was found in nine Shongwe isolates (*table 3.1*) and in none of the Helen Joseph isolates. This mutation alters the start codon preventing initiation of HBeAg translation (Laras *et al*., 1998). One of the eight isolates (S274A) with the initiation codon mutation was HBeAg positive (*table 3.1*), the initiation codon mutation completely abolishes HBeAg expression therefore; the HBeAg positivity may be explained by the presence of a mixed HBV infection, with the minor population providing a wild-type initiation codon.

In the BCP region, four isolates (HJH01A, HJH03A, HJH10A and HJH01B) from the Helen Joseph cohort (*figure 3.8*) and eight HBV isolates (*table 3.1*) from Shongwe had the A1762T G1764A mutations. These mutations are known to down regulate HBeAg expression and have been shown to increase viral replication and to be closely related to progression of chronic liver disease (Baptista *et al*., 1999, Hiroshi *et al*., 2002, Song *et al*., 2005, Tong *et al*., 2005, Buckwold *et al*., 1996, Baumert *et al*., 2007). Isolate HJH10A from Helen Joseph also had the T1753C mutation together with the A1762T G1764A was found in one Helen Joseph isolate (HJH10A) and five (S061A, S148A, S184A, S221A and S264A) Shongwe isolates. This combination of mutations have been described as markers for HCC (Yeh *et al*., 2010, Chen and Oon, 2000, Zhang *et al*., 2010).

Ten DNA sequences from Helen Joseph had the TCAT Kozak sequence at nt 1809-1812, which is found in all South African subgenotype A1, one isolate had TCTT instead of TCAT

and the remaining 2 had GCAC, which is found in subgenotypes other than A1. Thirty nine isolates had the TCAT and 10 had GCAC at position 1809-1812. Three nucleotide substitutions within this region were detected in seven of the 39 subgenotype A1 and two of subgenotype A2 isolates from Shongwe. These triple mutations may completely switch off HBeAg expression (Ahn *et al*., 2003). The point mutations around the Kozak sequence are generally found in subgenotype A1 and not in other genotypes, These mutations reduces a HBeAg translation by the leaky scanning mechanism (Kozak, 1999). This a process by which the 40s ribosomal unit locates the mRNA initiation codon, which is key step in translation and is required for the expression of almost all eukaryotic genes (Berthelot *et al*., 2004, Kozak, 1999). In a South African population, the Kozak sequence mutation in the 1809-1812 region was shown to occur in about 80% of South African HBV subgenotype A1 isolates (Baptista *et al*., 1999). This set of mutations is a characteristic of subgenotype A1 (Kimbi *et al*., 2004), decreasing HBeAg at the translational level (Kramvis and Kew, 2007c, Ahn *et al*., 2003). This is a possible alternative mechanism of early HBeAg seroconversion in patients infected with subgenotypes A1., .

When comparing the mutations with the HBsAg status, the G1862T mutation occurred in 23% of the HBsAg-negative patients but in none of the HBsAg-positive patients ($p<0.05$) (*figure 3.10.*). This mutation which interferes, with the post-translational modification of the HBeAg-precursor, together with the Kozak sequence mutation (1809-1812) and the A1762T G1764A may completely abolish HBeAg expression. This mutation, in combination with two of these mutations, could thus account for the HBeAg negativity of seven isolates in the present study.

The 1888 region had a single nucleotide substitution mutation in four of the Helen Joseph isolates and in 20 Shongwe isolates. The G1899A mutation occurred in 3 cases from Shongwe and in none of the Helen Joseph isolates. The 1888 G to A mutation introduces an out-of-frame AUG start codon, 13 nucleotides upstream of the core AUG start codon and a minicistron that can potentially be translated into seven amino acids: 'Met-Ala- Leu-Gly-His-Gly-His'. Therefore, the newly introduced start codon at 1888 may have an important role in the regulation of the translational efficiency of a downstream start codon (Kimbi *et al*., 2004).

4.3 COMPLETE S REGION ANALYSIS

Phylogenetic analyses of the complete S region was used to compare the subgenotype A1 isolates from Helen Joseph and Shongwe Hospitals, against subgenotype A1 HBV isolates from Asian and African countries. The sequences separated into two distinct clusters: the African (Congo, Rwanda, South Africa and Zimbabwe) and the "Asian" cluster (Bangladesh, India, Philippines, Japan, Nepal, Somalia, United Arab Emirates and South Africa) (figures *3.12A* and *3.12B).* The isolates from the African cluster showed a lot of variation compared to the "Asian" cluster, which comprised of mostly Asian subgenotype A1 isolates, some South African and Somalian isolates. The latter isolates did not show much variation in the preS1/preS2/HBsAg and spacer regions (summarised in *figures 3.12A* and *3.12B).* This concurs with the hypothesis that this subgenotype has been endemic in the African population for a long period of time (Kramvis and Kew, 2007c).

The complete S region has fundamental biological importance. It encodes for three distinct, related proteins LHBs, MHBs and SHBs, which share the same carboxyl terminal LHBs and MHBs encoded by the preS/S region of the HBV, have been found to mediate attachment of the virus to the host receptors (Neurath *et al*., 1986, Huy *et al*., 2003). Moreover, B-cell and T-cell epitopes are also located on the portion of the proteins coded for by the preS region. The HBsAg contains a cysteine rich "*a*" determinant, which is the major target of neutralising antibodies against HBV (Jilg *et al*., 1984). Of all the variations known to occur on the HBV genome, the mutations within the surface are some of the most relevant clinically. They are known to affect immunogenicity of the HBsAg, determined more specifically by the cysteine-rich '*a*' determinant. This is the region in which a cluster of epitopes targeted by neutralizing antibodies against HBV are raised , any alterations in this region are important because they allow the virus to avoid the neutralizing effect of the host's immunoglobulins (HBIg) (Jilg *et al*., 1984). Therefore, changes in the surface protein affect the antigenicity and immunogenicity of HBsAg, leading to immune escape and diagnostic difficulties, giving false negative results (Carman, 1997, Hsu *et al*., 1999). Mutations in the surface antigen may occur naturally as a result of the replication of HBV or may be induced by external factors such as vaccination and drugs against HBV. These mutants are able to infect vaccinated and unvaccinated individuals. Therefore in the context of HBV/HIV co-infection, and especially in the presence of high HBsAg-negativity seen in this patient population, it was of great importance that these mutations be defined.

In this study, eight isolates from Helen Joseph and 34 isolates from Shongwe Hospital were analysed. HBsAg status for Helen Joseph status could not be determined because of serum depletion. From the Shongwe study, 15 isolates were HBsAg-negative and 19 isolates were HBsAg positive. The three regions: preS1, preS2 and the HBsAg regions as well the

polymerase region overlapping the HBsAg region were analysed for mutations including immune/vaccine escape mutants, 'a' determinant mutations, drug resistant mutations and mutations that may be unique to HBV-HIV co-infected with subgenotype A1 HBV isolates. Furthermore, sequences were analyzed in an attempt to explain the HBsAg negativity in these patients (*summarised in table 3.1*).

Five isolates from the "Asian" cluster had preS deletion mutants: one Helen Joseph isolate and four HBsAg-positive Shongwe isolates had preS2 deletion mutants: (*figure 3.12A*). Deletion mutants in the preS region have been previously found to occur more frequently in HIV-coinfected patients (Audsley *et al*., 2010) and in HCC patients (Raimondo *et al*., 1991, Kew *et al*., 2005). Deletion mutants in the preS region have been reported to cause overproduction and accumulation of LHBs protein in the endoplasmic reticulum (ER), which causes significant ER stress that may induce DNA damage and genomic instability and hence play a possible role in hepatocarcinogenesis (Hsieh *et al*., 2004, Chen *et al*., 2006). A higher oncogenic potential of pre-S2 deletions has been found compared with that of pre-S1 deletion mutants (Yeung *et al*., 2011). The preS mutants may contribute to viral oncogenesis by transcriptional activation of the viral promoter elements (Hildt *et al*., 1996).

All sequences from both Helen Joseph and Shongwe Hospital had the 54Q and 91V, which have been shown to be a characteristic of subgenotype A1 in the preS1 region (Kimbi *et al*., 2004, Norder *et al*., 1996). Pres1 residues 21-96 contain the virus neutralising epitope and a hepatocellular binding site and the preS2 residues 1-11 and 21-47 are have been shown to mediate HBV attachment to the hepatocytes (Kim *et al*., 1997), therefore mutations in these regions may lead to conformational changes on the LHBs and MHBs, which may result in

HBsAg negativity. PreS1 mutations within the neutralising epitope and hepatocellular binding sites were detected: S5L, S6A/P, F25L, I48V, N40I (*summarised in table 3.1*) and in addition to this F25L, L54P/S L85M, V88L, T90V and P94T were found in isolates from Shongwe. The preS2 start codon mutation M1I was found in a statistically higher proportion of HBV from HBsAg-negative individuals compared to isolates from HBsAg-positive individuals. In the preS2 region, L32H substitution was found in 3 of the 8 isolates from Helen Joseph and in 8 of 34 isolates from Shongwe. This mutation is a unique subgenotype A1 determinant (Kimbi *et al*., 2004). R10Q was observed in 5 isolates from Shongwe. This mutation is in the major preS2 antigenic region known to carry numerous B cell, T-helper cell and cytotoxic T-lymphocyte epitopes (Paulij *et al*., 1999). Therefore this mutation may reduce the binding ability of the antibodies to the epitope and interfere with their neutralising effect.

All the participants of the study were over the age of 18. In South Africa vaccination against HBV was introduced into the Expanded Programme for Immunization (EPI) in 1995, therefore it is unlikely that any of the participants had received this vaccine. However, HBV immune/vaccine escape mutants in the HBsAg region were detected in three isolates (HJH01A, HJH12A and HJH12B) from Helen Joseph and 11 isolates from Shongwe. One Helen Joseph isolate (HJH07A) had escape mutant E164V. This mutation may interfere with recognition of HBsAg by antibodies, showing reduced binding to anti-HBs antibodies and has the potential of vaccine/immune escape (Torresi, 2002). The K122R mutation was detected in 2/8 isolates (HJH12A and HJH12B) from Helen Joseph. The amino acid at this position is used to determine the *d/y* specificities in the serological subtype and therefore this mutation results in the conversion of the *adw* subtype *to ayw* (Kramvis *et al*., 2008). The serological subtype *ayw* occurs in the minority of subgenotype A1 isolates (Kramvis *et al*,

2008) and is mainly confined to isolates mainly originating in Malawi (Sugauchi *et al*., 2003). Serological subtype *ayw* is mostly found in genotype D and have been isolated from genotype A isolates from South Africa (Bowyer et al., 1997a), Zimbabwe (Chirara and Chetsanga, 1994) and Cameroon (Norder et al., 1992b)

From the Shongwe Hospital study, five out of 15 HBsAg positive isolates and 6 out of 19 HBsAg negative isolates (*figure 3.15*) had immune/vaccine escape mutants. Six isolates had the vaccine escape mutant F129R and one with E164G/R. F120T occurred together with the E164G/R in two isolates. The F120T is a vaccine escape mutant which produces changes in the rtT128N in the polymerase protein and have been found to partially restore the in vitro replication phenotype of LMV resistant HBV (Torresi *et al*., 2002).

The following mutations in the 'a' determinant were found in different combinations in 8 isolates: C100Y, Q101H, M103L, T115R, S117N, T118K and N131K (*figure 3.14*). The HBsAg, which is the region containing the 'a' determinant (nt 99-150) is very significant for anti-HBIg binding and is targeted by antibodies used in standard HBV tests. Any alteration in this region may result in clinically significant mutations, which include, the vaccine/immune escape mutants, drug induced mutants and the 'a' determinant mutants. The mutations may result in false positive tests for HBsAg, which is used as one of the standard test to check for HBV. Therefore these mutations may be the reason for the HBsAg-negativity seen the patients infected with HBV isolates carrying these mutations.

Of great interest was the presence of previously identified reactivation markers (Henke-Gendo *et al*., 2008). The reactivation mutation markers V168A together with S174N occurred in 5/19 of the HBsAg negative patients and in none of the HBsAg positive patients ($P<0.05$). These have previously been shown to occur in patients who resolved HBV but in whom the infection was reactivated as a result of HIV immune suppression (Henke-Gendo *et al*., 2008).

The polymerase region is one of the most conserved regions since the polymerase plays a major role in the replication cycle of the virus. However, long term therapy with nucleotide(s) analogues can lead to selection of drug-resistant mutants, which over time may become dominant quasispecies. Because the polymerase region overlaps the HBsAg of the HBV genome, the drug-resistant mutations may also affect the antigenicity of the HBsAg and *vice versa*. Therefore these drug resistant mutants, selected during therapy, have the potential to become in addition immune/vaccine escape mutants (Sheldon *et al*., 2007, Torresi, 2002).

The overlapping polymerase region of HBV isolated from patients attending the Helen Joseph Hospital showed no drug resistant mutations. A few substitutions in the polymerase region were detected in HBV isolates from Shongwe, including, rtQ125E in 3 isolates and rtF109S, these mutations have not been previously described and their significance is unknown. Three Shongwe HBV isolates had drug resistant mutations. The rtV214A was detected in one HBsAg positive isolate and had the corresponding mutation Y206C in the HBsAg region. Lamivudine associated drug resistant mutants rtL180M together with rtM204V were detected in one HBsAg-negative isolate and results in the corresponding

HBsAg mutation, I195M. The I195M has been found to restore replication efficiency lost by the rtM204V in the polymerase region (Szomor *et al*., 2008, Yeh *et al*., 2000).

Another lamivudine-associated mutation, rtV173L occurred alone in one HBsAg negative isolate with the corresponding E164P mutation in the overlapping HBsAg region. The rtV173L together with rtL180M + rtM204V are lamivudine selected mutations which may cause vaccine escape mutants which may results in impaired secretion of the HBsAg (Sheldon *et al*., 2006).One stop codon mutation was detected at position sC69STOP of the HBsAg region in an isolate from a HBsAg-positive individual, who also had HBV with the rtV214A drug resistant mutation. Other lamivudine resistant mutations (rtA181T/sW172STOP, rtV191I/sW182STOP and rtV207I/sW199STOP) have been shown to cause premature stop codons in the HBsAg region, which may result in impairment of HBsAg secretion, resulting in HBsAg negativity (Sheldon and Soriano, 2008).

An unusual start codon mutation, rtE1D, was seen in eight isolates, which grouped in the "Asian" cluster following phylogenetic analysis. This mutation occurred together with rtS105T + rtH122N in 7 isolates and with rtQ125E in three isolates. After comparison of these isolates with 457 sequences from GenBank, the rtE1D mutation was found in isolates from genotype B and G from Asian countries. Glutamic acid (E) and aspartic acid (D) have similar chemical structures and properties; therefore this mutation is not expected to introduce a big functional change to the reverse transcriptase polymerase and the isolates are probably replicative.

All the mutations described occurred in genotype A. This is in agreement previous studies that demonstrated that compared to other genotypes, genotype A in HBV-HIV co-infected patients is more prone to drug associated mutations, immune/vaccine escape mutants, preS mutants associated with immune suppression and HCC (Thio *et al*., 2002, Audsley *et al*., 2010, Iacomi *et al*., 2009); and BCP mutations, which may result in HBeAg-negativity (Ahn et al., 2003, Kramvis et al., 1997b, Baptista et al., 1999, Laras et al., 1998). Considering that lamivudine is extensively used in South Africa as part of the ART regimen, the presence of these mutations, prior to the initiation of ART in HBV/HIV-infected individuals, is a significant observation, which needs to be investigated further.

Chapter 5: Conclusion

In conclusion, the study showed subgenotype A1 predominates in HBV-HIV co-infected individuals from both urban and rural South Africa. Subgenotype A1 HBV isolates have mutations that can affect HBeAg-expression at the transcriptional, translational and post-translational level (Kramvis and Kew, 1999) and these mutations can account for the HBeAg-negativity seen in the majority of HBV-HIV infected individuals from Shongwe. Although there were no significant differences between the mutations occurring in HBV from HBsAg positive and negative individuals, preS region mutations and deletions and "*a*" determinant, immune/vaccine escape mutants may account for HBsAg negativity seen in some patients. Moreover, the standard commercial tests being used currently in South Africa in the health services will be unable able to detect these HBsAg mutants and HBV infection will remain undetected. Nucleic acid detection of HBV can provide an alternative testing method for HBV because it can detect HBV DNA in the presence or absence of HBsAg in the serum. Deletion mutants have previously been shown to occur in HBV from HCC patients were detected in the present study. These could be a risk factor for the development of HCC in HIV patients, whose lifespan is being increased following the introduction of HAART. Thus more studies are necessary to functionally characterize these deletion mutants. The presence of drug resistant mutations in the polymerase region, prior to the initiation of HAART, which to date has contained Lamivudine, has important implications and repercussions. These mutants are replicative, can be transmitted and can cause liver disease including chronic hepatitis and eventually HCC. This study has provided important information on the molecular characteristics of HBV in HIV infected individuals prior to the initiation of ART, which may have important clinical relevance.

Chapter 6: REFERENCES

AHN, S. H., KRAMVIS, A., KAWAI, S., SPANGENBERG, H. C., LI, J., KIMBI, G., KEW, M., WANDS, J. & TONG, S. 2003. Sequence variation upstream of precore translation initiation codon reduces hepatitis B virus e antigen production. *Gastroenterology,* 125**,** 1370-1378.

ALLEN, M. I., DESLAURIERS, M., ANDREWS, C. W., TIPPLES, G. A., WALTERS, K.-A., TYRRELL, D. L., BROWN, N., GROUP, L. C. I. & CONDREAY, L. D. 1998. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. *Hepatology,* 27**,** 1670-1677.

ALTER, M. J. 2003. Epidemiology of hepatitis B in Europe and worldwide. *Journal of Hepatology,* 39.

ALTER, M. J. 2006. Epidemiology of viral hepatitis and HIV co-infection. *Journal of Hepatology,* 44**,** S6-S9.

ANDRÉ, F. 2000. Hepatitis B epidemiology in Asia, the Middle East and Africa. *Vaccine,* 18**,** S20-S22.

ARANKALLE, V. A., GANDHE, S. S., BORKAKOTY, B. J., WALIMBE, A. M., BISWAS, D. & MAHANTA, J. 2010. A novel HBV recombinant (genotype I) similar to Vietnam/Laos in a primitive tribe in eastern India. *Journal of Viral Hepatitis,* 17**,** 501-510.

ARAUZ-RUIZ, P., NORDER, H., ROBERTSON, B. H. & MAGNIUS, L. O. 2002. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol,* 83**,** 2059-2073.

ARAUZ-RUIZ, P., NORDER, H., VISONÁ, K. A. & MAGNIUS, L. O. 1997. Genotype F prevails in HBV infected patients of Hispanic origin in Central America and may carry the precore stop mutant. *Journal of Medical Virology,* 51**,** 305-312.

AUDSLEY, J., LITTLEJOHN, M., YUEN, L., SASADEUSZ, J., AYRES, A., DESMOND, C., SPELMAN, T., LAU, G., MATTHEWS, G. V., AVIHINGSANON, A., SEABERG, E., PHILP, F., SAULYNAS, M., RUXRUNGTHAM, K., DORE, G. J., LOCARNINI, S. A., THIO, C. L., LEWIN, S. R. & REVILL, P. A. 2010. HBV mutations in untreated HIV-HBV co-infection using genomic length sequencing. *Virology,* In Press, Corrected Proof.

BANCROFT, W. H., SNITBHAN, R., SCOTT, R. M., TINGPALAPONG, M., WATSON, W. T., TANTICHAROENYOS, P., KARWACKI, J. J. & SRIMARUT, S. 1977. Transmission of Hepatitis B Virus to Gibbons by Exposure to Human Saliva Containing Hepatitis B Surface Antigen. *The Journal of Infectious Diseases,* 135**,** 79-85.

BAPTISTA, M., KRAMVIS, A. & KEW, M. C. 1999. High prevalence of 1762^{T} 1764^{A} mutations in the basic core promoter of hepatitis B virus isolated from black africans with hepatocellular carcinoma compared with asymptomatic carriers. *Hepatology.*

BARTHOLOMEUSZ, A. & SCHAEFER, S. 2004. Hepatitis B virus genotypes: comparison of genotyping methods. *Reviews in Medical Virology,* 14**,** 3-16.

BAUMERT, T. F., THIMME, R. & VON WEIZSÄCKER, F. 2007. Pathogenesis of hepatitis B virus infection. *World J Gastroenterol,* 13.**,** 8.

BERTHELOT, K., MULDOON, M., RAJKOWITSCH, L., HUGHES, J. & MCCARTHY, J. E. G. 2004. Dynamics and processivity of 40S ribosome scanning on mRNA in yeast. *Molecular Microbiology,* 51**,** 987-1001.

BISCEGLIE A. M. DI, MASKEW MHAIRI, SCHULZE D, REYNEKE ANNE, MCNAMARA L & C, F. 2010. HIV–HBV coinfection among South African patients receiving antiretroviral therapy. *Antiviral Therapy,* 15 9.

BLOQUEL, B., JEULIN, H., BURTY, C., LETRANCHANT, L., RABAUD, C. & VENARD, V. 2010. Occult hepatitis B infection in patients infected with HIV: Report of two cases of hepatitis B reactivation and prevalence in a hospital cohort. *Journal of Medical Virology,* 82**,** 206-212.

BODSWORTH, N. J., COOPER, D. A. & DONOVAN, B. 1991. The Influence of Human Immunodeficiency Virus Type 1 Infection on the Development of the Hepatitis B Virus Carrier State. *The Journal of Infectious Diseases,* 163**,** 1138-1140.

BONHOEFFER, S. & SNIEGOWSKI, P. 2002. Virus evolution: The importance of being erroneous. *Nature,* 420**,** 367-369.

BORCHANI-CHABCHOUB, I., GARGOURI, A. & MOKDAD-GARGOURI, R. 2000. Genotyping of Tunisian hepatitis B virus isolates based on the sequencing of preS2 and S regions. *Microbes and Infection,* 2**,** 607-612.

BOTHA, J. F., DUSHEIKO, G. M., RITCHIE, M. J. J., MOUTON, H. W. K. & KEW, M. C. 1984. HEPATITIS B VIRUS CARRIER STATE IN BLACK CHILDREN IN OVAMBOLAND: ROLE OF PERINATAL AND HORIZONTAL INFECTION. *The Lancet,* 323**,** 1210-1212.

BOWYER, S., VAN STADEN, L., KEW, M. & SIM, J. 1997a. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. *J Gen Virol,* 78**,** 1719-1729.

BOWYER, S. M. & SIM, J. G. M. 2000. Relationships within and between genotypes of hepatitis B virus at points across the genome: footprints of recombination in certain isolates. *J Gen Virol,* 81**,** 379-392.

BOWYER, S. M., VAN STADEN, L., KEW, M. C. & SIM, J. G. 1997b. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. *J Gen Virol,* 78 (Pt 7)**,** 1719-29.

BUCKWOLD, V., XU, Z., CHEN, M., YEN, T. & OU, J. 1996. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. *Journal of Virology,* 70**,** 5845-5851.

BURNETT, R. J., FRANÇOIS, G., KEW, M. C., LEROUX-ROELS, G., MEHEUS, A., HOOSEN, A. A. & MPHAHLELE, M. J. 2005. Hepatitis B virus and human immunodeficiency virus co-infection in sub-Saharan Africa: a call for further investigation. *Liver International,* 25**,** 201-213.

BYUNG CHUL, Y., JOONG-WON, P., HYUNG JOON, K., DONG HO, L., YOUNG JU, C. & SILL MOO, P. 2003. Precore and core promoter mutations of hepatitis B virus and hepatitis B e antigen-negative chronic hepatitis B in Korea. *Journal of Hepatology,* 38**,** 98-103.

CARMAN, W. F. 1997. The clinical significance of surface antigen variants of hepatitis B virus. *Journal of Viral Hepatitis,* 4**,** 11-20.

CARMAN, W. F., HADZIYANNIS, S., MCGARVEY, M. J., JACYNA, M. R., KARAYIANNIS, P., MAKRIS, A. & THOMAS, H. C. 1989. MUTATION PREVENTING FORMATION OF HEPATITIS B e ANTIGEN IN PATIENTS WITH CHRONIC HEPATITIS B INFECTION. *The Lancet,* 334**,** 588-591.

CARMAN, W. F., KARAYIANNIS, P., WATERS, J., THOMAS, H. C., ZANETTI, A. R., MANZILLO, G. & ZUCKERMAN, A. J. 1990. Vaccine-induced escape mutant of hepatitis B virus. *The Lancet,* 336**,** 325-329.

CHANDRA, P. K., BISWAS, A., DATTA, S., BANERJEE, A., PANIGRAHI, R., CHAKRABARTI, S., DE, B. K. & CHAKRAVARTY, R. 2009. Subgenotypes of hepatitis B virus genotype D (D1, D2, D3 and D5) in India: differential pattern of mutations, liver injury and occult HBV infection. *Journal of Viral Hepatitis,* 16**,** 749-756.

CHEN & OON 2000. Mutations and deletions in core promoter and precore stop codon in relation to viral replication and liver damage in Singaporean hepatitis B virus carriers. *European Journal of Clinical Investigation,* 30**,** 787-792.

CHEN, B. F., LIU, C. J., JOW, G. M., CHEN, P. J., KAO, J. H. & CHEN, D. S. 2006. High Prevalence and Mapping of Pre-S Deletion in Hepatitis B Virus Carriers With Progressive Liver Diseases. *Gastroenterology,* 130**,** 1153-1168.

CHEN, C. Y., CROWTHER, C., KEW, M. C. & KRAMVIS, A. 2008a. A valine to phenylalanine mutation in the precore region of hepatitis B virus causes intracellular retention and impaired secretion of HBe-antigen. *Hepatology Research,* 38**,** 580-592.

CHEN, C. Y., CROWTHER, C., KEW, M. C. & KRAMVIS, A. 2008b. A valine to phenylalanine mutation in the precore region of hepatitis B virus causes intracellular retention and impaired secretion of HBe-antigen. *Hepatol Res,* 38**,** 580-92.

CHEN, M. T., BILLAUD, J. N., SALLBERG, M., GUIDOTTI, L. G., CHISARI, F. V., JONES, J., HUGHES, J. & MILICH, D. R. 2004. A function of the hepatitis B virus precore protein is to regulate the immune response to the core antigen. *Proc Natl Acad Sci U S A,* 101**,** 14913-8.

CHIRARA, M. M. & CHETSANGA, C. J. 1994. Variant of hepatitis B virus isolated in zimbabwe. *Journal of Medical Virology,* 42**,** 73-78.

COLIN, J.-F., CAZALS-HATEM, D., LORIOT, M. A., MARTINOT-PEIGNOUX, M., PHAM, B. N., AUPERIN, A., DEGOTT, C., BENHAMOU, J.-P., ERLINGER, S., VALLA, D. & MARCELLIN, P. 1999. Influence of human immunodeficiency virus infection on chronic hepatitis B in homosexual men. *Hepatology,* 29**,** 1306-1310.

COLLINSON, M. A., TOLLMAN, S. M. & KAHN, K. 2007. Migration, settlement change and health in post-apartheid South Africa: Triangulating health and demographic

surveillance with national census data1. *Scandinavian Journal of Public Health,* 35**,** 77-84.

COOLEY, L., AYRES, A., BARTHOLOMEUSZ, A., LEWIN, S., CROWE, S., MIJCH, A., LOCARNINI, S. & SASADEUSZ, J. 2003. Prevalence and characterization of lamivudine-resistant hepatitis B virus mutations in HIV-HBV co-infected individuals. *AIDS,* 17**,** 1649-1657.

DE MADDALENA, C., GIAMBELLI, C., TANZI, E., COLZANI, D., SCHIAVINI, M., MILAZZO, L., BERNINI, F., EBRANATI, E., CARGNEL, A., BRUNO, R., GALLI, M. & ZEHENDER, G. 2007. High level of genetic heterogeneity in S and P genes of genotype D hepatitis B virus. *Virology,* 365**,** 113-124.

DEVESA, M., LOUREIRO, C., RIVAS, Y., MONSALVE, F., CARDONA, N., DUARTE, M., POBLETE, F., GUTIERREZ, M., BOTTO, C. & PUJOL, F. 2008. Subgenotype diversity of hepatitis B virus American genotype F in Amerindians from Venezuela and the general population of Colombia. *Journal of Medical Virology,* 80**,** 20-26.

DIBISCEGLIE, A. M., KEW, M. C., DUSHEIKO, G. M., BERGER, E. L., SONG, E., PATERSON, A. C. & HODKINSON, H. J. 1986. Prevalence Of Hepatitis B Virus Infection Among Black Children In Soweto. *British Medical Journal (Clinical Research Edition),* 292**,** 1440-1442.

ESTACIO, R. C., CHAVEZ, C. C., OKAMOTO, H., LINGAO, A. L., REYES, M. T., DOMINGO, E. & MAYUMI, M. 1988. Nucleotide sequence of a hepatitis B virus genome of subtype adw isolated from a Philippine: Comparison with the reported three genomes of the same subtype. *Journal of Gastroenterology and Hepatology,* 3**,** 215-222.

FARES, M. A. & HOLMES, E. C. 2002. A Revised Evolutionary History of Hepatitis B Virus (HBV). *Journal of Molecular Evolution,* 54**,** 807-814.

FATTOVICH, G. 2003. Natural history of hepatitis B. *Journal of Hepatology,* 39**,** 50-58.

FIRNHABER, C., REYNEKE, A., SCHULZE, D., MALOPE, B., MASKEW, M., PATRICK, M., SANNE, I. & DI BISCEGLIE, A. 2008. The prevalence of hepatitis B co-infection in a South African urban government HIV clinic. *S Afr Med J* . 98:**,** 541-.

FIRNHABER, C., VIANA, R., REYNEKE, A., SCHULTZE, D., MALOPE, B., MASKEW, M., DI BISCEGLIE, A., MACPHAIL, P., SANNE, I. & KEW, M. 2009. Occult hepatitis B virus infection in patients with isolated core antibody and HIV co-infection in an urban clinic in Johannesburg, South Africa. *International Journal of Infectious Diseases,* 13**,** 488-492.

FIRNHABER, C. S. & IVE, P. 2009. HEPATITIS B AND HIV CO-INFECTION IN SOUTH AFRICA: JUST TREAT IT! *South African Journal of Medicine,* 2**,** 4.

FIX, O. K., LOCARNINI, S. A. & PETERS, M. G. 2007. Virology And Clinical Management of Hepatitis B And HIV Coinfection. *Physicians' Research Network,* 11**,** 8.

FORBI, J. C., VAUGHAN, G., PURDY, M. A., CAMPO, D. S., XIA, G.-L., GANOVA-RAEVA, L. M., RAMACHANDRAN, S., THAI, H. & KHUDYAKOV, Y. E. 2010. Epidemic History and Evolutionary Dynamics of Hepatitis B Virus Infection in Two Remote Communities in Rural Nigeria. *PLoS ONE,* 5**,** e11615.

FRANCOIS, G., DOCHEZ, C, MPHAHLELE, J.M, BURNETT, R, VAN HAL, G, MEHEUS, A 2008. Hepatitis B vaccination in Africa: mission accomplished? *The Southern African Journal of Epidemiology and Infection,* 23**,** 24-28.

FRANÇOIS, G., KEW, M., VAN DAMME, P., MPHAHLELE, M. J. & MEHEUS, A. 2001. Mutant hepatitis B viruses: a matter of academic interest only or a problem with far-reaching implications? *Vaccine,* 19**,** 3799-3815.

GRAY, D., L., WEBER, D. & LEMON, S. 1989. HORIZONTAL TRANSMISSION OF HEPATITIS B VIRUS. *The Lancet,* 333**,** 889-893.

GÜNTHER, S. 2006. Genetic variation in HBV infection: genotypes and mutants. *Journal of Clinical Virology,* 36**,** S3-S11.

GUNTHER, S., PIWON, N., IWANSKA, A., SCHILLING, R., MEISEL, H. & WILL, H. 1996. Type, prevalence, and significance of core promoter/enhancer II mutations in hepatitis B viruses from immunosuppressed patients with severe liver disease. *Journal of Virology,* 70**,** 8318-8331.

GUNTHER, S., SOMMER, G., VON BREUNIG, F., IWANSKA, A., KALININA, T., STERNECK, M. & WILL, H. 1998. Amplification of Full-Length Hepatitis B Virus Genomes from Samples from Patients with Low Levels of Viremia: Frequency and Functional Consequences of PCR-Introduced Mutations. *J. Clin. Microbiol.,* 36**,** 531-538.

HADLER, S. C., JUDSON, F. N., O'MALLEY, P. M., ALTMAN, N. L., PENLEY, K., BUCHBINDER, S., SCHABLE, C. A., COLEMAN, P. J., OSTROW, D. N. & FRANCIS, D. P. 1991. Outcome of Hepatitis B Virus Infection in Homosexual Men and Its Relation to Prior Human Immunodeficiency Virus Infection. *The Journal of Infectious Diseases,* 163**,** 454-459.

HANNOUN, C., NORDER, H. & LINDH, M. 2000. An aberrant genotype revealed in recombinant hepatitis B virus strains from Vietnam. *J Gen Virol,* 81**,** 2267-2272.

HARRISON, T. J. 2006. Hepatitis B Virus: Molecular Virology and Common Mutants. *Semin Liver Dis,* 26**,** 087,096.

HAZENBERG, M. D., OTTO, S. A., VAN BENTHEM, B. H., ROOS, M. T., COUTINHO, R. A., LANGE, J. M., HAMANN, D., PRINS, M. & MIEDEMA, F. 2003. Persistent

immune activation in HIV-1 infection is associated with progression to AIDS. *AIDS,* 17**,** 1881-1888.

HENKE-GENDO, C., AMINI-BAVIL-OLYAEE, S., CHALLAPALLI, D., TRAUTWEIN, C., DEPPE, H., SCHULZ, THOMAS F., HEIM, A. & TACKE, F. 2008. Symptomatic Hepatitis B Virus (HBV) Reactivation despite Reduced Viral Fitness Is Associated with HBV Test and Immune Escape Mutations in an HIV-Coinfected Patient. *The Journal of Infectious Diseases,* 198**,** 1620-1624.

HESS, G., ROSSOL, S., VOTH, R., WEBER, K. C. & MEYER ZUM BÜSCHENFELDE, K. H. 1989. Modification of the immune response against hepatitis B virus by the human immunodeficiency virus. *Rheumatology International,* 9**,** 175-179.

HILDT, E., SAHER, G., BRUSS, V. & HOFSCHNEIDER, P. H. 1996. The Hepatitis B Virus Large Surface Protein (LHBs) Is a Transcriptional Activator. *Virology,* 225**,** 235-239.

HIROSHI, Y., KUNIHIKO, H., EIICHI, T., JOJI, T., KIYOMI, Y. & SHIRO, I. 2002. Precore and core promoter mutations, hepatitis B virus DNA levels and progressive liver injury in chronic hepatitis B. *Journal of Hepatology,* 37**,** 355-363.

HORVATH, J. & RAFFANTI, S. P. 1994. Clinical Aspects of the Interactions Between Human Immunodeficiency Virus and the Hepatotropic Viruses. *Clinical Infectious Diseases,* 18**,** 339-347.

HOU, J., LIU, Z. & GU, F. 2005. Epidemiology and Prevention of Hepatitis B Virus Infection. *International Journal of Medical Sciences,* 2**,** 50-57.

HSIEH, Y.-H., SU, I.-J., WANG, H.-C., CHANG, W.-W., LEI, H.-Y., LAI, M.-D., CHANG, W.-T. & HUANG, W. 2004. Pre-S mutant surface antigens in chronic hepatitis B virus infection induce oxidative stress and DNA damage. *Carcinogenesis,* 25**,** 2023-2032.

HSU, H.-Y., CHANG, M.-H., LIAW, S.-H., NI, Y.-H. & CHEN, H.-L. 1999. Changes of hepatitis B surface antigen variants in carrier children before and after universal vaccination in taiwan. *Hepatology,* 30**,** 1312-1317.

HÜBSCHEN, J. M., MBAH, P. O., FORBI, J. C., OTEGBAYO, J. A., OLINGER, C. M., CHARPENTIER, E. & MULLER, C. P. 2010. Detection of a new subgenotype of hepatitis B virus genotype A in Cameroon but not in neighbouring Nigeria. *Clinical Microbiology and Infection***,** no-no.

HUY, T. T.-T., USHIJIMA, H., WIN, K. M., LUENGROJANAKUL, P., SHRESTHA, P. K., ZHONG, Z.-H., SMIRNOV, A. V., TALTAVULL, T. C., SATA, T. & ABE, K. 2003. High Prevalence of Hepatitis B Virus Pre-S Mutant in Countries Where It Is Endemic and Its Relationship with Genotype and Chronicity. *J. Clin. Microbiol.,* 41**,** 5449-5455.

HUY, T. T. T., NGOC, T. T. & ABE, K. 2008. New Complex Recombinant Genotype of Hepatitis B Virus Identified in Vietnam. *Journal of Virology,* 82**,** 5657-5663.

HUY, T. T. T., USHIJIMA, H., SATA, T. & ABE, K. 2006. Genomic characterization of HBV genotype F in Bolivia: genotype F subgenotypes correlate with geographic distribution and T¹⁸⁵⁸ variant. *Archives of Virology,* 151**,** 589-597.

IACOMI, F., VINCENTI, D., VAIRO, F., SOLMONE, M., MARIANO, A., PISELLI, P., PURO, V., CAPOBIANCHI, M. R. & ANTONUCCI, G. 2009. Effect of HIV co-infection on mutation patterns of HBV in patients with lamivudine-resistant chronic hepatitis B. *Journal of Medical Virology,* 81**,** 1151-1156.

IDOKO, J., MELONI, S., MUAZU, M., NIMZING, L., BADUNG, B., HAWKINS, C., SANKALÃ©, J. L., EKONG, E., MURPHY, R., KANKI, P. & THIO, CHLOEÂ L. 2009. Impact of Hepatitis B Virus Infection on Human Immunodeficiency Virus

Response to Antiretroviral Therapy in Nigeria. *Clinical Infectious Diseases,* 49**,** 1268-1273.

JILBERT, A. R., BURRELL, C. J., TRIANI, M. & KANN, M. 2002. Hepatitis B Virus Replication. *In:* LAI, C. L. & LOCARNINI, S. (eds.) *Hepatitis B Virus.* London: International Medical Press.

JILG, W., DELHOUNE, C., DEINHARDT, F., ROUMELIOTOU-KARAYANNIS, A. J., PAPAEVANGELOU, G. J., MUSHAHWAR, I. K. & OVERBY, L. R. 1984. Hepatitis B surface antigen (HBsAg) subtype-specific antibodies in persons vaccinated against hepatitis B. *Journal of Medical Virology,* 13**,** 171-178.

KANN, M. 2002. Structural and Molecular Virology. *In:* LAI, C. L. & LOCARNINI, S. (eds.) *Hepatitis B Virus.* London: International medical press.

KAO, J.-H. 2002. Hepatitis B viral genotypes: Clinical relevance and molecular characteristics. *Journal of Gastroenterology and Hepatology,* 17**,** 643-650.

KATO, H., ORITO, E., GISH, R. G., SUGAUCHI, F., SUZUKI, S., UEDA, R., MIYAKAWA, Y. & MIZOKAMI, M. 2002. Characteristics of Hepatitis B Virus Isolates of Genotype G and Their Phylogenetic Differences from the Other Six Genotypes (A through F). *Journal of Virology,* 76**,** 6131-6137.

KEW, M. C. 1996. Progress towards the comprehensive control of hepatitis B in Africa: a view from South Africa. *Gut,* 38**,** S31-S36.

KEW, M. C., KRAMVIS, A., YU, M. C., ARAKAWA, K. & HODKINSON, J. 2005. Increased hepatocarcinogenic potential of hepatitis B virus genotype A in Bantu-speaking sub-saharan Africans. *Journal of Medical Virology,* 75**,** 513-521.

KIDD-LJUNGGREN, K., MIYAKAWA, Y. & KIDD, A. H. 2002. Genetic variability in hepatitis B viruses. *J Gen Virol,* 83**,** 1267-1280.

KIM, H., RYU, C. & HONG, H. 1997. Hepatitis B virus preS1 functions as a transcriptional activation domain. *J Gen Virol,* 78**,** 1083-1086.

KIMBI, G. C., KRAMVIS, A. & KEW, M. C. 2004. Distinctive sequence characteristics of subgenotype A1 isolates of hepatitis B virus from South Africa. *J Gen Virol,* 85**,** 1211-1220.

KLEINSCHMIDT, I., PETTIFOR, A., MORRIS, N., MACPHAIL, C. & REES, H. 2007. Geographic Distribution of Human Immunodeficiency Virus in South Africa. *Am J Trop Med Hyg,* 77**,** 1163-1169.

KOZAK, M. 1999. Initiation of translation in prokaryotes and eukaryotes. *Gene,* 234**,** 187-208.

KRAMVIS, A., ARAKAWA, K., YU, M. C., NOGUEIRA, R., STRAM, D. O. & KEW, M. C. 2008. Relationship of serological subtype, basic core promoter and precore mutations to genotypes/subgenotypes of hepatitis B virus. *Journal of Medical Virology,* 80**,** 27-46.

KRAMVIS, A., BUKOFZER, S., KEW, M. C. & SONG, E. 1997a. Nucleic acid sequence analysis of the precore region of hepatitis B virus from sera of southern African black adult carriers of the virus. *Hepatology,* 25**,** 235-40.

KRAMVIS, A., BUKOFZER, S., KEW, M. C. & SONG, E. 1997b. Nucleic acid sequence analysis of the precore region of hepatitis B virus from sera of southern African black adult carriers of the virus. *Hepatology,* 25**,** 235-240.

KRAMVIS, A., KEW, M. & FRANCOIS, G. 2005a. Hepatitis B virus genotypes. *Vaccine,* 23**,** 2409-23.

KRAMVIS, A., KEW, M. & FRANÇOIS, G. 2005b. Hepatitis B virus genotypes. *Vaccine,* 23**,** 2409-2423.

KRAMVIS, A. & KEW, M. C. 1998. Structure and function of the encapsidation signal of hepadnaviridae. *Journal of Viral Hepatitis,* 5**,** 357-367.

KRAMVIS, A. & KEW, M. C. 1999. The core promoter of hepatitis B virus. *Journal of Viral Hepatitis,* 6**,** 415-427.

KRAMVIS, A. & KEW, M. C. 2005. Relationship of genotypes of hepatitis B virus to mutations, disease progression and response to antiviral therapy. *Journal of Viral Hepatitis,* 12**,** 456-464.

KRAMVIS, A. & KEW, M. C. 2007a. Epidemiology of hepatitis B virus in Africa, its genotypes and clinical associations of genotypes. *Hepatology Research,* 37**,** S9-S19.

KRAMVIS, A. & KEW, M. C. 2007b. Epidemiology of hepatitis B virus in Africa, its genotypes and clinical associations of genotypes. *Hepatol Res,* 37**,** S9-S19.

KRAMVIS, A. & KEW, M. C. 2007c. Molecular characterization of subgenotype A1 (subgroup Aa) of hepatitis B virus. *Hepatology Research,* 37**,** S27-S32.

KRAMVIS, A., KEW, M. C. & BUKOFZER, S. 1998a. Hepatitis B virus precore mutants in serum and liver of Southern African Blacks with hepatocellular carcinoma. *Journal of Hepatology,* 28**,** 132-141.

KRAMVIS, A., KEW, M. C. & BUKOFZER, S. 1998b. Hepatitis B virus precore mutants in serum and liver of Southern African Blacks with hepatocellular carcinoma. *J Hepatol,* 28**,** 132-41.

KRAMVIS, A., WEITZMANN, L., OWIREDU, W. K. B. A. & KEW, M. C. 2002. Analysis of the complete genome of subgroup A' hepatitis B virus isolates from South Africa. *J Gen Virol,* 83**,** 835-839.

KURBANOV, F., TANAKA, Y., FUJIWARA, K., SUGAUCHI, F., MBANYA, D., ZEKENG, L., NDEMBI, N., NGANSOP, C., KAPTUE, L., MIURA, T., IDO, E., HAYAMI, M., ICHIMURA, H. & MIZOKAMI, M. 2005. A new subtype

(subgenotype) Ac (A3) of hepatitis B virus and recombination between genotypes A and E in Cameroon. *J Gen Virol,* 86**,** 2047-2056.

LAI, C. & LOCARNINI, S. 2008. *Hepatitis B virus*, International Medical Press.

LAI, C. L., LOCARNINI,S. (ed.) 2002. *Hepatitis B virus,* London: international Medical Press.

LAPERCHE, S., GIRAULT, A., BEAULIEU, M. J., BOUCHARDEAU, F. & COUROUCÉ, A. M. 2001. Determination of hepatitis B virus subtypes by an enzyme immunoassay method using monoclonal antibodies to type-specific epitopes of HBsAg. *Journal of Viral Hepatitis,* 8**,** 447-453.

LARAS, A., KOSKINAS, J., AVGIDIS, K. & HADZIYANNIS, S. J. 1998. Incidence and clinical significance of hepatitis B virus precore gene translation initiation mutations in e antigen-negative patients. *Journal of Viral Hepatitis,* 5**,** 241-248.

LASKUS, T., RAKELA, J., NOWICKI, M. J. & PERSING, D. H. 1995. Hepatitis B virus core promoter sequence analysis in fulminant and chronic hepatitis B. *Gastroenterology,* 109**,** 1618-1623.

LIM, C. K., TAN,J.T. M, KHOO J.B.S, RAVICHANDRAN, A, MEI, H, LOW, Y.C, & **TON, A. S. H.** 2006. Correlations of HBV Genotypes, Mutations Affecting HBeAg Expression and HBeAg/anti-HBe Status in HBV Carriers. *International Journal of Medical Sciences,* 3**,** 14-20.

LINDH, M., ANDERSSON, A.-S. & GUSDAL, A. 1997. Genotypes, nt 1858 Variants, and Geographic Origin of Hepatitis B Virus: Large-Scale Analysis Using a New Genotyping Method. *The Journal of Infectious Diseases,* 175**,** 1285-1293.

LOCARNINI, S., MCMILLAN, J. & BARTHOLOMEUSZ, A. 2003. The Hepatitis B Virus and Common Mutants. *Semin Liver Dis,* 23**,** 005,020.

LOCARNINI, S. A. 1998. Hepatitis B virus surface antigen and polymerase gene variants: Potential virological and clinical significance. *Hepatology,* 27**,** 294-297.

LOK, A. S., AKARCA, U. & GREENE, S. 1994. Mutations in the pre-core region of hepatitis B virus serve to enhance the stability of the secondary structure of the pre-genome encapsidation signal. *Proceedings of the National Academy of Sciences of the United States of America,* 91**,** 4077-4081.

LUKHWARENI, A., BURNETT, R. J., SELABE, S. G., MZILENI, M. O. & MPHAHLELE, M. J. 2009. Increased detection of HBV DNA in HBsAg-positive and HBsAg-negative South African HIV/AIDS patients enrolling for highly active antiretroviral therapy at a Tertiary Hospital. *Journal of Medical Virology,* 81**,** 406-412.

MACDONALD, D. M., HOLMES, E. C., LEWIS, J. C. M. & SIMMONDS, P. 2000a. Detection of Hepatitis B Virus Infection in Wild-Born Chimpanzees (Pan troglodytes verus): Phylogenetic Relationships with Human and Other Primate Genotypes. *Journal of Virology,* 74**,** 4253-4257.

MACDONALD, D. M., HOLMES, E. C., LEWIS, J. C. M. & SIMMONDS, P. 2000b. Detection of Hepatitis B Virus Infection in Wild-Born Chimpanzees (Pan troglodytes verus): Phylogenetic Relationships with Human and Other Primate Genotypes. *J. Virol.,* 74**,** 4253-4257.

MAHONEY, F. J. 1999. Update on Diagnosis, Management, and Prevention of Hepatitis B Virus Infection. *Clin. Microbiol. Rev.,* 12**,** 351-366.

MAKUWA, M., SOUQUIÈRE, S., TELFER, P., APETREI, C., VRAY, M., BEDJABAGA, I., MOUINGA-ONDEME, A., ONANGA, R., MARX, P. A., KAZANJI, M., ROQUES, P. & SIMON, F. 2006. Identification of hepatitis B virus subgenotype A3 in rural Gabon. *Journal of Medical Virology,* 78**,** 1175-1184.

MARRONE, A., NIRO, G. A., ZAMPINO, R., COSTAGLIOLA, L., CIRILLO, G., COPPOLA, N., CAFARO, M., MEROLA, A., GUERRERA, B., CIOTTI, M., FONTANA, R., ADINOL, L. E., ANDRIULLI, A. & RUGGIERO, G. 2008. 566 HEPATITIS B VIRUS (HBV) CORE PROMOTER (CP), PRECORE (PC) AND SURFACE MUTATIONS IN HEPATITIS D VIRUS (HDV) INFECTION: A MOLECULAR EPIDEMIOLOGY STUDY. *Journal of Hepatology,* 48**,** S212.

MASSON, W. & LITTWIN, S. 2002. pathogenesis of hepadavirus infectios. *In:* LAI, C. L. & LOCARNINI, S. (eds.) *Hepatitis BVirus.* London: International Medical Press.

MAYERAT, C., MANTEGANI, A., SPERTINI, F. & FREI, P. C. 1999. Mutations in the Basal Core Promoter and Precore/Core Gene of Hepatitis B Virus in Patients with Chronic Active but not Acute Hepatitis B. *European Journal of Clinical Microbiology & Infectious Diseases,* 18**,** 871-878.

MCCORMACK, G. P. & CLEWLEY, J. P. 2002. The application of molecular phylogenetics to the analysis of viral genome diversity and evolution. *Reviews in Medical Virology,* 12**,** 221-238.

MCIVER, C. J., JACQUES, C. F. H., CHOW, S. S. W., MUNRO, S. C., SCOTT, G. M., ROBERTS, J. A., CRAIG, M. E. & RAWLINSON, W. D. 2005. Development of Multiplex PCRs for Detection of Common Viral Pathogens and Agents of Congenital Infections. *J. Clin. Microbiol.,* 43**,** 5102-5110.

MCMAHON, B. J., ALWARD, W. L. M., HALL, D. B., HEYWARD, W. L., BENDER, T. R., FRANCIS, D. P. & MAYNARD, J. E. 1985. Acute Hepatitis B Virus Infection: Relation of Age to the Clinical Expression of Disease and Subsequent Development of the Carrier State. *The Journal of Infectious Diseases,* 151**,** 599-603.

MCMAHON, B. J. & SPACH, D. H. 2005. *Natural History of Chronic Hepatitis B Infection* [Online]. Available:

http://depts.washington.edu/hepstudy/hepB/history/chronic/index.shtml [Accessed 07 November 2010 2010].

MILICH, D. & LIANG, T. J. 2003. Exploring the biological basis of hepatitis B e antigen in hepatitis B virus infection. *Hepatology,* 38**,** 1075-1086.

MOOLLA, N., KEW, M. & ARBUTHNOT, P. 2002. Regulatory elements of hepatitis B virus transcription. *Journal of Viral Hepatitis,* 9**,** 323-331.

MPHAHLELE, M. J., LUKHWARENI, A., BURNETT, R. J., MOROPENG, L. M. & NGOBENI, J. M. 2006. High risk of occult hepatitis B virus infection in HIV-positive patients from South Africa. *Journal of Clinical Virology,* 35**,** 14-20.

MULDERS, MICK N., VENARD, V., NJAYOU, M., EDORH, A. P., BOLA OYEFOLU, AKEEB O., KEHINDE, M. O., MUYEMBE TAMFUM, J. J., NÉBIE, YACOUBA K., MAÏGA, I., AMMERLAAN, W., FACK, F., OMILABU, SUNDAY A., LE FAOU, A. & MULLER, CLAUDE P. 2004. Low Genetic Diversity despite Hyperendemicity of Hepatitis B Virus Genotype E throughout West Africa. *The Journal of Infectious Diseases,* 190**,** 400-408.

NASSAL, M. & SCHALLER, H. 1993. Hepatitis B virus replication. *Trends in Microbiology,* 1**,** 221-228.

NAUMANN, H., SCHAEFER, S., YOSHIDA, C. F. T., GASPAR, A. M. C., REPP, R. & GERLICH, W. H. 1993. Identification of a new hepatitis B virus (HBV) genotype from Brazil that expresses HBV surface antigen subtype adw4. *J Gen Virol,* 74**,** 1627-1632.

NEURATH, A. R., KENT, S. B. H., STRICK, N. & PARKER, K. 1986. Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus. *Cell,* 46**,** 429-436.

NORDER, H., COUROUCE, A.-M. & MAGNIUS, L. O. 1992a. Molecular basis of hepatitis B virus serotype variations within the four major subtypes. *J Gen Virol,* 73**,** 3141-3145.

NORDER, H., COUROUCÉ, A.-M. & MAGNIUS, L. O. 1994. Complete Genomes, Phylogenetic Relatedness, and Structural Proteins of Six Strains of the Hepatitis B Virus, Four of Which Represent Two New Genotypes. *Virology,* 198**,** 489-503.

NORDER, H., EBERT, J. W., FIELDS, H. A., MUSHAHWAR, I. K. & MAGNIUS, L. O. 1996. Complete Sequencing of a Gibbon Hepatitis B Virus Genome Reveals a Unique Genotype Distantly Related to the Chimpanzee Hepatitis B Virus. *Virology,* 218**,** 214-223.

NORDER, H., HAMMAS, B., LOFDAHL, S., COUROUCE, A.-M. & MAGNIUS, L. O. 1992b. Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. *J Gen Virol,* 73**,** 1201-1208.

NUNEZ, M., RAMOS, B., DIAZ-POLLAN, B., CAMINO, N., MARTIN-CARBONERO, L., BARREIRO, P., GONZALEZ-LAHOZ, J. & SORIANO, V. 2006. Virological Outcome of Chronic Hepatitis B Virus Infection in HIV-Coinfected Patients Receiving Anti-HBV Active Antiretroviral Therapy. *AIDS Research and Human Retroviruses,* 22**,** 842-848.

OKAMOTO, H., IMAI, M., TSUDA, F., TANAKA, T., MIYAKAWA, Y. & MAYUMI, M. 1987. Point mutation in the S gene of hepatitis B virus for a d/y or w/r subtypic change in two blood donors carrying a surface antigen of compound subtype adyr or adwr. *Journal of Virology,* 61**,** 3030-4.

OKAMOTO, H., TSUDA, F., SAKUGAWA, H., SASTROSOEWIGNJO, R. I., IMAI, M., MIYAKAWA, Y. & MAYUMI, M. 1988. Typing Hepatitis B Virus by Homology in

Nucleotide Sequence: Comparison of Surface Antigen Subtypes. *J Gen Virol,* 69**,** 2575-2583.

OLINGER, C. M., JUTAVIJITTUM, P., HÜBSCHEN, J. M., YOUSUKH, A., SAMOUNTRY, B., THAMMAVONG, T., TORIYAMA, K. & MULLER, C. P. 2008. Possible New Hepatitis B Virus Genotype, Southeast Asia. Centers for Disease Control and Prevention.

OLINGER, C. M., VENARD, V., NJAYOU, M., OYEFOLU, A. O. B., MAIGA, I., KEMP, A. J., OMILABU, S. A., LE FAOU, A. & MULLER, C. P. 2006. Phylogenetic analysis of the precore/core gene of hepatitis B virus genotypes E and A in West Africa: new subtypes, mixed infections and recombinations. *J Gen Virol,* 87**,** 1163-1173.

ORITO, E., ICHIDA, T., SAKUGAWA, H., SATA, M., HORIIKE, N., HINO, K., OKITA, K., OKANOUE, T., IINO, S., TANAKA, E., SUZUKI, K., WATANABE, H., HIGE, S. & MIZOKAMI, M. 2001. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology,* 34**,** 590-594.

ORITO, E., MIZOKAMI, M., INA, Y., MORIYAMA, E. N., KAMESHIMA, N., YAMAMOTO, M. & GOJOBORI, T. 1989. Host-independent evolution and a genetic classification of the hepadnavirus family based on nucleotide sequences. *Proceedings of the National Academy of Sciences of the United States of America,* 86**,** 7059-7062.

OWIREDU, W. K. B. A., KRAMVIS, A. & KEW, M. C. 2001. Molecular analysis of hepatitis B virus genomes isolated from black African patients with fulminant hepatitis B. *Journal of Medical Virology,* 65**,** 485-492.

PATIENT, R., HOURIOUX, C. & ROINGEARD, P. 2009. Morphogenesis of hepatitis B virus and its subviral envelope particles. *Cellular Microbiology,* 11**,** 1561-1570.

PATIENT, R., HOURIOUX, C., SIZARET, P.-Y., TRASSARD, S., SUREAU, C. & ROINGEARD, P. 2007. Hepatitis B Virus Subviral Envelope Particle Morphogenesis and Intracellular Trafficking. *Journal of Virology,* 81**,** 3842-3851.

PAULIJ, W. P., DE WIT, P. L. M., SUNNEN, C. M. G., VAN ROOSMALEN, M. H., PETERSEN-VAN ETTEKOVEN, A., COOREMAN, M. P. & HEIJTINK, R. A. 1999. Localization of a unique hepatitis B virus epitope sheds new light on the structure of hepatitis B virus surface antigen. *J Gen Virol,* 80**,** 2121-2126.

PÉREZ-OLMEDA, M., NÚÑEZ, M., GARCÍA-SAMANIEGO, J., RÍOS, P., GONZÁLEZ-LAHOZ, J. & SORIANO, V. 2003. Distribution of Hepatitis B Virus Genotypes in HIV-Infected Patients with Chronic Hepatitis B: Therapeutic Implications. *AIDS Research and Human Retroviruses,* 19**,** 657-659.

PETERS, M. G. 2007. Diagnosis ans Management of Hepatitis B Virus and HIV Co-infection. *International AIDS Society-USA.* San Francisco: Topics in HIV Medicine.

POURKARIM, M. R., LEMEY, P., AMINI-BAVIL-OLYAEE, S., MAES, P. & VAN RANST, M. 2010. Novel hepatitis B virus subgenotype A6 in African-Belgian patients. *Journal of Clinical Virology,* 47**,** 93-96.

QUARLERI, J., MORETTI, F., BOUZAS, M. B., LAUFER, N., CARRILLO, M. G. M., GIULIANO, S. F. N., PÃ©REZ, H., CAHN, P. & SALOMON, H. 2007. Hepatitis B Virus Genotype Distribution and Its Lamivudine-Resistant Mutants in HIV-Coinfected Patients with Chronic and Occult Hepatitis B. *AIDS Research and Human Retroviruses,* 23**,** 525-531.

RAIMONDO, G., CAMPO, S., SMEDILE, V., RODINO, G., SARDO, M. A., BRANCATELLI, S., VILLARI, D., PERNICE, M., LONGO, G. & SQUADRITO, G. 1991. Hepatitis B virus variant, with a deletion in the preS2 and two translational stop

codons in the precore regions, in a patient with hepatocellular carcinoma. *Journal of Hepatology,* 13**,** S74-S77.

RAMOS, B. N., NÃºÃ±EZ, M., MARTÃ-N-CARBONERO, L., SHELDON, J., RIOS, P., LABARGA, P., ROMERO, M., BARREIRO, P., GARCÃ-A-SAMANIEGO, J. & SORIANO, V. 2007. Hepatitis B Virus Genotypes and Lamivudine Resistance Mutations in HIV/Hepatitis B Virus-Coinfected Patients. *JAIDS Journal of Acquired Immune Deficiency Syndromes,* 44**,** 557-561 10.1097/QAI.0b013e3180314b46.

REVILL, P., YUEN, L., WALSH, R., PERRAULT, M., LOCARNINI, S. & KRAMVIS, A. 2010. Bioinformatic analysis of the hepadnavirus e-antigen and its precursor identifies remarkable sequence conservation in all orthohepadnaviruses. *Journal of Medical Virology,* 82**,** 104-115.

RODRIGUEZ-FRIAS, F., BUTI, M., JARDI, R., COTRINA, M., VILADOMIU, L., ESTEBAN, R. & GUARDIA, J. 1995. Hepatitis B virus infection: Precore mutants and its relation to viral genotypes and core mutations. *Hepatology,* 22**,** 1641-1647.

SAKAMOTO, T., TANAKA, Y., ORITO, E., CO, J., CLAVIO, J., SUGAUCHI, F., ITO, K., OZASA, A., QUINO, A., UEDA, R., SOLLANO, J. & MIZOKAMI, M. 2006. Novel subtypes (subgenotypes) of hepatitis B virus genotypes B and C among chronic liver disease patients in the Philippines. *J Gen Virol,* 87**,** 1873-1882.

SALISSE, J. & SUREAU, C. 2009. A Function Essential to Viral Entry Underlies the Hepatitis B Virus "a" Determinant. *Journal of Virology,* 83**,** 9321-9328.

SÁNCHEZ, L. V., MALDONADO, M., BASTIDAS-RAMÍREZ, B. E., NORDER, H. & PANDURO, A. 2002. Genotypes and S-gene variability of Mexican hepatitis B virus strains. *Journal of Medical Virology,* 68**,** 24-32.

SBAI, A., BENNANI, A., BENJOUAD, A. & HASSAR, M. 2007. HBV genotypes in Morocco. *Journal of Clinical Virology,* 38**,** 184-185.

SCAGLIONI, P. P., MELEGARI, M. & WANDS, J. R. 1996. Recent advances in the molecular biology of hepatitis B virus. *Bailliére's Clinical Gastroenterology,* 10**,** 207-225.

SCHÄDLER, S. & HILDT, E. 2009. HBV Life Cycle: Entry and Morphogenesis. *Viruses,* 1**,** 185-209.

SCHAEFER, S. 2005. Hepatitis B virus: significance of genotypes. *Journal of Viral Hepatitis,* 12**,** 111-124.

SEEGER, C. & MASON, W. S. 2000. Hepatitis B Virus Biology. *Microbiol. Mol. Biol. Rev.,* 64**,** 51-68.

SELABE, S. G., LUKHWARENI, A., SONG, E., LEEUW, Y. G. M., BURNETT, R. J. & MPHAHLELE, M. J. 2007. Mutations associated with lamivudine-resistance in therapy-naïve hepatitis B virus (HBV) infected patients with and without HIV co-infection: Implications for antiretroviral therapy in HBV and HIV co-infected South African patients. *Journal of Medical Virology,* 79**,** 1650-1654.

SHELDON, J., RAMOS, B., GARCIA-SAMANIEGO, J., RIOS, P., BARTHOLOMEUSZ, A., ROMERO, M., LOCARNINI, S., ZOULIM, F. & SORIANO, V. 2007. Selection of Hepatitis B Virus (HBV) Vaccine Escape Mutants in HBV-Infected and HBV/HIV-Coinfected Patients Failing Antiretroviral Drugs With Anti-HBV Activity. *JAIDS Journal of Acquired Immune Deficiency Syndromes,* 46**,** 279-282 10.1097/QAI.0b013e318154bd89.

SHELDON, J., RODÈS, B., ZOULIM, F., BARTHOLOMEUSZ, A. & SORIANO, V. 2006. Mutations affecting the replication capacity of the hepatitis B virus. *Journal of Viral Hepatitis,* 13**,** 427-434.

SHELDON, J. & SORIANO, V. 2008. Hepatitis B virus escape mutants induced by antiviral therapy. *J. Antimicrob. Chemother.***,** dkn014.

SHIBAYAMA, T., MASUDA, G., AJISAWA, A., HIRUMA, K., TSUDA, F., NISHIZAWA, T., TAKAHASHI, M. & OKAMOTO, H. 2005. Characterization of seven genotypes (A to E, G and H) of Hepatitis B virus recovered from Japanese patients infected with human immunodeficiency virus type 1. *Journal of Medical Virology,* 76**,** 24-32.

SHIRE, N. J., ROUSTER, S. D., STANFORD, S. D., BLACKARD, J. T., MARTIN, C. M., FICHTENBAUM, C. J. & SHERMAN, K. E. 2007. The Prevalence and Significance of Occult Hepatitis B Virus in a Prospective Cohort of HIV-Infected Patients. *JAIDS Journal of Acquired Immune Deficiency Syndromes,* 44**,** 309-314 10.1097/QAI.0b013e31802e29a9.

SIEGEL, D. 2007. *VIRUSES, HIV, PRIONS, AND RELATED TOPICS* [Online]. Winchester. Available: http://www.stanford.edu/group/virus/1999/tchang/replication.htm [Accessed 11 November 2010].

SONG, L. H., DUY, D. N., BINH, V. Q., LUTY, A. J. F., KREMSNER, P. G. & BOCK, C. T. 2005. Low frequency of mutations in the X gene, core promoter and precore region of hepatitis B virus infected Vietnamese. *Journal of Viral Hepatitis,* 12**,** 160-167.

SORIANO, V., MOCROFT, A., PETERS, L., ROCKSTROH, J., ANTUNES, F., KIRKBY, N., DE WIT, S., MONFORTE, A. D. A., FLISIAK, R. & LUNDGREN, J. 2010. Predictors of hepatitis B virus genotype and viraemia in HIV-infected patients with chronic hepatitis B in Europe. *Journal of Antimicrobial Chemotherapy,* 65**,** 548-555.

SORIANO, V., RIVAS, P. & NUÃ±EZ, M. 2008. Editorial Commentary: Risks and Benefits of Using Antiretroviral Therapy in HIVâ€ Infected Patients with Chronic Hepatitis B in Developing Regions. *Clinical Infectious Diseases,* 47**,** 1486-1489.

STUYVER, L., DE GENDT, S., VAN GEYT, C., ZOULIM, F., FRIED, M., SCHINAZI, R. F. & ROSSAU, R. 2000. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol,* 81**,** 67-74.

SU, H. & YEE, J. K. 1992. Regulation of hepatitis B virus gene expression by its two enhancers. *Proceedings of the National Academy of Sciences of the United States of America,* 89**,** 2708-2712.

SUGAUCHI, F., CHUTAPUTTI, A., ORITO, E., KATO, H., SUZUKI, S., UEDA, R. & MIZOKAMI, M. 2002. Hepatitis B virus genotypes and clinical manifestation among hepatitis B carriers in Thailand. *Journal of Gastroenterology and Hepatology,* 17**,** 671-676.

SUGAUCHI, F., KUMADA, H., ACHARYA, S. A., SHRESTHA, S. M., GAMUTAN, M. T. A., KHAN, M., GISH, R. G., TANAKA, Y., KATO, T., ORITO, E., UEDA, R., MIYAKAWA, Y. & MIZOKAMI, M. 2004. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. *J Gen Virol,* 85**,** 811-820.

SUGAUCHI, F., MIZOKAMI, M., ORITO, E., OHNO, T., HAYASHI, K., KATO, T., TANAKA, Y., KATO, H. & UEDA, R. 2000. Molecular Evolutionary Analysis of the Complete Nucleotide Sequence of Hepatitis B Virus (HBV) in a Case of HBV Infection Acquired through a Needlestick Accident. *Clinical Infectious Diseases,* 31**,** 1195-1201.

SUGAUCHI, F., ORITO, E., KATO, H., SUZUKI, S., KAWAKITA, S., SAKAMOTO, Y., FUKUSHIMA, K., AKIBA, T., YOSHIHARA, N., UEDA, R. & MIZOKAMI, M. 2003. Genotype, serotype, and phylogenetic characterization of the complete genome sequence of hepatitis B virus isolates from Malawian chronic carriers of the virus. *Journal of Medical Virology,* 69**,** 33-40.

SULKOWSKI, M., S. 2008. Viral hepatitis and HIV coinfection. *Journal of Hepatology,* 48**,** 353-367.

SZOMOR, K., DENCS, Á., GARAI, E., RUSVAI, E., BERENCSI, G. & TAKÁCS, M. 2008. Mutation spectra of the surface-protein-coding region of the HBV genome in HBV-vaccinated and non-vaccinated individuals in Hungary. *Archives of Virology,* 153**,** 1885-1892.

TAKAHASHI, K., AOYAMA, K., OHNO, N., IWATA, K., AKAHANE, Y., BABA, K., YOSHIZAWA, H. & MISHIRO, S. 1995. The precore/core promoter mutant (T1762A1764) of hepatitis B virus: clinical significance and an easy method for detection. *J Gen Virol,* 76**,** 3159-3164.

TAKAHASHI, K., BROTMAN, B., USUDA, S., MISHIRO, S. & PRINCE, A. M. 2000. Full-Genome Sequence Analyses of Hepatitis B Virus (HBV) Strains Recovered from Chimpanzees Infected in the Wild: Implications for an Origin of HBV. *Virology,* 267**,** 58-64.

TANAKA, Y., ORITO, E., YUEN, M.-F., MUKAIDE, M., SUGAUCHI, F., ITO, K., OZASA, A., SAKAMOTO, T., KURBANOV, F., LAI, C.-L. & MIZOKAMI, M. 2005. Two subtypes (subgenotypes) of hepatitis B virus genotype C: A novel subtyping assay based on restriction fragment length polymorphism. *Hepatology Research,* 33**,** 216-224.

TATEMATSU, K., TANAKA, Y., KURBANOV, F., SUGAUCHI, F., MANO, S., MAESHIRO, T., NAKAYOSHI, T., WAKUTA, M., MIYAKAWA, Y. & MIZOKAMI, M. 2009. A Genetic Variant of Hepatitis B Virus Divergent from Known Human and Ape Genotypes Isolated from a Japanese Patient and Provisionally Assigned to New Genotype J. *Journal of Virology,* 83**,** 10538-10547.

THAKUR, V., GUPTAN, R. C., KAZIM, S. N., MALHOTRA, V. & SARIN, S. K. 2002. Profile, spectrum and significance of HBV genotypes in chronic liver disease patients in the Indian subcontinent. *Journal of Gastroenterology and Hepatology,* 17**,** 165-170.

THIO, C. L. 2003. Hepatitis B in the Human Immunodeficiency Virus-Infected Patient: Epidemiology, Natural History, and Treatment. *Semin Liver Dis,* 23**,** 125-136.

THIO, C. L. 2009. Hepatitis B and human immunodeficiency virus coinfection. *Hepatology,* 49**,** S138-S145.

THIO, C. L., SEABERG, E. C., SKOLASKY JR, R., PHAIR, J., VISSCHER, B., MUÑOZ, A. & THOMAS, D. L. 2002. HIV-1, hepatitis B virus, and risk of liver-related mortality in the Multicenter Cohort Study (MACS). *Lancet,* 360**,** 1921-1926.

TIOLLAIS, P., POURCEL, C. & DEJEAN, A. 1985. The hepatitis B virus. *Nature,* 317**,** 489-495.

TONG, S., KIM, K. H., CHANTE, C., WANDS, J. & LI, J. 2005. Hepatitis B Virus e Antigen Variants. *Int J Med Sc,* 2**,** 6.

TORRESI, J. 2002. The virological and clinical significance of mutations in the overlapping envelope and polymerase genes of hepatitis B virus. *Journal of Clinical Virology,* 25**,** 97-106.

TORRESI, J., EARNEST-SILVEIRA, L., DELIYANNIS, G., EDGTTON, K., ZHUANG, H., LOCARNINI, S. A., FYFE, J., SOZZI, T. & JACKSON, D. C. 2002. Reduced Antigenicity of the Hepatitis B Virus HBsAg Protein Arising as a Consequence of Sequence Changes in the Overlapping Polymerase Gene That Are Selected by Lamivudine Therapy. *Virology,* 293**,** 305-313.

UNAIDS 2009. AIDS Epidemic update. Geneva: WHO.

USUDA, S., OKAMOTO, H., IWANARI, H., BABA, K., TSUDA, F., MIYAKAWA, Y. & MAYUMI, M. 1999. Serological detection of hepatitis B virus genotypes by ELISA

with monoclonal antibodies to type-specific epitopes in the preS2-region product. *Journal of Virological Methods,* 80**,** 97-112.

YEH, C.-T., CHIEN, R.-N., CHU, C.-M. & LIAW, Y.-F. 2000. Clearance of the original hepatitis B virus YMDD-motif mutants with emergence of distinct lamivudine-resistant mutants during prolonged lamivudine therapy. *Hepatology,* 31**,** 1318-1326.

YEH, C. T., SO, M., NG, J., YANG, H. W., CHANG, M. L., LAI, M. W., CHEN, T. C., LIN, C. Y., YEH, T. S. & LEE, W. C. 2010. Hepatitis B virus–DNA level and basal core promoter A1762T/G1764A mutation in liver tissue independently predict postoperative survival in hepatocellular carcinoma. *Hepatology***,** n/a-n/a.

YEUNG, P., WONG, D. K.-H., LAI, C.-L., FUNG, J., SETO, W.-K. & YUEN, M.-F. 2011. Association of Hepatitis B Virus Pre-S Deletions with the Development of Hepatocellular Carcinoma in Chronic Hepatitis B. *Journal of Infectious Diseases,* 203**,** 646-654.

YOKOSUKA, O. & ARAI, M. 2006. Molecular biology of hepatitis B virus: effect of nucleotide substitutions on the clinical features of chronic hepatitis B. *Medical Molecular Morphology,* 39**,** 113-120.

YU, H., YUAN, Q., GE, S.-X., WANG, H.-Y., ZHANG, Y.-L., CHEN, Q.-R., ZHANG, J., CHEN, P.-J. & XIA, N.-S. 2010. Molecular and Phylogenetic Analyses Suggest an Additional Hepatitis B Virus Genotype "I". *PLoS ONE,* 5**,** e9297.

ZEKRI, A.-R., HAFEZ, M., MOHAMED, N., HASSAN, Z., EL-SAYED, M., KHALED, M. & MANSOUR, T. 2007. Hepatitis B virus (HBV) genotypes in Egyptian pediatric cancer patients with acute and chronic active HBV infection. *Virology Journal,* 4**,** 74.

ZENG, G., WANG, Z., WEN, S., JIANG, J., WANG, L., CHENG, J., TAN, D., XIAO, F., MA, S., LI, W., LUO, K., NAOUMOV, N. V. & HOU, J. 2005. Geographic

distribution, virologic and clinical characteristics of hepatitis B virus genotypes in China. *Journal of Viral Hepatitis,* 12**,** 609-617.

ZENTGRAF, H. W. 2002. *Morphology & Genome Organization* [Online]. Available: http://www.klinikum.uni-heidelberg.de/1-Morphology-Genome-Organization.104932.0.html [Accessed 04 November 2010].

ZHANG, D., MA, S., ZHANG, X., ZHAO, H., DING, H. & ZENG, C. 2010. Prevalent HBV point mutations and mutation combinations at BCP/preC region and their association with liver disease progression. *BMC Infectious Diseases,* 10**,** 271.

ZOULIM, F. & LOCARNINI, S. 2009. Hepatitis B Virus Resistance to Nucleos(t)ide Analogues. *Gastroenterology,* 137**,** 1593-1608.e2.

Appendix A: Patient's Informed Consent

PARTICIPANT INFORMATION LEAFLET AND ENROLLMENT INFORMED CONSENT

The molecular and functional characterization of hepatitis B virus (HBV) genotypes isolated from human immunodeficiency virus (HIV) infected Southern Africans
(Shongwe Hospital HIV / HBV Study)

Version 1.5a
May 2009

INVESTIGATORS

Prof. Anna Kramvis
Hepatitis Virus Diversity Research Programme
Department of Internal Medicine
Faculty of Health Sciences
University of the Witwatersand
Tel: (011) 488-3100
Email: Anna.Kramvis@wits.ac.za

Dr Neil A Martinson
Perinatal HIV Research Unit
Wits Health Consortium
University of the Witwatersrand
and
Chris Hani Baragwanath Hospital
Old Potch Road, Soweto
Tel: (011) 989 9703
E-mail: martinson@hivsa.com

INTRODUCTION

Good day, my name is **Agatha Nkosi**. I am a research nurse assisting in a study being carried out by the University of the Witwatersrand in Johannesburg. I would like to invite you be part of a research study that will assess whether or not you have been exposed to hepatitis B virus (HBV).

Before you decide whether to take part in the study, we would like to explain why we are doing the study, the risks and benefits, and what would be expected of you if you agree to be in the study. This study is sponsored by the Medical Research Council (MRC) of South Africa.

PURPOSE OF THE STUDY

This study is researching hepatitis B virus (HBV). A virus is a very small germ that gets caught up in the cells of the person who has it and can cause severe illness or maybe no illness at all. HBV is a very common infection in some parts of Africa and HBV causes liver damage in some people who are infected by it. People who have been exposed to HBV can either cure themselves of the infection over time, or be unable to cure

themselves and will continue to be infected with HBV. Ongoing HBV infection may cause serious liver problems later, including liver cancer. Because HIV-infection is also very common, we want to look at people who are infected with both HIV and HBV.

At the first visit we want to:

- Count how many HIV-infected people (people who have the virus that causes AIDS) also have been exposed to HBV
- Count how many of the ones who are HBV infected also have HBV that could be transmitted to other people
- How many people have cleared the infection themselves
- Check how many people have the genetic makeup that allows liver cancer

If you have HBV, we also would like to see you another 4 times: 3, 6, 12 and 18 months after you started antiretroviral (ARV) treatment, to see what happens to HBV when HIV is treated with ARVs. If we can find hepatitis B virus (HBV) in your blood, we will see what family or type of HBV it is. We will ask about 250 people to give blood at the first visit and we estimate that about 30 of you will have hepatitis B infection and will be followed up while taking ARVs.

VOLUNTARY PARTICIPATION

It is important that you know the following:

- You don't have to be in this study if you do not want to. You will still receive medical care at the HIV clinic here.
- You may decide to withdraw from the study at any time, without affecting your normal medical care.

STUDY PROCEDURES

This study will not give you better care than if you were not part of it. We are only studying HBV. Your doctor or nurse here at the clinic will still decide what is the best treatment for you. They may use some of the results we find to decide.

At the first visit and and at each scheduled visit you will:

- Have an interview with the research nurse or doctor. You will be asked to answer questions about yourself and your health.
- You will have blood taken for HIV viral load and CD4 count at enrollment and at the last study visit 6 months later, either as part of routine care or research participation.
- We will take blood for studies of HBV and to see how your liver is working at the first visit. If your blood shows that you have active HBV, we will let the doctor in charge know and we will ask you if we can see you again after 3, 6, 12 and 18 months after you started ARVs. At each of these visits we will take blood for HBV studies and to check how your liver is working. We will store your samples at the Medical School in Johannesburg, but will only do tests on them related to HBV and HIV.
- Sometimes HBV causes liver cancer. One of the tests we may do in the future will be a test of the chemicals that determine how your body works (the chromosomes). This test checks if you may be more likely to get liver cancer related to hepatitis B infection (in medical terms, we will be looking to see if you have the p53 gene on chromosome 17).

- You will be asked for updated information on where you live and how to keep in contact with you. If you miss a visit, the study staff will try to contact you by telephone. They also may visit your home to find you. They will try to reach you through the contact people that you provide. If they talk to these people that know you, they will not tell them why they are trying to reach you.

RISKS AND/OR DISCOMFORTS
We will take about 5 teaspoons (25ml) of blood from you. This may make you feel discomfort or pain when your blood is drawn. Occasionally people may feel dizzy or faint. You may get a bruise where the needle goes in.

BENEFITS
You will get no direct health benefit from being in this study, but we will be informing your doctor or nurse if you have active hepatitis B infection and that may be of indirect benefit to you. The doctor may change the HIV antiretrovirals you receive if he/she thinks that this is required.

LEAVE THE STUDY
You can leave this study at any time without causing a problem. If you decide to leave the study, please tell the research nurse or clinic staff why you wish to leave.

COSTS TO YOU
There is no cost to you for being part of this study. Study investigations are free of charge. You will be reimbursed R25 for your travel costs if you agree to be part of the study and you will receive R50 each time when you come to your follow up visits.

CONFIDENTIALITY
We will keep your information private and confidential. Your information may only be disclosed if required by law. With your permission, study staff may visit you at home to check on your health or to contact you if you are late for a study visit. Any publication of this study will not use your name or identify you personally. Your name will not be stored in any electronic form in a database or computer. Your study records may be reviewed by study staff and people who check that we are actually doing this study the way we said we would do it.

RESEARCH-RELATED INJURY
If you are injured as a result of participation in this study, the study clinic will give you immediate necessary treatment for your injuries. The cost of this treatment will not be charged to you.

PERSONS TO CONTACT FOR PROBLEMS OR QUESTIONS
If you ever have questions about this study or in case you are injured as a result of participation in this research study, you should contact Dr Precious Gabashane at Shongwe Hospital (013 781 0219) or Dr Neil Martinson at the PHRU (011 989 9703 during office hours).

If you have questions about your rights as a research subject you can contact Professor Cleaton Jones on 011 717 2301, Wits Research Ethics Committee, 10th Floor, Senate House, University of the Witwatersrand.

STATEMENT OF CONSENT AND SIGNATURES

I have read this form in its entirety, or I have had it read to me in the event that I am not able to read. I have discussed the information with study staff. My questions have been answered. I understand that my decision whether or not to take part in the study is my own. I understand that I may withdraw at any time. A witness has been present for the entire consent process.

Participant Name	Participant Signature or Thumb-print	Date

Interviewer Name	Interviewer Signature	Date

Consent Discussion Witness	Witness Signature or Thumb-print	Date

Appendix B: ETHICS APPROVAL CERTIFICATES

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Ms Euphodia Makondo

CLEARANCE CERTIFICATE	**M090414**
PROJECT	Genotyping and Molecular Characterisation of Hepatitis B Virus (HBV) from Human Immuno-deficiency Virus (HIV) Infected Individuals in Southern Africa
INVESTIGATORS	Ms Euphodia Makondo.
DEPARTMENT	Department of Internal Medicine
DATE CONSIDERED	09.04.29
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 09.05.29 **CHAIRPERSON** .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof A Kramvis

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Kramvis

CLEARANCE CERTIFICATE	PROTOCOL NUMBER M080450
PROJECT	The molecular and functional characteristics of Hepatitis B virus (HBV) genotypes isolated from HIV infected South Africans
INVESTIGATORS	Prof A Kramvis
DEPARTMENT	Department of Medicine
DATE CONSIDERED	08.04.25
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 08.05.20 CHAIRPERSON .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor :

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. I agree to a completion of a yearly progress report.

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Samo/Firnhaber

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M050620**
PROJECT	Pilot Study on Effect of Anti-Retroviral Therapy (ART) on Hepatitis B Viral (HBV) Infection
INVESTIGATORS	Drs I/C Samo/Firnhaber
DEPARTMENT	Clinical HIV Research Unit
DATE CONSIDERED	05.06.24
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 05.08.01 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr P Ive

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

.....

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX C: SOLUTIONS, CHEMICALS AND REAGENTS

10X TBE Buffer

108 g Tris base

55 g Boric acid

9.3 g EDTA

Make up to 1 L volume with additional distilled water and autoclave

1X TBE Buffer

1 ml of 10X TBE

Fill up with distilled water to final volume of 1 L

1% agarose

1 g agarose

100 ml 1X TBE

Microwave until agarose as completely dissolved,

Add Ethidium Bromide and pour on the gel casting tray with a comb and allow to set

APENDIX D: BCP/PC MUTATION ANALYSIS.