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# Molecular characterization and genotyping of hepatitis B virus (HBV) from sudanese end stage renal disease (ESRD) patients with overt and occult HBV infection

**Nekhwevha, Khudani**

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# Molecular Characterization and Genotyping of Hepatitis B Virus (HBV) from Sudanese End Stage Renal Disease (ESRD) Patients with Overt and Occult HBV infection

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**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, August 2018**

**Declaration**

I, Khudani Nekhwevha 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 …… 2018**

**Presentations/Posters**

**Molecular Characterisation and Genotyping of Hepatitis B Virus (HBV) from Sudanese End Stage Renal Disease (ESRD) Patients with Overt and Occult HBV infection**

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**Abstract**

Hepatitis B virus (HBV), which causes infection globally, is endemic in sub-Saharan Africa. Currently, nine genotypes (A to I), distributed geographically, are recognised. In Sudan, located in north-east Africa, HBV prevalence ranges from 4% to 26%, and genotypes D and E predominate. Patients suffering from end-stage renal disease (ESRD) exhibit poor kidney function and require regular haemodialysis. HBV exposure in haemodialysis patients in Sudan is high, as the immunosuppressive nature of renal disease increases risk from HBV infection. The aim of this study was to molecularly characterise HBV from ESRD patients in Sudan undergoing haemodialysis and also to use phylogenetic analysis to determine if HBV infection was occurring in the four haemodialysis centres from which HBV DNA was successfully amplified. We undertook polymerase chain reaction (PCR) amplification and DNA sequencing of the basic core promoter/pre-core (BCP/PC) and the complete S (*preS1/preS2/S*) regions of 68 HBV DNA positive ESRD patients undergoing haemodialysis in four different treatment centres (Salma, Ibn Sena, Elturki and Alnaw) in Khartoum. In Hepatitis B surface antigen (HBsAg) positive (overt) HBV-mono-infected samples, PCR amplicons from the BCP/PC region were obtained from 28/46 (60.9%) of samples, with DNA sequences obtained from 24/46 (52.2%). Results for the S region were 23/46 (50%) and 15/46 (32.6%), for PCR amplification and sequencing, respectively. BCP/PC PCR amplification from 12 occult HBV-mono-infected samples resulted in 2/12(16.7%) amplifications of low density bands. Hence no DNA was obtained for sequencing. However, 4/12 (33.3%) of these samples yielded S region amplicons, with DNA obtained from 3/12 (25%). Ten HBV/HCV co-infected samples yielded 4/10 (40%) positive samples in the BCP/PC, with 3/10 sequenced (30%) in the complete S, 2/10 (20%) amplified with low DNA quality. Neighbour-joining phylogenetic analysis using 1000 bootstrap indicated that genotype D predominated at 11/15 (73.3%), followed by genotypes E and A, at 3/15 (20%) and 1/15

(6.7%), respectively. Genotype D was found in all centres and mostly detected at Salma 6/15 (40%), whereas genotype A was only detected at Elturki. Moreover, Genotype E was only detected in Salma and Ibn Sena. We identified a six nucleotide insertion occurring in the core region of HBV from four individuals from Salma centre. This was a novel finding in genotype D isolates as this insertion was previously found in genotype A. The *preS1* mutation, ps1I48V, which occurs mostly in genotype A isolates, was found in one genotype A isolate from Sudan. We further identified a preS2 deletion in one genotype E isolate. The deletions in the pre S contribute to the development of hepatocellular carcinoma (HCC). The HBV reactivation marker, S174N was found in one HBsAg negative (occult) individual. We further identified HBV drug resistance mutations, rtS213 and rtQ215H occurring in two different individuals from Ibn Sena centre. Both of these individuals were not vaccinated. In conclusion, in Sudanese ESRD patients, genotype distribution varies between treatment centres, although genotype D predominates. In addition, drug resistance and vaccine escape similar to the ones found in South Africa exist in Sudan. The small sample size did not allow us to compare the mutations found in HBV isolates from individuals with overt or occult infection. Hence, further studies to identify distinguishing mutations between occult and overt samples, as well as in HBV/HCV co-infected individuals in Sudan are still needed.

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**List of abbreviations**

3TC - Lamivudine

Aa- Australian antigen

Aa- Amino acid

Anti-HBeAg - anti-Hepatitis B e-antigen

Anti-HBs- Hepatitis B immune status/Anti-Hepatitis B surface antibody

ASC- Asymptomatic chronic carrier

ASHV- Arctic Squirrel Hepatitis Virus

Bp- Base pair

BCP- Basic core promoter

BQW- Best quality water

cccDNA- Covalently closed circular DNA

CHB- Chronic Hepatitis B virus infection

DNA- Deoxyribonucleic acid

ELISA- Enzyme-linked immunosorbent assay

ESRD- End Stage Renal Disease

ER- Endoplasmic reticulum

EtBr- Ethidium bromide

EtOH- Ethanol

FTC- Emtricitabine

GFR- Glomerular filtration rate

GSHV- Ground Squirrel Hepatitis Virus

HBcAg- Hepatitis B core antigen

HBeAg- Hepatitis B e antigen

HBsAg- Hepatitis B surface antigen

HBV- Hepatitis B virus

HCC- Hepatocellular carcinoma

HCV- Hepatitis C virus

HIV- Human immunodeficiency virus

Kb- Kilobase

LHBs- Large surface protein

MHBs- Medium surface protein

mRNA- Messenger ribonucleic acid

NA- Nucleoside/nucleotide analogue

NEIGHBOUR- Neighbour-joining

nt- Nucleotide

OBI - Occult HBV infection

ORF- Open reading frame

PC- Precore gene

PCR- Polymerase chain reaction

pgRNA- Pre-genomic RNA

PHYLIP- Phylogeny inference package

rcDNA- Relaxed circular DNA

RNA- Ribonucleic acid

RT- Reverse transcriptase

S gene- Surface gene

SHBs- Small surface protein

TBE- Tris Boric acid EDTA

TAF- Tenofovir alafenamide

TDF- Tenofovir disoproxil fumarate

UV- Ultraviolet light

WHO- World Health Organization

WMHBV- Woolly Monkey Hepatitis B virus

YMDD- Tyrosine, Methionine, Aspartic acid, Aspartic acid

## Chapter 1: Introduction

Hepatitis is the inflammation of the liver, which is characterised by major symptoms such as fatigue, yellowing of eyes and dark urine. The Hepatitis B virus (HBV) has a genome of approximately 3200 base pairs, which makes it the smallest DNA virus infecting humans. Furthermore, it is one of the most important human pathogens, causing major health problems globally. HBV belongs to the family *Hepadnaviridae* and is endemic in several parts of the world, including sub-Saharan Africa. It is estimated that around 257 million people are chronically infected with HBV world-wide (WHO, 2017) and its prevalence varies globally.

Using the prevalence carrier rates of hepatitis B surface antigen (HBsAg), the major envelope protein, in a population, HBV endemic zones can be classified as low (HBsAg < 2%), intermediate (HBsAg 2-7%) and high (HBsAg > 8%) endemicity (Hou et al., 2005, Mahoney, 1999) (Figure 1.1). Sub-Saharan Africa is an area in Africa that lies in the south of Sahara desert. According to World Health Organization (WHO), sub-Saharan Africa has the highest prevalence rate of HBV compared to other continents (WHO, 2015). The prevalence rates vary between the different countries and also within countries. For example, the prevalence of HBV in Sudan ranges from 3.9% to 26% (Yousif et al., 2014). Sub-Saharan Africa also has the highest prevalence of human immunodeficiency virus (HIV) infection globally, with 26 of the 37 million HIV-infected individuals residing in this region. Thus HBV/HIV co-infection is frequent, with HBV exposure rate in HIV-infected individuals in sub-Saharan Africa being as high as 44% (Matthews et al., 2014).

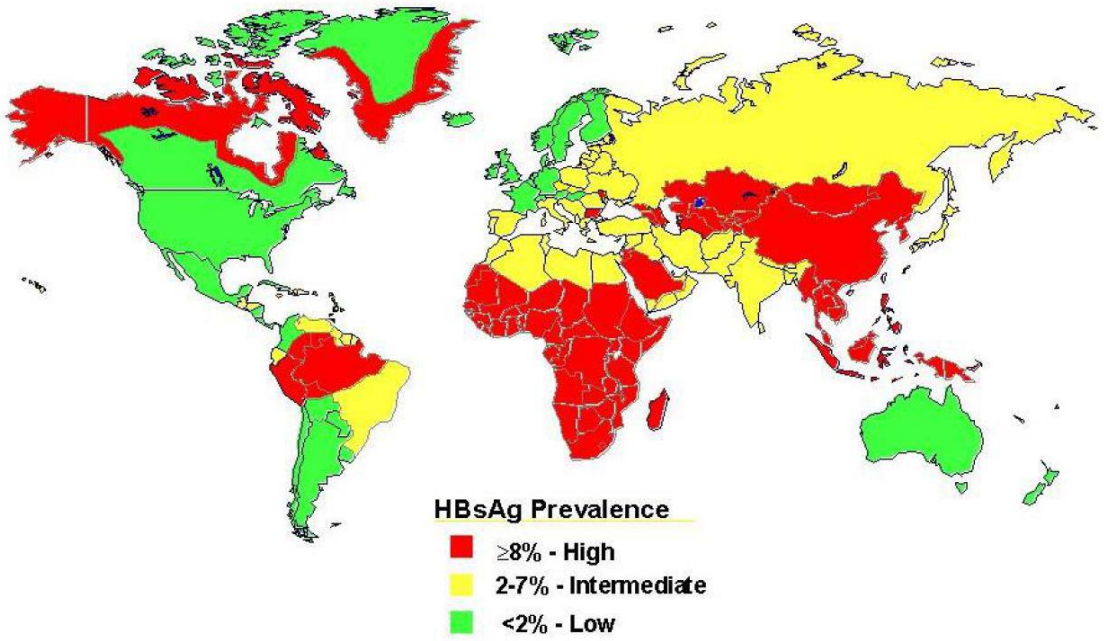



**Figure 1.1:** The worldwide prevalence of hepatitis B surface antigen (HBsAg**)**. Source: http://virology-online.com/viruses/HepatitisB.htm. Accessed on January 10, 2018. Reproduced with permission from Online Virology. HBV primary infection in susceptible hosts can either be asymptomatic or symptomatic, and in adults, this is usually self-limiting with viral clearance and the development of immunity (Wright and Lau, 1993). Symptoms may be specific, such as liver enlargement, or non-specific such as fatigue and nausea. Acute HBV clinical incubation is usually between 2 to 3 months. During this period, the serum Alanine aminotransferase levels rise and HBV DNA and HBsAg levels are high (Liang, 2009). Chronic symptoms of HBV infection are characterised by the presence of HBsAg for longer than six months. This infection is normally a result of horizontal transmission between siblings or perinatal (vertical) transmission from infected mothers to neonates. Children infected when they are younger than six years old are more likely to develop chronic infection, which can later cause liver disease and cirrhosis. Interactions between virus and host factors contribute to the diverse nature of chronic HBV infection (He et al., 2006). In approximately 1% of patients, acute liver failure occurs (Berk and Popper, 1978). The presence of fulminant hepatitis is marked by a sudden appearance of fever, abdominal pain, vomiting, jaundice, confusion and coma (Liang, 2009).

HBV DNA may be detected in HBsAg-negative individuals. This is referred to as occult HBV infection (OBI) (Raimondo et al., 2008) The chronic hepatitis is divided into six stages, and they are high replicative, low inflammatory, immune clearance, Hepatitis B e antigen (HBeAg)-negative chronic hepatitis, the low replicative, the reactivation and HBsAg loss or occult phases (Gish et al., 2015). The possible reasons of HBV related tumourigenesis are numerous and include possible effects of host immune response to HBV, HBV DNA integration, high HBV replication rates, HBV genotypic variations and the effect of certain viral mutations (Tufon et al., 2016). Interestingly, patients with chronic HBV are more likely to develop long term complications of chronic infection such as cirrhosis, hepatocellular carcinoma (HCC) and liver disease (Yousif et al., 2014). Moreover, HBV and HIV co-infection in sub-Saharan Africa has been shown to cause high mortality and morbidity of cases that are not as a consequence acquired immunodeficiency disease syndrome (AIDS) (Lewden et al., 2005). In addition, this co-infection contributes rapidly towards liver disease progression among infected patients (Di Martino et al., 2002, Thio, 2009) Recent studies undertaken in sub-Saharan countries such as Sudan (Mudawi et al., 2014, Yousif et al., 2013) and Cameroon (Tufon et al., 2016), indicate increased HBV viral loads may play a role in disease progression, which can lead to severe liver damage in patients co-infected with HBV/HIV.

With regard to HBV treatment, the primary goal for patients is to prevent progression of the disease, particularly to cirrhosis, liver failure, and HCC (Sorrell et al., 2009). The prevention of HCC often includes the use of antiviral treatment using pegylated interferon or nucleos(t)ide analogues (NA) (Kim et al., 2012). For best prognosis of the hepatitis, a synergistic approach of suppressing viral load and boosting the patient's immune response with immunotherapeutic interventions is required (Nebbia et al., 2012). Currently, HBV vaccination programmes are ongoing in endemic and less endemic areas, in 184 of 192

countries globally, with encouraging results. According to WHO, vaccine must be given to all infants after birth or within 24 hours (WHO, 2017).

Sudan, which is a republic country in the north-east of Africa, is divided into North and South regions. In south Sudan, the HBV exposure, as measured by the anti-HBc marker, was detected to be between 47% and 78% (McCarthy et al., 1994). In the general population, HBsAg-positivity was between 3.9% to 26% (Mahgoub et al., 2011, McCarthy et al., 1994). The majority of previous studies carried out in Sudan determined HBV infection using HBsAg as the marker by Enzyme-linked immunosorbent assay (ELISA) (Mudawi et al., 2007), although some of researchers used Immunochromatography tests, followed by ELISA for confirmation. For HIV infection, most of the studies followed the WHO recommendations for the diagnosis of HIV (WHO, 2006). All the studies carried out between 1989 and 2012, to determine the prevalence of HBV, HIV and coinfection in Sudan, are summarised in Table 1.1 (Abbas et al., 2010, Abdelrahim, 2010, Abou et al., 2009, Elsheikh et al., 2007, Hyams et al., 1989, Mahgoub et al., 2011, McCarthy et al., 1989, McCarthy et al., 1995, Mohammed et al., 2011, Mudawi et al., 2007).

In HIV-positive patients in Sudan, this exposure to HBV infection, was found to be 62.8% and the prevalence of HBsAg 11.7%, indicating chronic HBV infection (Mudawi et al., 2014, Yousif et al., 2014). Moreover, 15.1% of HBsAg-negative individuals were found to be HBV DNA positive, indicating OBI (Yousif et al., 2014).

ESRD patients undergoing dialysis have been shown to have a high risk of HBV infection but no studies have been carried out in this population in Sudan. Thus, this was the aim of the present study.

**Table 1.1:** Prevalence of HBV, HIV and coinfection in Sudan (Modified from Yousif 2014, PHD thesis)

| | Year | Geographical area | Participants | Number of participants | HBsAg % | Anti-HBc % | Anti-HBs % | HIV % | HIV/HBV % | References |
|---|---|---|---|---|---|---|---|---|---|---|
| HBV studies | 1989 | Gezira- central Sudan | Villagers | 851 | 18.7 | 16.2 | 63.9 | NA[¶] | NA | (Hyams *et al.*, 1989) |
| | 1994 | Juba- south Sudan | Out patients | 651 | 26 | 67 | NM[€] | NA | NA | (McCarthy *et al.*, 1994) |
| | 2007 | Omduman, central Sudan | Pregnant women | 728 | 5.6 | NM | NM | NA | NA | (Elsheikh *et al.*, 2007) |
| | 2008 | Shendi, north Sudan | Blood donors | 78 | 5.1 | NM | NM | NA | NA | (Nail *et al.*, 2008) |
| | 2009 | Darfur, western Sudan | Blood donors | 400 | 6.5 | NM | NM | NA | NA | (Abou *et al.*, 2009) |
| | 2010 | Gezira- central Sudan | Out patients | 404 | 6.9 | 47.5 | 47.5 | NA | NA | (Mudawi *et al.*, 2007) |
| | 2011 | Khartoum | hospital staff | 245 | 4.9 | NM | NM | NA | NA | (Elduma and Saeed, 2011) |
| | 2011 | Khartoum | Blood donors | 404 | 3.9 | 36 | 50.4 | NA | NA | (Mahgoub *et al.*, 2011) |
| | 2012 | Khartoum | Haemodialysis patients | 353 | 4.5 | NM | NM | NA | NA | (Gasim *et al.*, 2011) |
| HIV studies | 1995 | Juba- south Sudan | Out patients | 401 | NA | NA | NA | 6.2 | NA | (McCarthy *et al.*, 1995) |
| | 1997 | Khartoum | Children | 1118 | NA | NA | NA | 1.2 | NA | (Hashim *et al.*, 1997) |
| | 2006 | Different sites | TB patients | 1797 | NA | NA | NA | 4.0 | NA | (El-Sony, 2006) |
| | 2006 | Khartoum maternity hospital | Pregnant women | 305 | NA | NA | NA | 1 | NA | (Gassmelseed *et al.*, 2006) |
| | 2008 | NA | NA | NA | NA | NA | NA | 1.4 | NA | {UNAIDS., 2008 #1267} |
| | 2010 | Khartoum | Children | 106 | NA | NA | NA | 5.7 | NA | (Abbas *et al.*, 2010) |
| | 2010 | Khartoum maternity hospital | Female sex workers | 321 | NA | NA | NA | 0.9 | NA | (Abdelrahim, 2010) |
| | 2011 | Eastern Sudan | pregnant women | 430 | NA | NA | NA | 0.23 | NA | (Mohammed *et al.*, 2011) |
| HBV/HIV co-infection studies | 1989 | North east | Sexually active heterosexuals | 536 | 14 | 63 | 49 | 0 | 0 | (McCarthy *et al.*, 1989a) |
| | 1989 | North east, east and south | Soldiers | 773 | 25 | 52 | 52 | 1.7 | 0.8 | (McCarthy *et al.*, 1989b) |

¶ NA: Not applicable, [€]ND: Not measured

## 1.1 The hepatitis B virus

### 1.1.1 History of HBV

Hepatitis has been known for centuries. The identification of Australia antigen (Aa) in 1963 led to a major breakthrough in the understanding of hepatitis viruses (Blumberg et al., 1965). However, eventhough the Aa had been identified, the relationship between it and hepatitis was still unclear. The link between the virus and Aa was confirmed by Alfred Prince and co-workers, who were studying patients with transfusion-associated hepatitis (Prince et al., 1964). The antigen was later identified as HBsAg (Prince et al., 1970). The identification of the causative agent by these studies, gave a platform to the investigations of HBV and its association with liver disease. The prevalence of HBV infection varies by geographical region (Carey, 2009) and is widely studied worldwide.

### 1.1.2 Classification: Family *Hepadnaviridae*

HBV is a partially double stranded DNA virus, with a complete minus strand and an incomplete plus strand, belonging to the family *Hepadnaviridae* (Figure 1.2). This family is divided into two genera, the *Orthohepadnaviridae* (hepadnaviruses infecting mammals) and the *Avihepadnaviridae* (hepadnaviruses infecting birds). HBV is the prototype of the *Orthohepadnaviridae*. Non-human primates, including chimpanzees (*Pan troglodytes*), White handed gibbons (*Hylobates lar*), Orangutans (*Pongo Pygmaeus pygmaeus*) and Gorillas (*Gorilla gorilla*), are also susceptible to infection with orthohepadnaviruses (Grethe et al., 2000, Norder et al., 1996, Vaudin et al., 1988). Moreover, *Avihepadnaviruses* have been documented in ducks, herons, geese, storks and cranes (Seeger and Mason, 2000). Although *avihepadnaviruses* show little nucleic acid sequence similarity with HBV, the duck hepatitis B virus has been used as a model system to study hepadnavirus replication, and Woodchuck hepatitis virus infections have been used to study chronic liver disease and HCC (Seeger and Mason, 2000).

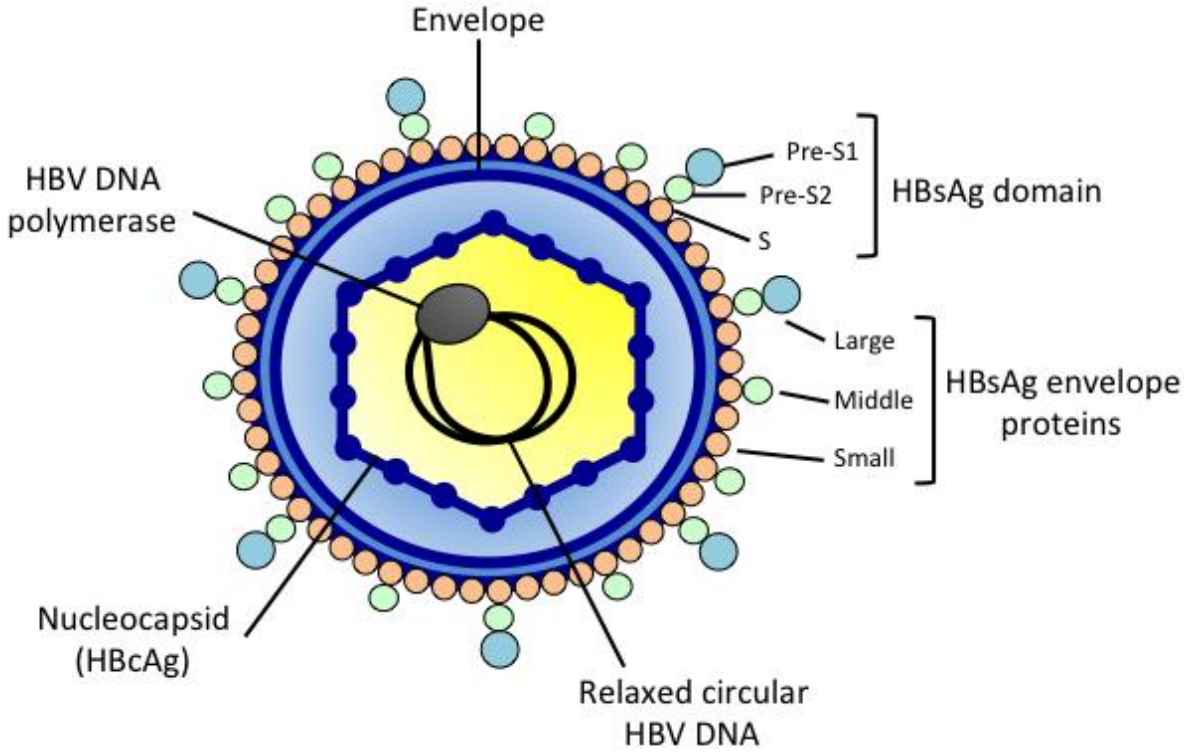



**Figure 1.2:** The hepatitis B virus, 42 nm small, with L-, M- and S-proteins attached to a lipid bilayer. Inside is the capsid containing the partially double-stranded releaxed DNA including the polymerase. Modified from Gonzalex, 2006, with permission from *Microbes*.

### 1.1.3 HBV structure

The HBV genome is organised into four open reading frames (ORF), which are termed preCore/Core, for the precore and capsid proteins (*PreC/C*), Polymerase (*P*), Surface (*S*) for surface antigens and (*X)* for X proteins (Figure 3). These ORFs encode the seven HBV proteins (Figure 1.3), which are translated from four transcripts using ccc DNA as the template. The LHBs protein is translated from the 2.4 kb mRNA and is referred to as the *preS1* transcript (Hildt et al., 2002) . The MHBs is translated from the 2.1 kb mRNAs, which

is referred to as the *preS2* transcript and SHBs is translated from the 2.1 kb *preS2/S* (Hildt et al., 2002). However, the X protein, 0.8 kb, which stimulates virus replication, is not found in mature virus particles.

The reverse transcirbed DNA is encapsidated together with the polymerase into a capsid composed of core protein (HBcAg), which matures by being enveloped in the lipid membrane in three envelope glycoproteins, namely HBV large surface protein (LHBs), HBV medium surface protein (MHBs) and HBV small surface protein (SHBs) are embedded. The mature virion is referred to as the Dane particle. Additionally, HBV e antigen (HBeAg), which shares a part of the core protein (amino acids 1 to 149) (Lin et al., 2010) is not found in mature virus particles, but is secreted by the infected cell in a soluble form (Kramvis et al., 1997).

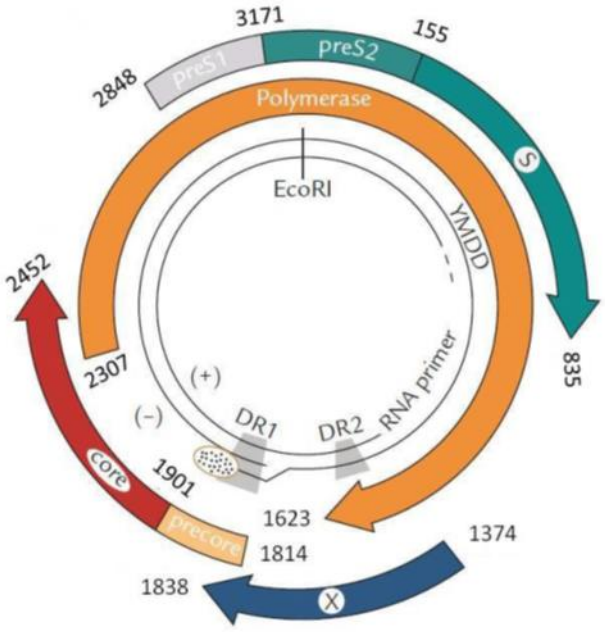



**Figure 1.3**: The four open reading frames of the HBV genome (*C*, *X*, *P* and *S*). The inner circles represent the minus (–) and positive (+) DNA strands. The outer circles represent the four open reading frames (ORFs). The above structure was adapted from HBV **genotype D**. Modified from (Lin and Kao, 2010), with permission from *Elsevier*.

### 1.1.4 HBV replication

The first step of HBV infection (Figure 1.4) involves the binding of the HBV particle to hepatocytes. This step is accomplished through a functional receptor called the sodium taurocholate cotransporting polypeptide (NTCP) (Yan et al., 2012). There is a high affinity between the *preS1*-peptide and NTCP, and this receptor is only expressed in hepatocytes, located on the sinusoidal/basolateral surface, explaining the replicaton of HBV in the liver. (Baumert et al., 2014). Upon entry of the virus into the cell through endocytosis, the viral nucleocapsid is released into the cytoplasm and later transported to the nucleus through microtubules. In the nucleus, the relaxed circular DNA (rcDNA) is repaired by the viral polymerase, leading to completion of positive strand synthesis (Nassal, 2008). This is

followed by ligation of both positive and negative strands. As a result the covalently closed circular DNA (cccDNA) is formed (Nassal, 2008), which is a template for the different viral transcripts (Sohn et al., 2009).

The cccDNA provides a transcriptional template for RNA polymerase II to produce genomic and subgenomic RNA transcripts, which are: 0.7 kb (kilobase) X mRNA, 2.1 kb S mRNA, 2.1 kb *preS2* mRNA, 2.4 kb *preS1*, 3.5 kb pre-C/C mRNA and 3.5 kb pre-genomic RNA (pg RNA) (Beck and Nassal, 2007, Moolla et al., 2002, Yaginuma and Koike, 1989). The *cis* acting elements in conjunction with four promoters and two enhancers control the synthesis of four RNA transcripts (Kramvis and Kew, 1999). The heterogeneous (due to lack of distinct TATA box in the BCP region) transcription start site (5') of pre C mRNA is mapped into nucleotides 1785 to 1786 and 1791 to 1797 (Kramvis and Kew, 1999). Moreover, the transcription start site (5') of the pgRNA is mapped into nucleotide region 1815. The transcription of the two 3.5 kb transcripts is controlled by the core promoter, which is 232 bp long (1591 to 1822 from *Eco*RI site) (Kramvis and Kew, 1999). Once transcription is done, all the transcripts are transported to the cytoplasm. This leads to translation of seven proteins (Urban et al., 2010). The presence of four major mRNA transcripts, transcribed from cccDNA, which are translated into 7 HBV proteins, makes the 3.2 kb HBV genome an extremely compact and efficient entity. Control of gene expression and the subsequent translation of HBV proteins must therefore be an organised and regulated process.

The two 3.5 kb are the most abundant and important transcripts. Additionally, the functions of these two transcripts are distinct. The pgRNA encodes for the core and polymerase, whereas, the pre-C mRNA encodes the precore-core protein, the precursor of HBeAg (Baumert et al., 2014). The pgRNA is more than full-length copy of the genome. It contains

regions of direct repeat, DR1 and DR2, 10 bp each. Near both the 5' and the 3' ends an encapsidation signal (ε) is present, where the 5' end ε enables encapsidation of pgRNA into virus nucleocapsids.

The transcription of the *preS1* mRNA (2.4 kb) is controlled by *preS1* promoter, which is located 2710 to 2800 bp from *Eco*RI site. Whereas, the transcription of *preS2* mRNA is controlled by the *preS2* promoter (Moolla et al., 2002) Lastly, transcription of 0.7 kb transcript is regulated by the X promoter in the X open reading frame (X ORF) (Beck and Nassal, 2007, Guo et al., 1991). 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). HBx relieves the transcriptional inhibition of the Smc5/6 complex to allow productive hepatitis B virus gene expression (Decorsiere et al., 2016). However, 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 (Wang et al., 1994).

The reverse transcription takes place inside the capsid (Beck and Nassal, 2007). A specialised reverse transcriptase viral encoded polymerase (pol) is crucial during the replication of HBV (Jones and Hu, 2013). Pol uses the viral pgRNA as the template to synthesise minus-strand viral DNA via its RNA-dependent DNA polymerisation activity (Jones and Hu, 2013). The Pol then further use the minus-strand DNA as the template for plus-strand DNA synthesis (Jones and Hu, 2013). The RNase H activity in the Pol is required during the degradation of pgRNA template during minus-strand DNA synthesis (Jones and Hu, 2013). For its competence in protein-primed initiation of reverse transcription, Pol has to bind to a short RNA structure termed epsilon (ε) on the 5′ end of pgRNA in a host chaperone-dependent reaction (Jones et al., 2012).

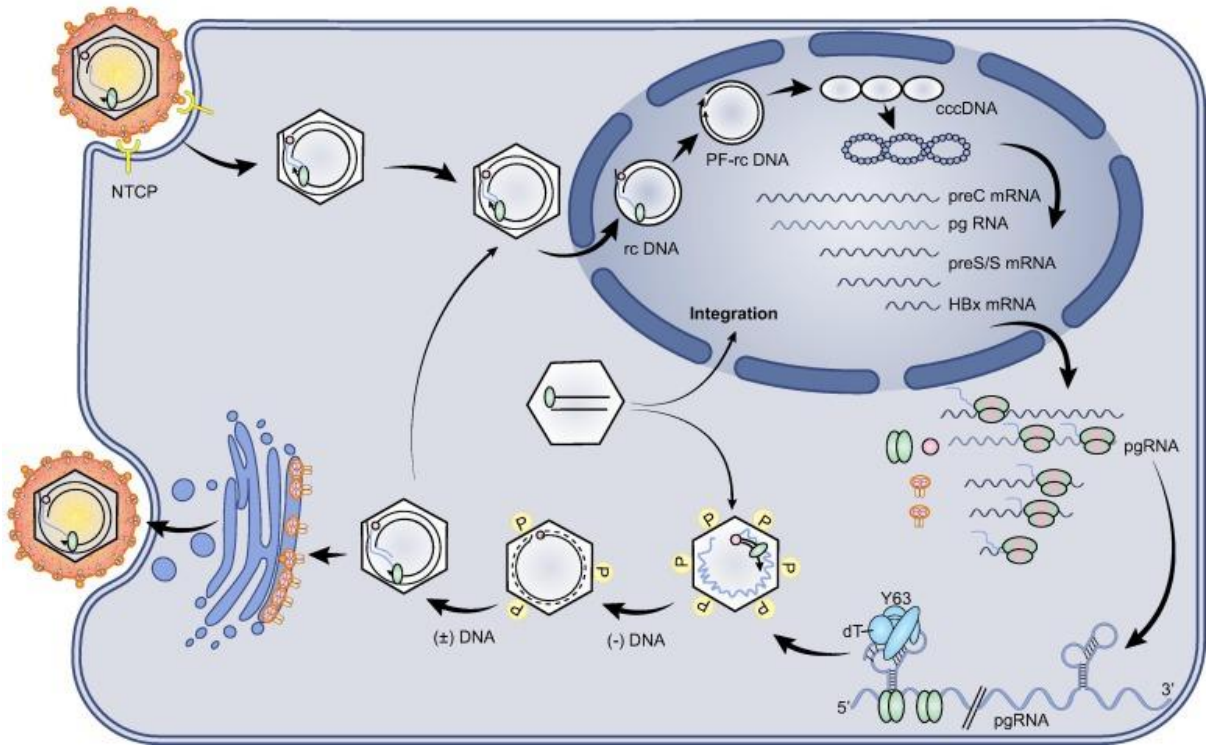



**Figure 1.4:** Life Cycle of HBV. Upon attachment of virions to NTCP the cycle starts. Then uncoating occurs. Uncoating is followed by relocation of the nucleocapsid into the nucleus where the rcDNA is converted into cccDNA. The cccDNA then serves as template for transcription of mRNA and the pgRNA. The mRNAs are then translated into viral proteins in the cytoplasm (Tong and Revill, 2016). Reproduced with permission from *Journal of Hepatology*.

## 1.2 HBV Genotypes

As a result of the HBV RT lacking proof-reading ability, HBV displays sequence heterogeneity. As such, HBV recombinants, sub-genotypes and genotypes emerge (Kramvis et al., 2005, Sunbul, 2014). In relation to viruses, genotypes can be defined as the genomic sequence of the virus that has stabilised for a period of time (Okamoto et al., 1988) . Using intergroup divergence of the complete sequence of 7.5% and divergence within the S gene of HBV, nine genotypes (A-I) have been identified (Hannoun et al., 2000, Kramvis et al., 2008, Norder et al., 1992, Stuyver et al., 2000) (Figure 1.5). These genotypes are distributed worldwide (Table 1.2; Figure 1.5). In 2009, a tenth genotype (J) was proposed (Tatematsu et al., 2009), but this genotype has not been widely accepted, as it was isolated from a single Japanese individual. Using 4 to 8% intergroup nucleotide difference in the HBV genome, subgenotypes of genotypes A, B, C, D, F and I have been determined (Kramvis, 2014).

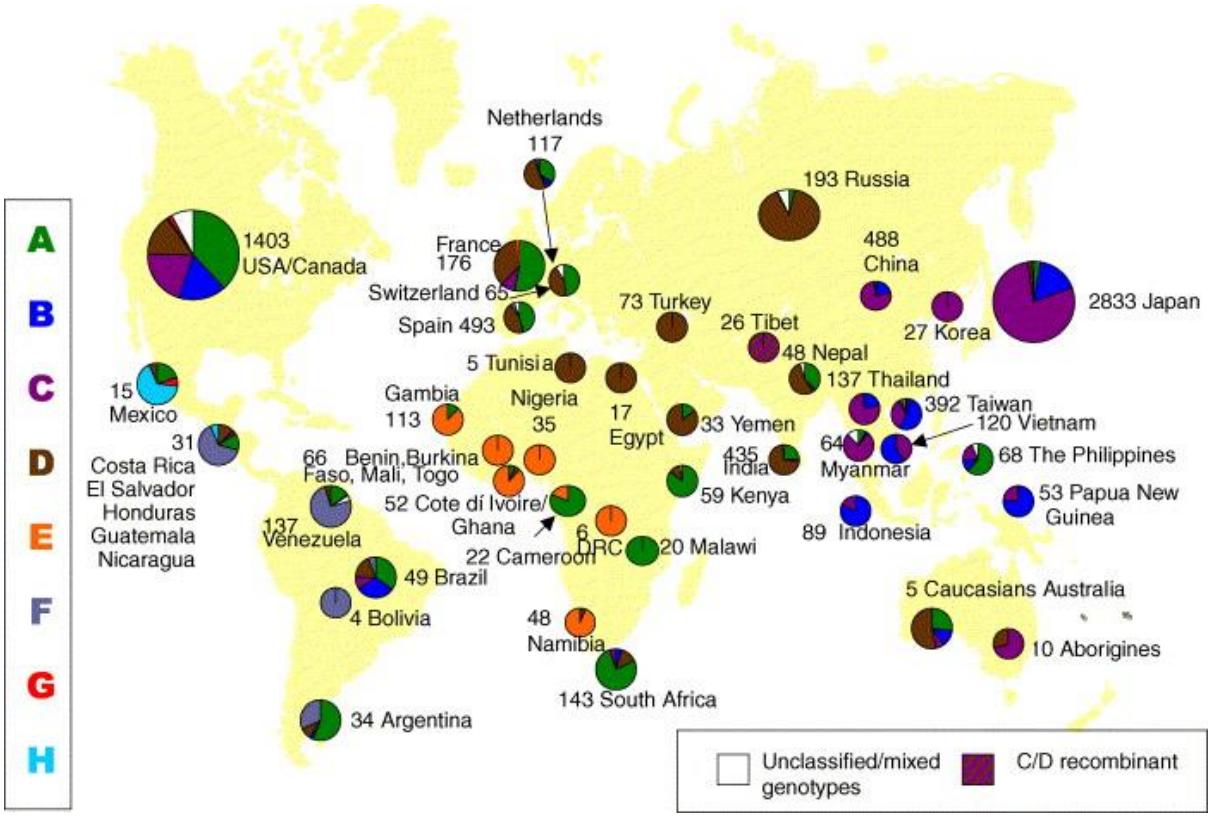



**Figure 1.5**: The nine HBV genotypes which are distributed worldwide. Each genotype is shown in a different colour and the geographic distribution of the genotypes can be seen (Kramvis et al., 2005). Reproduced with permission from *Elservier*.

Four serological subtypes of HBV have been identified, termed: *adw*, *adr*, *ayw* and *ayr*. These subtypes are determined by amino acid variations in the major antigenic domain (the "a"determinant) of the S ORF, as well as by two mutually exclusive sub-determinants at positions 122 and 160 (Bancroft et al., 1972, Le Bouvier, 1971). In addition, the four subtypes can be classified into ten serological subtypes based on further variations at residues. These ten subtypes are; *ayw*1, *ayw*2, *ayw*3, *ayw*4, *ayr*, *adw*2, *adw*3, *adw*4, *adrq+* and *adrq-* (Arauz-Ruiz et al., 2002, Norder et al., 1992). The association between serotypes and genotypes is shown in Table 1.2. Using computer and statistical analysis, a highly statistically significant association between serological subtypes and genotypes has been found [$\chi^2$-test for association, $p<0.0001$ (Kramvis et al., 2008)]. The serological subtype frequencies for genotypes A, B, F, G, and H are similar ($\chi^2$-test for association, $p=0.58$), with

the frequencies for genotypes D and E also being similar ($\chi^2$- test for association, p=1.0) (Kramvis et al., 2008).

**Table 1.2**: Worldwide Distribution of HBV Genotypes.

| Genotype | Serotype | Geographical distribution | References |
|---|---|---|---|
| A | *adw*2, *ayw*1, *ayw*2, *adw*4 | North western Europe, USA, sub-Saharan Africa, East Africa and central Africa , Japan, and the Philippines | (Kimbi et al., 2004, Sugauchi et al., 2003, Swenson et al., 2001, Usuda et al., 1999) |
| B | *adw*2, *ayw*1, *adr* | Indonesia, South and East Asia, Vietnam, Brazil, Indonesia, the Philippines and Venezuela | (Blitz et al., 1998, Swenson et al., 2001, Usuda et al., 1999) |
| C | *adr, adrq+, adrq- ayr, adw,* *adw*2, *ayw*2, *ayw*3, *adwr* | Venezuela, Korea, China, Japan Polynesia, Vietnam, East Asia, Japan, the Philippines, Tibet Australia | (Sugauchi et al., 2003, Usuda et al., 1999) |

| | | | |
|---|---|---|---|
| | | [Aborigines] and Japan | |
| D | *ayw*, *ayw*3, *ayw*4, *adw*3 | West, Central and, East Africa, India<br>Mediterranean area, Middle East, USA and<br>Central America | (Arankalle et al., 2003, Arauz-Ruiz et al., 2002, Borchani-Chabchoub et al., 2000, Flodgren et al., 2000, Norder et al., 1993, Swenson et al., 2001) |
| E | *ayw*4 | West Africa, Nigeria, Western and Central<br>Africa | (Mulders et al., 2004, Odemuyiwa et al., 2001) |
| F | *adw*4, *adw*4*q*−, *adw*2, *ayw*4 | South and Central American Hispanics, France,<br>Alaska, | (Arauz-Ruiz et al., 1997, Blitz et al., 1998, Norder et al., 1992, Quintero et al., 2002) |
| G | *adw*2 | France, Germany, USA and the United | (Stuyver et al., 2000) |

| | | | |
|---|---|---|---|
| | | Kingdom | |
| H | *adw*4 | Northern part of Latin America, Central America and Mexico | (Arauz-Ruiz et al., 1997) |
| I | *adw*2 | Vietnam and Laos | (Arankalle et al., 2010, Tong et al., 2013) |

HBV genotypes are distributed worldwide (Table 1.2; Figure 1.5). For example, genotype A is dominant in Europe, Africa and India. Genotypes B and C are dominant in Asia, whereas, genotypes D and E are dominant in the middle east, Latin America, West Africa and Colombian populations (Alvarado Mora et al., 2010, Kramvis et al., 2005, Odemuyiwa et al., 2001). Genotype F is dominant in Central and South America (Pineiro y Leone et al., 2003, Torres et al., 2011). Genotypes G and H are dominant in France, Mexico and the United States of America (Stuyver et al., 2000, Torres et al., 2011). The last HBV genotype, genotype I was found in Vietnam and Laos (Olinger et al., 2008, Tran et al., 2008).

The distinct geographic distribution of HBV genotypes can be used to trace the dispersal patterns of HBV infection as a result of migrations. This is also crucial in linking the genotypes to different clinical outcomes within regions because HBV genotypes have been shown to affect the clinical outcomes of infection and response to antiviral therapy (Kramvis, 2014, Lin and Kao, 2010).

HBV endemicity is very high in Africa (Figure 1.6). Three genotypes A, D and E predominate (Kramvis and Kew, 2007); Figure 1.6), whereas, genotypes B and C are detected

sporadically (Zekri et al., 2007). Genotype A has a total length of 3221 bp (eg: AY233275, an accession number of sub-genotype A1 sequence). It is characterised by a 6 nucleotide insertion at the 3' end of the core gene, which results in two amino acids at positions 153 and 154 at the core gene. This insertion and the amino acids have not been found in other genotypes (Kramvis et al., 2005). Genotype A has been classified into subgenotypes A1, A2, quasi-subgenotype A3 (A3, A4, A5 and A7) and A4 (Pourkarim et al., 2011). In sub-Saharan Africa, subgenotype A1 is the predominant subgenotype (Bowyer et al., 1997, Kimbi et al., 2004, Kramvis et al., 2002) and has been associated with high risk of HCC (Kew et al., 2005). Patients infected with subgenotype A1 have a higher rate of HBeAg negativity, low HBV DNA levels (Tanaka et al., 2004) and develop HCC at a younger age compared to patients infected with other genotypes (Kew et al., 2005).

Genotype D has a worldwide distribution. In Africa, it has been described in Egypt, Morocco, Tunisia, West and South Africa (Borchani-Chabchoub et al., 2000, Sbai et al., 2007, Yousif et al., 2013, Zekri et al., 2007). The total length of Genotype D is 3182 bp nucleotides. It is characterised by a 33-nucleotide deletion at the N terminus of the *preS1* region. Genotype D has been divided into six subgenotypes: D1 to D6 (Yousif and Kramvis, 2013). These subgenotypes are diverse. For example, Subgenotype D1 is found mainly in the Middle East, subgenotype D2 in Europe and Asia, subgenotype D3 in South Africa, Northern America and Europe, subgenotype D4 in Northern and Central Americas and Australia, subgenotype D5 only in India, subgenotype D6 in Papua New Guinea, Indonesia, Morocco and Tunisia (Yousif and Kramvis, 2013). Most patients infected with genotype D generally seroconvert to anti-Hepatitis B e-antigen (anti-HBe) positivity at a more advanced age, which means they may undergo multiple hepatitis flares, which accelerates the progression of chronic hepatitis to cirrhosis (Lin and Kao, 2010, McMahon et al., 2001).

Genotype E has a total length of 3212 bp. It is mostly found in Africa, especially in central and West Africa (Kao, 2002, Mulders et al., 2004). It is characterised by a 3 nucleotide deletion in the pre S1 region (Kramvis et al., 2005, Norder et al., 1996). Furthermore, the amino acids of the *preS1*, *preS2* and S genes are well conserved, with signature motifs specific for genotype E: Leu, Ser, Trp, Thr, Val, Pro, Leu, Glu and Trp at positions 3 to 11 of the *preS1* (Kramvis et al., 2005). However, Genotype E does not separate from genotype D in the X and C ORFs (Kidd-Ljunggren et al., 2002, Kramvis et al., 2005), and its existence was initially questioned (Bowyer et al., 1997, Kidd-Ljunggren et al., 2002). Moreover, this genotype has been found to have a low genetic diversity in a large geographical area. This suggests that this genotype emerged recently (Andernach et al., 2009).

Interestingly, recombination has been reported between genotype A and D over the non-overlapping regions of the S and P ORFs (De Maddalena et al., 2007, Odemuyiwa et al., 2001). Genotype D/E recombinant has also been detected in Sudan (Yousif et al., 2013). This could have resulted from the recombination of two or more genotypes co-circulating in the geographical region. When two or more genotypes circulate at the same time, co-infection with more than one genotype may occur and lead to the recombination events between these genotypes and provide a variation within individuals and in the population in general (Kramvis et al., 2005). Recombination may also help the virus evade the host immune response by changing antigenetic configurations (Shi et al., 2012). Recombination events increase genetic diversity in the HBV population and lead to the designation of an increased number of genotypes/subgenotypes. Different studies that determined the HBV genotypes and serotypes worldwide are listed in table 1.2 below (Arankalle et al., 2010, Arankalle et al., 2003, Arauz-Ruiz et al., 2002, Arauz-Ruiz et al., 1997, Blitz et al., 1998, Borchani-Chabchoub et al., 2000, Flodgren et al., 2000, Kimbi et al., 2004, Mulders et al., 2004,

Norder et al., 1993, Norder et al., 1992, Odemuyiwa et al., 2001, Stuyver et al., 2000, Sugauchi et al., 2003, Swenson et al., 2001, Usuda et al., 1999).

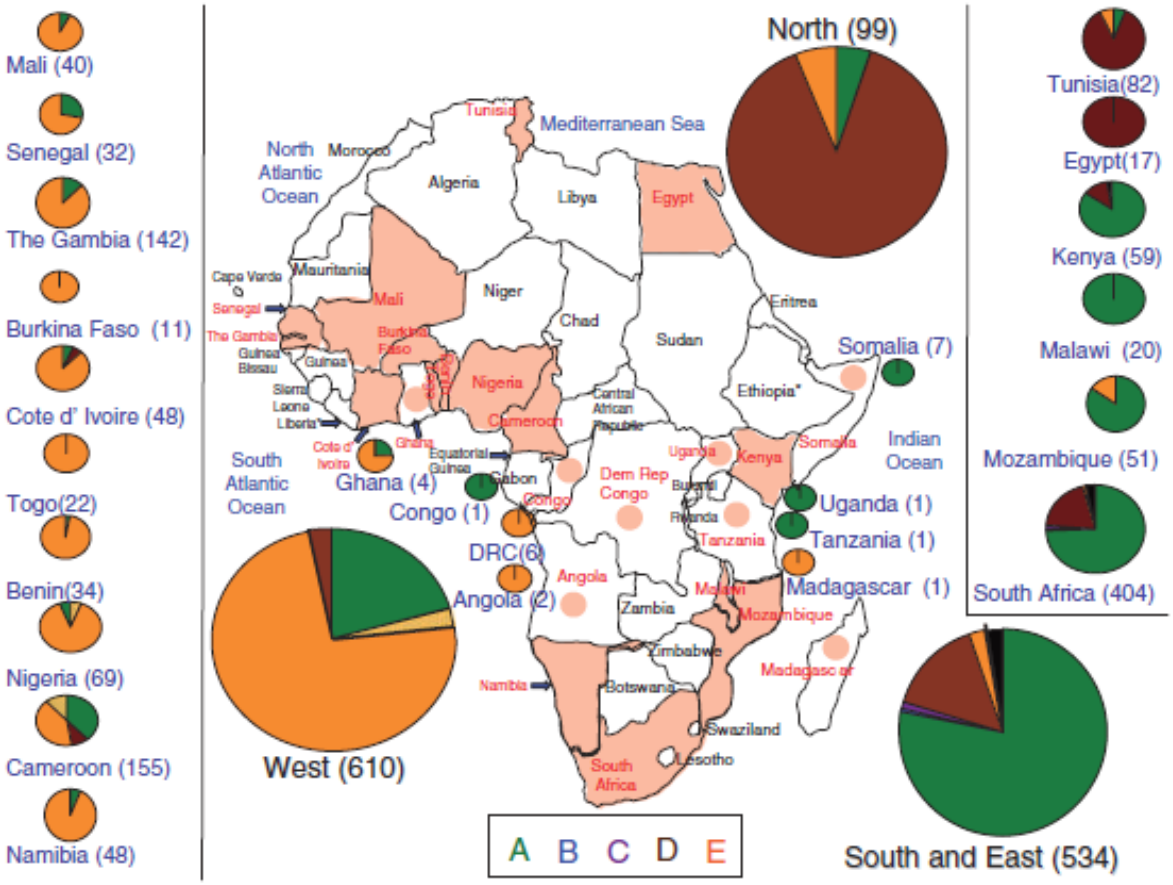



**Figure 1.6:** HBV Genotypes Distribution on the African continent (Kramvis and Kew, 2007). Each genotype is coloured differently. Genotype A is predominant in South and East of Africa, genotype E is predominant in the West Africa and genotype D is predominant in the North. Accessed on November 29, 2017. Reproduced with permission from *Hepatology Research*.

### 1.3 HBV mutants: distribution and effect on disease progression

Mutations are random changes in the genome, which result in genetically different individuals. The mutation rate of HBV is extremely high due to the absence of proof reading ability of HBV reverse transcriptase (Paraskevis et al., 2013). Changes in the viral genome

due to mutations play a crucial role in virus adaptation to the environment and to antiviral treatments resulting in viral evolution.

### 1.3.1 Mutations that can affect expression of HBeAg

The basic core promoter (BCP) controls the transcription of precore and pre-genomic mRNA. BCP mutations were discovered by Okamoto and colleagues (Okamoto et al., 1994). Precore and BCP mutations are the two main mutations that affect HBeAg expression. In the precore region, not a full precore protein, a G to A mutation at position 1896 results in a stop codon (TGG to TAG) in the second last codon of precore region not full precore protein. This mutation, *"truncates the HBeAg precursor at 28 amino acids, affecting the HBeAg expression"* (Carman et al., 1989). The position of this mutation overlaps with the encapsidation signal (ε), which is a stem-loop structure of the pregnomic RNA (Li et al., 1993, Lok et al., 1994). To maintain stability of the ε signal, the G1896A mutation, requires a U at position 1858, to form a Watson-Crick base-pair. As such, this mutation is genotype specific because T1858 occurs in genotypes B, D, E G and some strains of C, while C1858 only occurs in genotypes A, F, H and some strains of genotype C. This explains the absence of G1896A in genotypes A, F, H and some strains of genotype C (Kramvis et al., 2005, Locarnini et al., 2003, Lok et al., 1994, Tong et al., 2013).

Further mutations at positions 1816, 1817, 1862, 1874 and 1897 can also affect HBeAg expression (Gunther et al., 1999, Kramvis et al., 1998). HBeAg translation can be aborted by precore start codon mutations at 1814, 1815 and 1816 (Gunther et al., 1999). In addition, mutations at positions 1817, 1874 and 1897 cause HBeAg truncation (Gunther et al., 1999). Moreover, the silent mutation, G1899A frequently occurs together with the precore stop codon mutation, G1896A (Gunther et al., 1999). Lastly, G1862T mutation, which occurs in the bulge of the ε signal (Kramvis et al., 1997, Kramvis et al., 1998), results in a valine to

phenylalanine mutation in the precore protein. This interferes with post-translational modification of the HBeAg precursor, resulting in intracellular retention and impaired secretion of HBeAg (Chen et al., 2008). G1862T has been detected more frequently in genotype A, particularly in subgenotype A1 (Kramvis et al., 1997).

The main mutant in the BCP region is the double mutation, A1762T/G1764A. This mutation results in transcriptional down-regulation of pre-core mRNA. Hence, decreasing HBeAg production and increasing the pre-genomic RNA packaging (Buckwold et al., 1996, Hunt et al., 2000). Interestingly, this type of mutation has been associated with HBV isolated from HCC patients (Kramvis and Kew, 1999). Furthermore, this mutation in conjunction with the T1753C mutations, may be used as a marker for advanced liver disease (Kramvis and Kew, 1999).

In the BCP region, variation at position 1809 to 1812 within the Kozak sequence occur, which is critical in the initiation of translation (Kozak) (Ahn et al., 2003, Baptista et al., 1999, Kozak, 1989). Mutations in this sequence affect HBeAg expression at the translational level and reduce HBeAg synthesis (Ahn et al., 2003). These variations occur mainly in subgenotype A1, which can be differentiated from subgenotype A2 by TCAT, instead of GCAC in the Kozak sequence, and 1888A instead of 1888G in the precore region (Kramvis et al., 2008).

### 1.3.2 Mutations in the complete S region

The surface gene has an important role in the HBV life cycle and infectivity of the virus. The highest heterogeneity in the HBV genome is found in the *preS1* and *preS2* regions. Mutations in these regions have been identified in HBV isolated from chronic hepatitis B patients

(Gunther et al., 1999). Deletions and mutations at the *preS2* start codon prevent *preS2* protein synthesis, indicating that the expression of *preS2* protein is not essential for viral replication, virion morphogenesis, and secretion (Yokosuka and Arai, 2006).

HBsAg contains the "a" determinant, which is the initial target of the Hepatitis B immune status/Anti-Hepatitis B surface antibody (anti-HBs) immune response (Carman et al., 1990). Mutations of the surface gene at the "a" determinant region lead to variations in the antigenicity and immunogenicity of the HBsAg, which may result in the inability of the immune system to detect the virus, "immune escape" (Torresi, 2002). Immune/vaccine escape mutants occur following vaccination and the treatment of liver transplantation recipients with HBV immunoglobulin (Carman et al., 1990). Moreover, there is a potential risk of false negative HBsAg results because commercially available monoclonal or polyclonal antibody may not detect the mutant HBV strains, "detection escape" (Yokosuka and Arai, 2006). The most common detection escape mutation is G to R at 145 in the S protein (sG145R), which minimises the binding of antibodies raised against S protein, to virions and subviral particles. This can moreover lead to breakthrough infection (Cooreman et al., 1999, Tong et al., 2013). Another mutation at the same position, sG145A, leads to diagnostic failure (Cooreman et al., 1999, Tong et al., 2013). Mutations sP120L, sT123N, sT126N, sQ129R, sM133L, sM133T and sD144A also have been reported to lead to vaccine escape (Carman et al., 1990, Yokosuka and Arai, 2006).

### 1.3.3 Drug resistance mutations and drugs active against HBV

Once patients test positive for HBV, they require treatment. Immunomodulatory agents, interferon-α and pegylated interferon and NA antiviral agents, such as nucleoside reverse transcriptase inhibitors (NRTIs) have been used for the treatment of chronic HBV infection

(Ching-Lung Lai, 2002). The following NRTIs have activity activity against HBV: Emtricitabine (FTC), Lamivudine (3TC), Tenofovir disoproxil fumarate (TDF), and Tenofovir alafenamide (TAF). FTC and 3TC drugs are similar and analogues of cytidine. They work by inhibiting RT of HBV. This interferes with replication of the virus and reduces its viral load. However, 3TC-resistant HBV emerges in approximately 40% and 90% of patients after 2 and 4 years on 3TC, respectively when used alone (Benhamou et al., 1999). Thus, it must be used in combination with other anti-HBV drugs. TDF also works by inhibiting viral RT, as such, preventing viral replication, with a limited inhibition of other enzymes such as DNA polymerases α, β, and mitochondrial DNA polymerase γ (Murakami et al., 2015). TAF also inhibits the viral RT, but it must first be converted to a pharmacological form Tenofovir diphosphate (Murakami et al., 2015).

Long-term use of NAs is associated with the evolution of viral resistance mutations in the HBV polymerase gene, resulting in treatment failure (Figure 1.7a) (Locarnini et al., 2003). The most common mutation, which confers lamivudine resistance and occurs in the RT domain, is the rtM204V/I at the YMDD motif (Torresi, 2002). This mutation can occur together with mutations: rtV173L or rtL180M (Ogata et al., 1999). Lamivudine resistance increases progressively during treatment at rates between 14 and 32% annually, approaching 70% after 48 months of treatment (Lai et al., 2003).

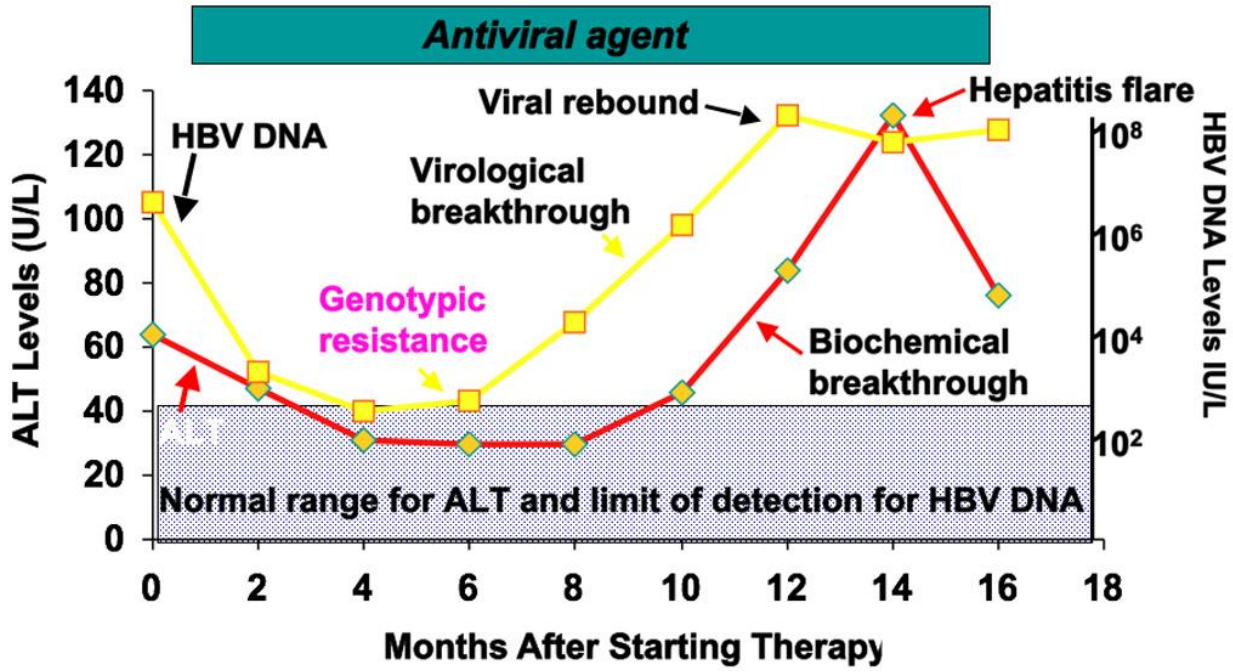



**Figure 1.7a:** The course of chronic hepatitis B patients treated with an antiviral agent who develops genotypic resistance, followed by virological and biochemical breakthrough and viral rebound with a flare of hepatitis (Ghany and Doo, 2009). Reproduced with permission from *Hepatology*.

Mutations associated with adefovir resistance are rtA181V/T and rtN236T (Tong et al., 2013). Entecavir has a higher genetic barrier to resistance because three mutations are needed to generate resistance (Gish et al., 2012). After 5 years of therapy with the NAs, resistance to lamivudine is 80%, 29% to adefovir and only 1.2% to entecavir (Zoulim and Locarnini, 2009).

Moreover, because the polymerase ORF overlaps HBsAg, amino acid changes in HBsAg selected by NAs can alter its antigenicity (Torresi, 2002). HBsAg silent mutations overlapping the polymerase region mutations selected by NAs include the mutants, sE164D and sM198I (Torresi, 2002). Mutations in HBsAg small envelope protein from HBsAg-negative (OBI) are shown in figure 1.7b.

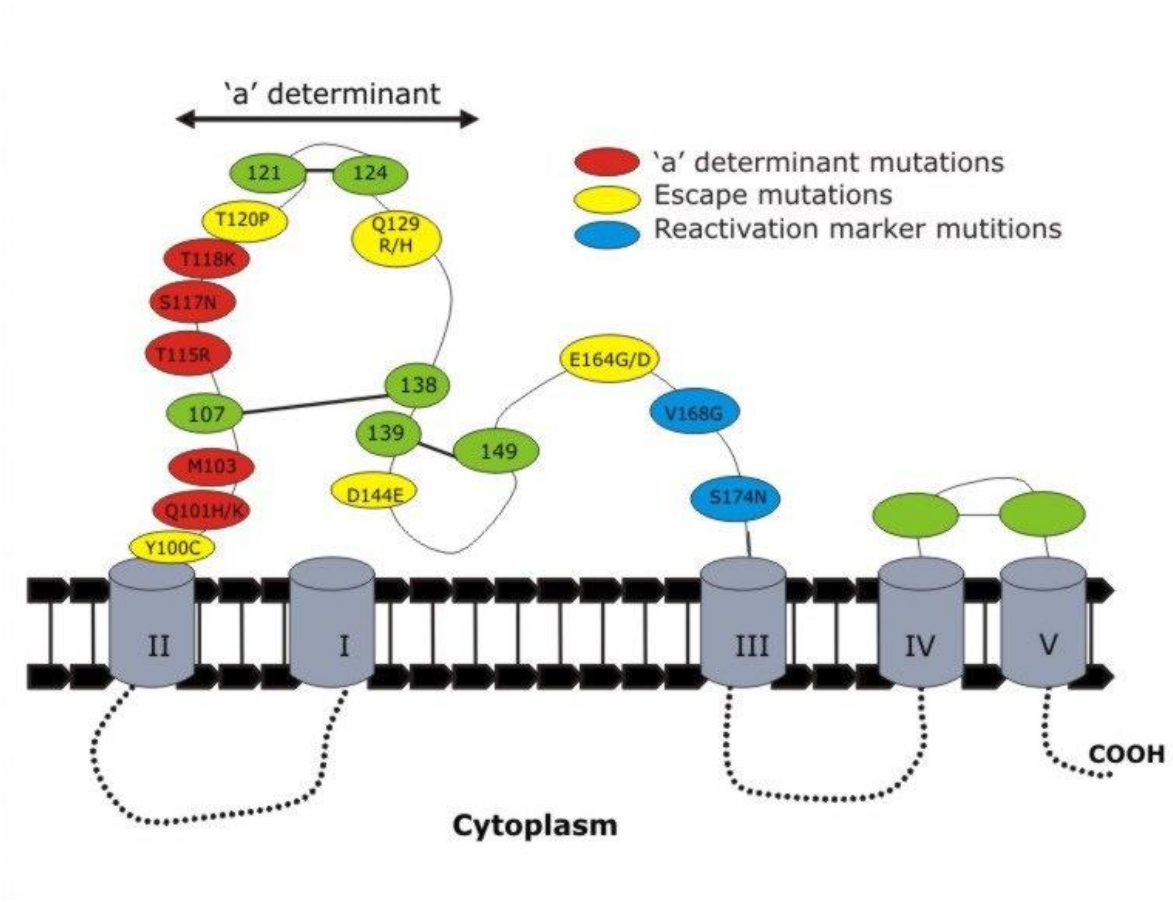



**Figure 1.7b:** Graphical Representation of the mutations found on the HBsAg small envelope protein from the HBsAg-negatives (occults). The red circles show the mutations found in the HBsAg "a" determinant epitope, yellow circles show the immune/vaccine escape mutants and the blue circles represents the HBV reactivation mutation markers. Accessed on November 21, 2017 from (Makondo et al., 2012). Reproduced with permission of *PLOS One*.

### 1.4 Chronic kidney disease and Haemodialysis

Kidneys are the organs responsible for waste filtering in the body. A normal individual is born with two functional kidneys. However, an individual can live with only one functioning kidney. The loss of both kidneys would lead to a rapid accumulation of wastes and death within a few days.

Chronic kidney disease is a condition that damages the kidneys and decreases their ability to keep an individual healthy (Sun et al., 2017)**.** If kidney disease progresses, wastes in the blood accumulate to high levels and make an individual feel unwell. Complications, such as high blood pressure, anaemia (low red blood cell count), weak bones, poor nutritional health and nerve damage may also develop (Hill et al., 2016). Kidney disease increases the risk of heart and blood vessel disease. These problems may develop slowly over a long period of time (Sun et al., 2017).

Glomerular filtration is the process by which the kidneys filter the blood, removing excess wastes and fluids (Robinson et al., 2016). The stages of chronic kidney disease are determined using the Glomerular Filtration Rate (GFR). (Figure 1.8). GFR is a calculation that determines how well the blood is filtered by the kidneys (Robinson et al., 2016). It is one way to measure kidney function. It estimates how much blood passes through the glomeruli each minute. GFR usually calculated using a formula that includes a person's age, gender, race, and serum creatinine levels (Garg and Bakris, 2002). A GFR under 60 mL/min/1.73 $m^2$ may indicate kidney disease (Garg and Bakris, 2002). The GFR value is directly proportional to kidney status. The lower the GFR number, the worse the kidney function. GFR is an estimate, it may not be a good measure of kidney health in some people, such as the very young or very old, amputees, or obese people (Garg and Bakris, 2002). In some cases, GFR

may also be estimated with a 24-hour urine collection (Robinson et al., 2016). Chronic kidney disease is defined as either kidney damage or GFR of less than 60 for longer than 3 months (Garg and Bakris, 2002). When GFR is below 15, patients require haemodialysis.

**Stages of chronic kidney disease**

| Stage | Description | GFR | What this means to you |
|---|---|---|---|
| 1 | Kidney damage with normal or high GFR | 90 or above | • Your doctor will try to find the cause of your kidney disease and begin treatment.<br>• Manage other health problems, such as diabetes and high blood pressure.<br>• See your doctor regularly to monitor your condition. |
| 2 | Kidney damage with mildly low GFR | 60-89 | • Your doctor will estimate how quickly your disease is progressing.<br>• Manage other health problems, such as diabetes and high blood pressure.<br>• Continue regular monitoring. |
| 3 | Kidney damage with moderately low GFR | 30-59 | • Your doctor will check you for complications, such as anemia and bone disease, and begin treatment if needed.<br>• Continue monitoring. |
| 4 | Kidney damage with severely low GFR | 15-29 | • Decide what type of treatment you want if kidney failure develops.<br>• Continue treatment and monitoring. |
| 5 | Kidney failure | Below 15 | • Start dialysis, have a kidney transplant, or choose palliative care.<br>• Continue to see your doctor for treatment and testing. |

**Figure 1.8:** The stages of Chronic kidney disease. Modified from http://Webmed.com. Accessed on November 6, 2017. Reproduced with permission from National Kidney Foundation.

## 1.5 HBV and Kidney Disease

The association between HBV infection with kidney disease is mostly detected in areas where infection occurs at an early stage either in childhood or during infancy (Chow et al., 2006). This association increases the frequency of chronic carriers in endemic areas. Due to the lower likelihood of childhood infection and lower prevalence rate, the frequency of HBV-related renal disease is relatively low in the USA and Europe compared to Africa (Chow et al., 2006). Through the determination of hepatitis B antigen-antibody complexes in the renal lesions using immunofluorescence microscopy, the pathogenicity of HBV in kidney infection were shown (Ribot et al., 1979). Despite the fact that HBV DNA has been shown in tubular infected kidney cells, its exact role is yet to be confirmed (Wang et al., 2005). Chronic HBV infection is an etiologic factor in secondary glomerular diseases (Chan, 2010). To date, only three renal diseases have been associated with HBV infection: Membranoproliferative glomerulonephritis, Membranous nephropathy and Polyarteritis nodosa. HBV can contribute towards renal disease via the host immune response to the infection, which plays a huge role in pathogenesis of HBV and HBV-associated nephropathy (Jones et al., 2002, Vo Thi Diem et al., 2005, Kilic et al., 2010). The deposition of HBV antigen-antibody immune complexes leads to glomerular and interstitial tubular damage, eventually resulting in the decline of renal function (Vo Thi Diem et al., 2005). Furthermore, HBV replication may promote apoptosis of renal tubular cells and lead to a decline in renal function (Chan, 2010). The treatment of HBV-related renal diseases consists of antiviral therapy in most patients. However, the data of HBV-related renal disease treatment is limited due to a few cases and uncontrolled observations, as well as no randomised trials (Chan et al., 2016).

The incidence of HBV infection in patients undergoing haemodialysis was reported to be 3.5% in 2001 (CDC, 2001). HBV infection in patients undergoing haemodialysis has

declined in recent years (Somi and Hajipour, 2012). However, as levels are still unacceptably high in developing countries, further work is required. The higher HBV infection rates within dialysis units in the developing world may be due to the higher background prevalence of HBV in the general population, as well as difficulties following infection control strategies against HBV, such as "standard" precautions, routine hemodialysis precautions, cohorting of HBsAg-positive patients by rooms, machines and staff and vaccination against HBV (Martin and Friedman, 1995).

The widespread use of hepatitis B vaccination has decreased the incidence of HBV-related renal diseases, providing evidence of the probable pathogenic role of HBV. Through the use of the vaccine, the HBV infection can be controlled. However, outbreaks of infection continue to occur in dialysis units, and prevalence rates are still high (Edey et al., 2010). The rate of antibody sero-conversion is lower in ESRD patients (DaRoza et al., 2003). The double-dose vaccination is recommended for ESRD patients before they undergo dialysis (Somi and Hajipour, 2012). If the dose is not effective, adjuvanted vaccines must be used for better response (Somi and Hajipour, 2012). However, more research is still needed to find effective adjuvants with less negative effects on patients.

### 1.6 Haemodialysis and HBV Infection in Sudan

If the GFR (mL/min/1.73m$^2$) is less than 15, then the individual is diagnosed with ESRD and requires haemodialysis. Haemodialysis is a treatment, which involves the use of a machine to filter out wastes in the blood using a solution. This usually occurs three times a week.

HBV infection among ESRD patients undergoing haemodialysis was first identified in the late 1960s in United Kingdom (CDC, 1977). According to statistics released in 1974, the

incidence of newly acquired HBV infection in haemodialysis patients was found to be 6.2%, which was relatively lower than the 30% reported in selected centres (CDC, unpublished data, 2001). However, the incidence rate has drastically decreased, with only 3.5% reported in all centres by 2001 (CDC, unpublished data 2001). This is mainly due to HBV vaccination, infection control measurements and screening for HBsAg and hepatitis B core antibody (anti-HBc). The prevalence of HBsAg in ESRD patients undergoing haemodialysis has been found at different frequencies worldwide. For example, Asian Pacific countries (1.3% to 14.6%) (Oguchi et al., 1992) has one of the highest prevalence rates of HBsAg in haemodialysis patients compared to 5.9% found in Italy (Mioli et al., 1992) and 1% found in USA (Finelli et al., 2005), as well as 12% found in Brazil (Teles et al., 1999).

Patients with ESRD are prone to OBI, which later results in distinct clinical problems among these patients (Said, 2011). This is mainly due to the immunosuppressive nature of renal failure and exposure to invasive procedures as well as sharing the same dialysis machine, and receiving more transfusions than the general population (Kwak and Kim, 2014). This in turn promotes chronicity of the virus and increases the likelihood of nosocomial spread of infection among patients on haemodialysis (Alavian, 2010). Moreover, the rate of chronicity following post-acute infection is 80% in ESRD patients relative to 3% in healthy individuals (Harnett et al., 1988).

Patients undergoing haemodialysis should be vaccinated against the virus. However, some patients have low acceptance and response rates to the HBV vaccine. This contributes to OBI transmission in haemodialysis patients (Motta et al., 2010). The prevalence of OBI transmission in haemodialysis patients varies from 0% to 54% according to the diagnostic techniques or HBV endemicity (Minuk et al., 2004, Yoo et al., 2013). Several other studies

suggest that OBI could be a source of viral spread both to other patients and staff within the haemodialysis units (Minuk et al., 2004, Motta et al., 2010). Therefore, patients and staff need HBV vaccine boosts to maintain levels of protective (anti-HBs) (Edey et al., 2010). Patients undergoing haemodialysis are also given HBV treatment drugs such as Lamivudine (Edey et al., 2010). Lamivudine suppresses the viral infection as well as improving the liver histology in patients suffering from HBV (Leung, 2008) To avoid nosocomial infections, strict dialysis-specific infection-control programmes, including avoidance of dialyser reuse and use of dedicated dialysis rooms and machines, should be implemented (Kwak and Kim, 2014). Moreover, education about HBV transmission methods in dialysis units and other preventative methods should be given to the staff (Kwak and Kim, 2014). Furthermore, regular screening for HBV DNA with sensitive PCR-based assays in all dialysis patients should be considered (Kwak and Kim, 2014) Lastly, more attention should be given to patients who receive immunosuppressive drugs after renal transplantation (Kwak and Kim, 2014).

HBsAg prevalence in haemodialysis patients from Sudan was reported at 4.5% (Gasim et al., 2012). A similar study in Kenya, which included anti-HBs and anti-HBc serology, reported a 8% HBsAg prevalence (Otedo et al., 2003). HBV prevalence may be under-reported in the only Sudanese study (Gasim et al., 2012) relative to the Kenyan one, which found 8% of adults to be HBsAg-positive after six months on dialysis (Otedo et al., 2003). Therefore to elucidate HBV infection and prevalence in Sudanese ESRD patients, additional studies, which include other serological markers and sensitive nucleic acid testing, are required. Sudan is an underdeveloped country, thus have poor maintenance of haemodialysis infrastructure for patients with ESRD because of lack of resources and therefore there may be high risk of HBV infection among these patients (Gasim et al., 2012).

## 1.7 Rationale

HBV genotypes have been found worldwide and they vary depending on the region. (Kramvis et al., 2005). Genotypes A, D and E are predominant in Africa (Kramvis et al., 2005). In Sudan, the predominance of genotype D or E depends on the patient population In Sudanese blood donors genotype E was prevalent at 55%, over genotype D (39%) (Mahgoub et al., 2011), whereas in liver disease patients and asymptomatic HBV carriers, genotype D was prevalent at 63% compared to genotype E at 29% (Yousif et al., 2013). On the hand, in HBV/HIV co-infected individuals, it was reported that genotype E was present at 21% and genotype D at 41% respectively (Yousif et al., 2014). This gives an indication that the distribution of genotypes may be linked to the clinical manifestation of HBV infection.

All previous studies in Sudan were either in blood donors, liver disease patients and HBV/HIV co-infected patients (Mahgoub et al., 2011, Yousif et al., 2013, Yousif et al., 2014). As such, it is important to study other patients suffering from HBV and other diseases. In the present study we examined the prevalence of HBV in patients diagnosed with ESRD, with or without hepatitis C virus (HCV) co-infection, who are undergoing haemodialysis. We also determined the predominant genotype in ESRD patients, in an attempt to give an indication that the distribution of the genotypes may be influenced by the clinical manifestation of HBV infection.

Since, HBV is transmitted via parentally from mother to child, horizontally between individuals, sexually and inatrogenieally. Since dialysis involves the use of needles there is an increased risk of parenteral transmissioon of HBV is transmitted via the parenteral route, ESRD patients have an increased chance of HBV infection (Vladutiu et al., 2000). Furthermore, the rate of developing chronic hepatitis is high in patients with other diseases

and these patients are a potential reservoir for virus transmission (Harnett et al., 1988). In addition, risk factors such as blood transfusion, have been shown to be associated with HBV positivity, which in turn suggests a possible nosocomial infection influencing the spread of HBV in haemodialysis units (Souza et al., 2003). Therefore, in this study, amplification of HBV DNA, followed by sequencing and phylogenetic analyses were used to check whether patients with ESRD on regular haemodialysis are infected with HBV in the selected nine haemodialysis centres in Khartoum.

## 1.8 Aims and objectives

### 1.8.1 Aim

The aim of this study is to molecularly characterise HBV from ESRD patients in Sudan undergoing haemodialysis and also to use phylogenetic analysis to determine if HBV infection is occurring in the four selected haemodialysis centres.

### 1.8.2 Objectives

(i) To molecularly characterise and genotype HBV in HBsAg-positive and HBsAg-negative Sudanese ESRD patients by sub-genomic sequence analysis.

(ii) To identify mutations in the complete S and BCP/PC regions of HBV genome in ESRD patients.

(iii) To identify HBV markers in ESRD patients infected with HBV alone when compared to those co-infected with HBV and HCV.

(iv) To investigate the possibility of a nosocomial infection contributing to the HBV-positivity of ESRD patients in the four selected haemodialysis centres in Sudan.

# Chapter 2: Materials and methods

## 2.1 Ethics clearance

The use of the serum samples was approved by the National Health Research Ethics Committee, Federal Ministry of Health in Sudan (14/10/2014) with proposal number 132-7-10. The samples were imported by the University of the Witwatersrand, Department of Internal Medicine, Hepatitis Virus Diversity Research Unit (HVDRU) with registration number 9699. The use of HBV samples in Africa was granted to Professor Anna Kramvis by the University of the Witwatersrand Human Research Ethics Committee, with medical clearance certificate number M150158. The study was approved University of the Witwatersrand Human Research Ethics Committee, with medical clearance certificate number:M170164 (see Appendix C).

## 2.2 Study samples and patient allocation

The samples were collected for a period between 3rd of December 2014 to 28th of January 2016 in nine centres at Khartoum, Sudan. The patients were chosen based on the fact they were HBV positive, having ESRD and undergoing Haemodialysis. Serum aliquots of 1 ml were stored at - 80°C. A total of 541 patients were recruited, with 448 negative for HCV antibodies and 93 positive for HCV antibodies. From the 448 HCV negative patients, 54 tested positive, with 394 testing negative for HBsAg. From the 394 HBsAg negative patients, 12 tested positive and 382 negative for HBV DNA. Of the 93 HCV positive patients, 2 tested positive and 91 tested negative for HBsAg. From the 91 HBsAg negative patients, 8 tested positive and 83 negative for HBV DNA. So in total, 76 patients, were found to be HBV DNA-positive in Sudan. Of the 76, 8 were excluded since their tubes were empty. Of the remaining 68 patients, 46 were Overt (HBsAg positive), 12 Occult (HBsAg negative and HBV DNA positive) and 10 HBV/HCV co-infected (2 overt:8 occult) (Figure 2.1).

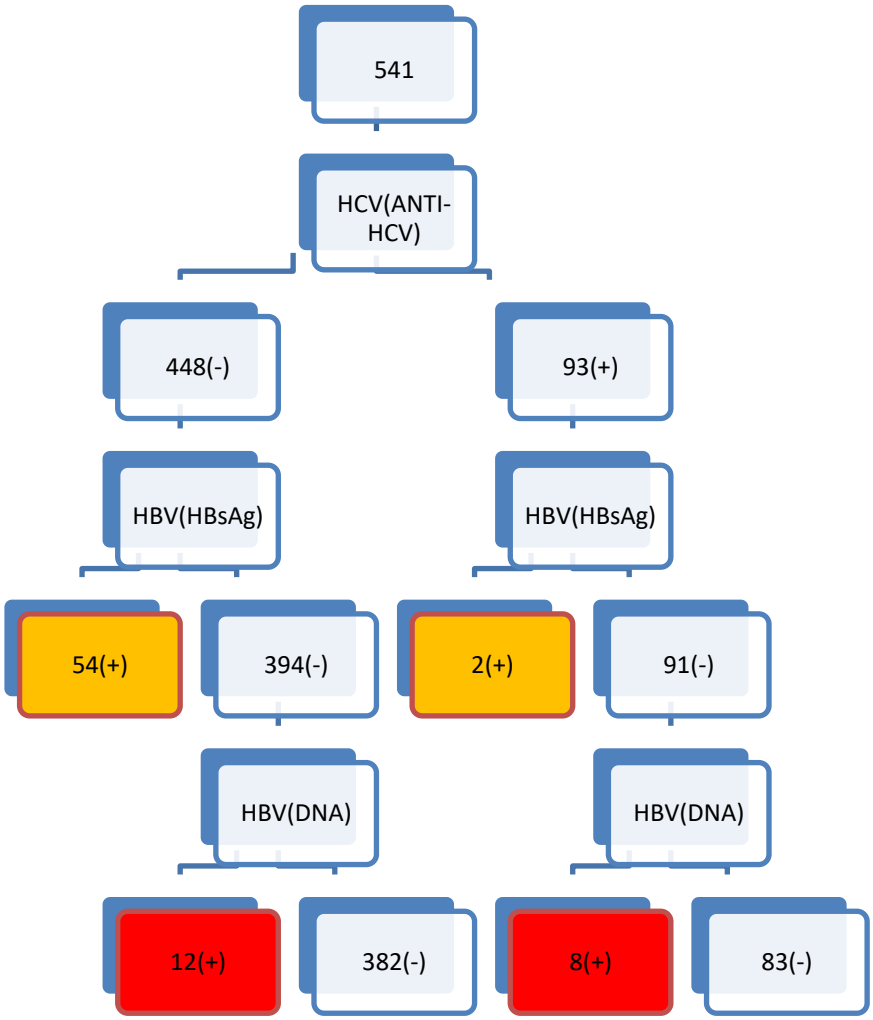



**Figure 2.1**: A flow diagram of sample acquisition (76) from Khartoum, Sudan. The samples used in the present study are highlighted in yellow (overt) and red blocks (occults) of the flow diagram**.**

## 2.3 Methodology

### 2.3.1 Nucleic Acid Extraction

To extract nucleic acids, two extraction methods were used: Maxwell® 16 Viral Total Nucleic Acid Purification Kit (Promega, Madison, WI, USA) and Qiagen kits (QIAamp®, DNA Blood Mini Kit, GmbH, Hilden, Germany) was used. The Maxwell® 16 Viral Total Nucleic Acid Purification Kit was used with a low positive amplification and then in order to improve the positivity of samples, Qiagen kit was used.

#### 2.3.1.1 Maxwell® 16 Viral Total Nucleic Acid Purification Kit

HBV DNA from serum samples was extracted using the Maxwell® 16 Viral Total Nucleic Acid Purification Kit (Promega, Madison, WI, USA), where the Maxwell® 16 Instrument (Cat.# AS2000 ) was used as per manufactures instructions. The instrument was configured to a low elution volume of 50 µl. After configuration of the instrument, samples were mixed with the lysis solution to form a lysate. The latter was then transferred to the cartridges. Purified high quality viral nucleic acid was obtained from 200 µl of serum after 45 minutes.

#### 2.3.1.2 QIAGEN Kit

Serum obtained from Sudanese patients was used to extract HBV DNA using the QIAamp® DNA Blood Mini Kit (QIAGEN, GmbH, Hilden, Germany), as per manufacturer's instructions. The samples 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 microcentrifuge tube, together with 20 µl QIAGEN Protease (Proteinase K). Two hundred microliters of 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 (3,000×g, A-4) 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. This was followed by the addition of 10 µl of elution buffer AE directly to the column. DNA was eluted by centrifugation for 1 min at 8 000 rpm and stored at -20$^{o}$C until needed. Best quality water (BQW) was included in the extraction used as a negative control.

### 2.4 Real time PCR

Real time polymerase chain reaction (Real time PCR) is a technique used to monitor the amplification of a targeted DNA molecule during the polymerase chain reaction (PCR), in real-time, not at its end, as in conventional PCR (Higuchi et al., 1993). Real time PCR uses an increase in the intensity of a fluorescent signal generated by an intercalating dye-labelled probe during amplification of a target sequence to detect nucleic acids either for their presence or absence. It is divided into three stages and visualised on a graph. The first stage is the exponential amplification where the doubling of the DNA product will occur with each cycle. The second stage is the leveling off stage in which the reaction slows down due to the loss of activity of the DNA polymerase and the consumption of reagents. The third stage is the plateau stage in which no more products accumulate due to a complete exhaustion of dNTPs and reagents.

The protocol was followed from (Weinberger et al., 2000). In this study, HBV-Taq 1 and HBV-Taq 2 primers, which amplify the BCP/PC and complete surface regions of HBV were used to quantify HBV DNA in an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, Ca, USA). These HBV Taq 1/2 primers were ordered from the University of CapeTown, school of molecular biology (www.mcb.uct.ac.za/mcb/services/oligonucleotides). The BS-1probe is ordered from Thermo Fisher Scientific. To generate a standard curve, the cloned diluted plasmid DNA, which contained a single genome of HBV DNA at concentrations ranging from $2 \times 10^1$ to $2 \times 10^{11}$ IU/ ml was used as a template. The final concentration of $10^6$ IU/ml was used as an internal control. To ensure validity of the results, all tests were done in duplicate in a 96 well plate. The Ct (Cycle threshold) was later used to measure the reading of PCR interpolated against linear regression of standard curve. After PCR run was complete, the 96 well skirted plates from (Bio-Rad CFX 96 TouchTM Real-Time PCR) were removed and the data was exported to excel format, for further analysis.

### 2.5 Complete S region amplification

PCR is a highly specific and sensitive method designed to amplify large quantities of a DNA sequence without the use of cloning (Mullis et al., 1986). 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 step occurs at different temperatures.

Nested PCR was performed on the complete surface gene using HotStarTaq Master Mix Kit (QIAGEN, GmbH, Hilden, Germany). The reaction mixture for the first round consisted of 12.5 µl of 3 x 0.85 ml HotStarTaq master mix. The 3 x 0.85 ml HotStarTax master mix consisted of (250 units HotStarTaq DNA Polymerase, PCR Buffer with 3 Mm $MgCl_2$, and 400 µM of each dNTP). The HotStarTaq master mix was mixed with 0.375 µl each of 20 µM

primer 2410 (+) and 1314 (-) and 2.5 µl of DNA, with the reaction topped up to 25 µl with distilled water. The reaction mixture for the second round consisted of 25 µl HotStar Taq master mix, 0.75 µl of each 20 µM primer 2451 (+) and 1280 (-), 5 µl of first round PCR product and topped up to 50 µl with distilled water. Primer sequences and PCR conditions are listed in (Appendix A)**.**

### 2.6 BCP/PC region amplification

Nested PCR was performed on the BCP/PC region (1742 to 1900 from the *Eco*RI site) using BIOLINE PCR kits (London, UK). The reaction mixture for the first round consisted of 2.5 µl of 10× $NH_4$ PCR buffer, 0.75 µl of 50mM $MgCl_2$, 2 µl of 10 mM dNTPs, 1.25 µl of each 20 µM primer 1606 (+) and 1974 (-), 0.1 µl of Taq DNA polymerase, 2.5 µl of DNA and topped up to 25 µl with distilled water. The reaction mixture for the second round consisted of 5 µl of 10× $NH_4$ PCR buffer, 1.5 µl of 50mM $MgCl_2$, 4 µl of 10 mM dNTPs, 2.5 µl of each 10 µM primer 1606 (+) and 1974 (-), 0.2 µl of Taq DNA polymerase, 5 µl of DNA and topped up to 50 µl with distilled water. Primer sequences and PCR conditions are listed in (Appendix A).

### 2.7 P7/P8 (Short S fragment) amplification

Nested PCR was performed on the Short S region (459–710 from *Eco*RI site) using BIOLINE PCR kits (London, UK). Primers P7 (256 to 278 from *Eco*RI site) and P8 (796 to 776 from *Eco*RI site) were used to amplify nucleotides 256–796 of the Short S region. The reaction mixture for the first round consisted of 2.5 µl of 10× $NH_4$ PCR buffer, 0.75 µl of 50mM $MgCl_2$, 2 µl of 10 mM dNTPs, 1.25 µl of each 20 µM primer 255 (+) and 759 (-), 0.1 µl of Taq DNA polymerase, 2.5 µl of DNA and topped up to 25 µl with distilled water. The reaction mixture for the second round consisted of 5 µl of 10× $NH_4$ PCR buffer, 1.5 µl of 50mM $MgCl_2$, 4 µl of 10mM dNTPs, 2.5 µl of each 10 µM primer 1606 (+) and 1974 (-), 0.2

µl of Taq DNA polymerase, 5 µl of DNA and topped up to 50 µl with distilled water. Primer sequences and PCR conditions are listed on (Appendix A).

### 2.8 Detection of Amplicons (Gel Electrophoresis)

A 5 µl aliquot of the amplicon was analysed by agarose gel electrophoresis. The percentage of the agarose used varied between 0.8% and 1.5%, depending on the expected size of amplicon. Commercially available DNA ladders of 100 bp or 1 kb (Promega Madison, USA) were used to determine the size of the amplicon. HBV DNA from HBsAg-positive, DNA positive patients was used as a positive control. BQW or DNA extracted from HBV DNA negative patients was used as a negative control.

Gels were electrophoresed in 1X TBE (Appendix B) at 70 mA for 30 to 60 minutes. Ethidium bromide (Appendix B) was included in the preparation of the gel at a final concentration of 1 µg/ml to allow for viewing of DNA under UV illumination.

### 2.9 Gel Purification of the PCR product

The PCR products (BCP/PC and complete S, respectively) with single bands were sent directly for sequencing unpurified. PCR products showing multiple bands were gel purified before being sent for sequencing. PCR amplicons were electrophoresed on a 1% agarose gel without Ethidium bromide (Appendix B) together with a 1 kb molecular weight marker (Promega, Madison, WI, USA) for complete S and 300 bp molecular weight marker for BCP/PC.

An aliquot of 40 µl of the PCR product was loaded onto the gel with 10 µl Loading Buffer containing Invitrogen™ SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, 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 halfway down the gel.

To excise the PCR product, the buffer (TBE) was poured off and the gel placed on a fluorescent light box to help visualise 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 gel with DNA was estimated by weighing the gel (1mg ~ 1µl), and three 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 to 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 µl of wash buffer was added to the column then spun for 30 sec. After repeating the wash step, the Zymo-Spin column was placed on to a clean 1.5 ml eppendorf tube and 10 µl of elution buffer was added on the column matrix and spun down to elute the DNA. The PCR products were stored at -20°C until sent for sequencing.

### 2.10 Sequencing

Sequencing was undertaken by Inqaba Biotech ™ using an ABI 3130XL genetic analyser (Applied Biosystems, Foster City, CA) and Big Dye Terminator v3.0 cycler Sequencing Ready Reaction kit (Applied Biosystems, Foster City, USA). The amplficiation and sequencing primers for the BCP/PC and complate S regions are shown in Appendix A. Once the sequencinges of the overlapping fragments of the complete S and of the BCP/PC regions were obtained, they were individually assembled using the Fragment Merger Tool (Bell and Kramvis, 2013a).

### 2.11 Bioinformatics and phylogenetic analyses

Both complete S and the BCP/PC DNA sequences were assembled and aligned using in GeneDoc (http://evolution.genetics.Washington.edu, 1995). The assembled Complete S and

BCP/PC sequences were compared with corresponding genotype D and E consensus sequences of HBV from GenBank (NCBI). For genotype D, a consensus was prepared using sequences with accession numbers, AB033558, AB048701 and AB033559 (GenBank, NCBI). For genotype E, a consensus was prepared using sequences with accession numbers EAB091255, ECHIMPAB032431 and EAB091255. Multiple sequence alignments and nucleotide divergence calculations were carried out using HVDRU tools (Bell and Kramvis, 2013b). Neighbour joining tree were prepared in Mega 7.2 software (Kumar et al., 2016). The nucleotide divergence calculations were determined using HVDRU divergence tool (Bell et al., 2016).

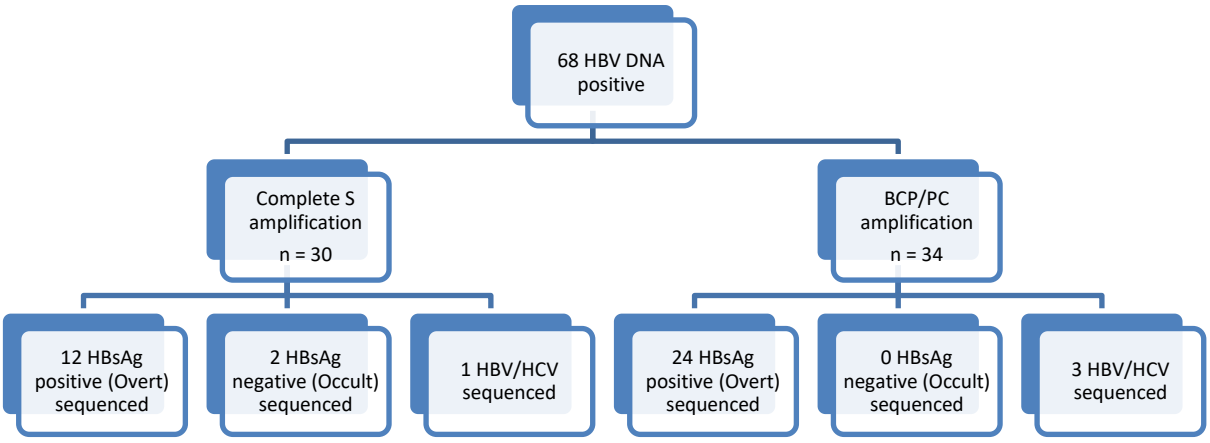
### 2.12 Statistical analysis

All p-values were determined using Anova single factor test and t-test: Two-Sample Assuming Equal Variances from Microsoft Excel 2013 v15.0 (https://products.office.com/en-us/microsoft-excel-2013).

# Chapter 3: Results

## 3.1 Overview

A total of 541 ESRD patients undergoing haemodialysis from South Sudan, enrolled in the study (Table 1.3). The patients attended haemodialysis in nine different centres (Table 3.1). The viral loads of these patients and their HBV DNA status were analysed (Table 3.1). From the initial five hundred and forty one patients who enrolled, only 76 were HBV DNA positive. This represented a prevalence of 14%. Eight serum samples were not included for further analyses because insufficient serum was available. Therefore, only 68 HBV samples were analysed downstream (Figure 3.1). Amplification of the BCP/PreC region and/or the complete S region was carried out (Figure 3.1). Out of the nine centres from which patients were enrolled, only samples from four centres, namely, Salma, Ibn Sena, Elturki and Alnaw amplified successfully. Thus the other five centres could not be included in the study. All vaccinated patients were HBsAg negative ($p<0.05$). There was statistical difference ($p<0.05$) between the viral loads and HBV positivity in the four highlighted centres (Table 3.1).




* 15 Complete S and 7 BCP/PC samples did not sequence well.

**Figure 3.1**: Flow diagram showing the protocol used to molecularly characterise HBV isolated from 68 HBV DNA positive samples from four haemodialysis centres in Khartoum.

**Table 3.1:** The overview of ESRD patients in nine centres at Khartoum, Sudan

| Center | Total | Vaccinated (Yes) | HBsAg positive | HBsAg negative | Number of HBV DNA Positive | Overt | Occult | Average Viral loads (log Iu/ml)* |
|---|---|---|---|---|---|---|---|---|
| Salma | 127 | 52 | 18 | 109 | 18 | 18 | 0 | 8.66 |
| Ibn Sena | 117 | 54 | 30 | 87 | 31 | 30 | 1 | 7.87 |
| Elturki | 110 | 41 | 0 | 110 | 6 | 0 | 6 | 3.99 |
| Alnaw | 26 | 11 | 2 | 24 | 9 | 2 | 7 | 12.05 |
| Soba | 23 | 5 | 0 | 23 | 1 | 0 | 1 | 0.2 |
| Elakadeemi | 121 | 54 | 2 | 119 | 5 | 2 | 3 | 3.91 |
| Tropical hospital | 9 | 8 | 0 | 9 | 1 | 0 | 1 | 2.59 |
| Police hospital | 4 | 3 | 0 | 4 | 1 | 0 | 1 | 3.32 |
| Elshurta | 4 | 0 | 4 | 0 | 4 | 4 | 0 | 4.23 |

* Centres yielding sufficient positive PCR results for downstream analysis are highlighted in orange.

** Viral loads between the centres were statistically significantly different ($p<0.05$).

### 3.2 Extraction and Viral loads

Initially, the Maxwell® 16 Viral Total Nucleic Acid Purification Kit was used for DNA extraction. However, only 16/46 (34.8%) samples tested positive for HBV DNA using real time PCR. Due to the low amplification of overt samples, we repeated the extraction using the Qiagen extraction kit, which yielded, 30/46 (65.2%) samples positive for HBV DNA

(Appendix D). Using the Maxwell® 16 Viral Total Nucleic Acid Purification Kit and the Qiagen kit, the average viral load in overt samples was measured to be 3.08 log IU/ml and 5.7 log IU/ml, respectively (Appendix D). Viral loads were measured in 11/12 (92%) of occult samples when using the Qiagen extraction method. The average viral load in the occult samples was 2.5 log IU/ml (Appendix D). Lastly, using Qiagen extraction kit, ten samples co-infected with HBV/HCV were extracted. Viral loads were measured in all 10/10 (100%) samples, giving an average viral load of 5.1 log IU/ml (Appendix D). Box and whiskers plots were prepared to compare the difference in viral loads between overt HBV mono-infected versus overt HBV/HCV co-infected, with no statistical difference ($p>0.05$) and occult HBV mono-infected versus occult HBV/HCV co-infected, with statistical difference ($p<0.05$) (Appendix G).

## 3.3 Detection of amplified PCR products

### 3.3.1 BCP/PC amplicons

The BCP/PC region of HBV was successfully amplified from DNA extracted from overt (HBsAg positive), occult (HBsAg negative) and HBV/HCV co-infected patients suffering from ESRD, as shown by a 307 bp bands on an agarose gel (Figure 3.2). Only 28/46 (60.9%) overt samples amplified in the BCP/PC, with 1/12 (8.3%) of the occult samples and 3/10 (10%) of the HBV/HCV co-infected amplifying (Appendix D). Figures 3.2 shows an example of the BCP/PC amplicons.

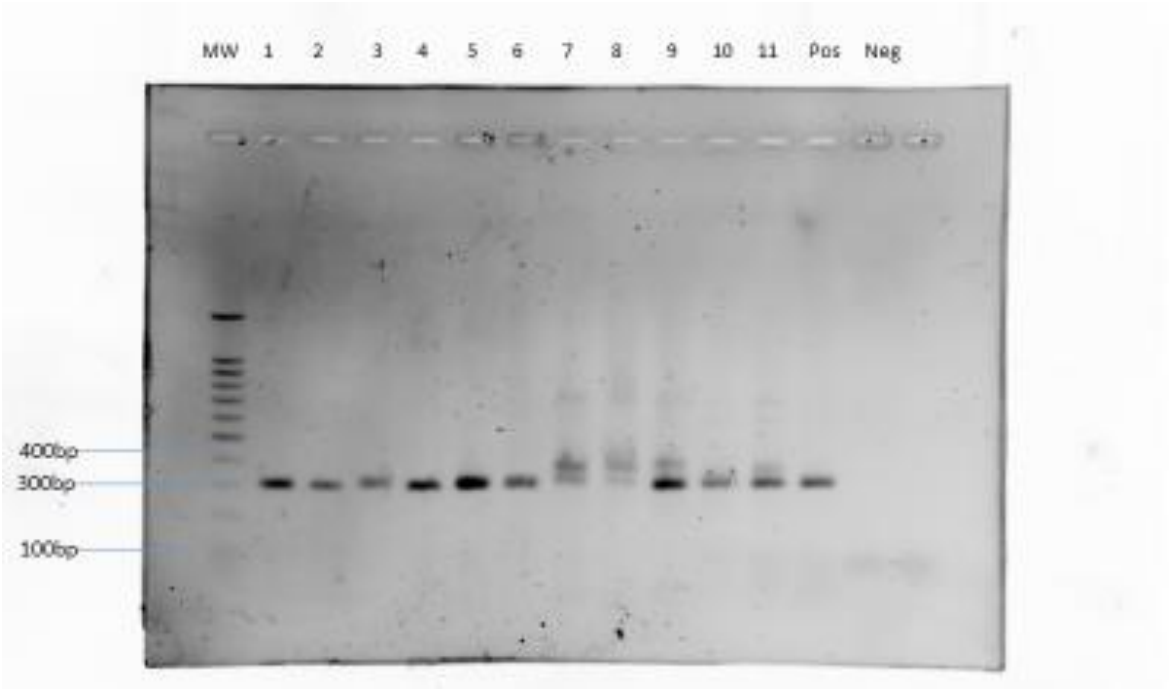



**Figure 3.2**: Detection of the amplified partial BCP/PC region of the HBV genome (1742 to 1901 from the *Eco*RI site) using primers covering the region 1653 to 1959 (Appendix A), hence the PCR product of 307 bp, visualised on an ethidium bromide stained 1 % agarose gel (inverted image).

Lane MW= 100 bp molecular weight marker (Promega).

Lanes 1 to 11 = successfully amplified BCP/PC gene Sudanese ESRD infected samples.

Lane 12 = PCR positive control (Pos).

Lane 13 = Negative control (BQW water) (Neg).

### 3.3.2 Complete Surface gene amplicons

The complete surface gene (complete S) amplification was successful in 28/51 (54.9%) samples with HBV viral loads (Appendix D). However, amplification in the complete S region was also successful in one sample (SDB35), which had a viral load of zero. Other samples with viral loads of zero did not amplify in either BCP/PC or complete S regions. The average viral load for samples that amplified was 8.34 log IU/ml compared to a lower viral load of 1.65 log IU/ml to samples that did not amplify ($p<0.05$). Successful amplification was shown by detection of a 2.1 kb DNA band (Figure 3.3). Only 23/46 (50%) overt samples amplified in the complete S, with 4/12 (33.3%) of the occult samples and 1/10 (10%) of the

HBV/HCV samples amplifying (Appendix D). An example of complete S region amplicons is shown in figure 3.3.

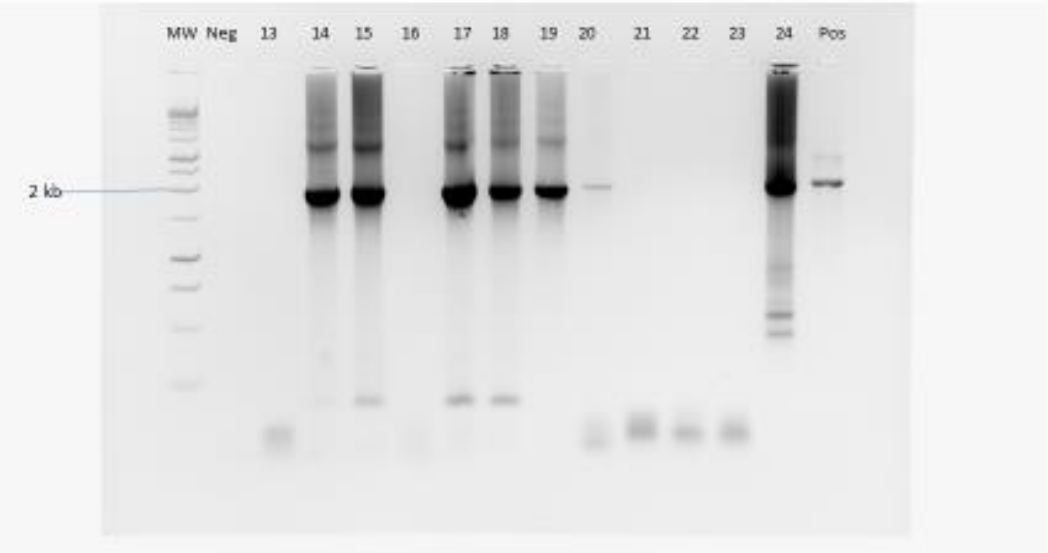



**Figure 3.3**: Visualisation of HBV complete surface gene amplicon (2854 to 835 from the *Eco*RI site) using primers covering the region 2410 to 1286 (Appendix A), hence the PCR product of 2.1Kb.

Lane MW = 1 kb molecular weight.

Lane 2 = BQW Negative control (Neg).

Lanes 13, 16, 21, 22, 23 = unsuccessful amplification of the complete surface antigen.

Lanes 14, 15, 17, 18, 19, 20, 24 = successfully amplified complete surface gene ESRD infected samples.

Lane 15 = PCR Positive control (Pos).

### 3.4 DNA sequencing analysis

PCR products of both the BCP/PC and the complete surface gene were sent for direct sequencing at the Central Analytical Facility, Stellenbosch University and Inqaba Biotech™. The data of HBsAg, vaccination, HBV DNA and whether sequencing was successful is listed in Appendix E.

### 3.4.1 Chromatogram analysis

A representative chromatogram of the Kozak sequence of BCP/PC amplicon is shown in Appendix F. Some samples were found to have mixed genotype sequences (Appendix F).

## 3.5 Phylogenetic analysis

Mega 7.2 software was used to construct neighbour joining trees of all ESRD Sudanese patients complete S sequences and sequences from other countries derived from GenBank belonging to genotypes A-I (Yousif et al., 2013) as references. Eleven isolates clustered with genotype D sequences from Sudan and from other countries, three isolates clustered with genotype E sequences and one isolate clustered with genotype A sequences (Figure 3.4). Each genotype was analysed separately with other sequences from Sudan (Figures 3.5-3.7). Among the genotype D isolates in this study, nine were considered to be subgenotype D1, as they clustered with D1 samples from Sudan (Yousif and Kramvis, 2013). The other two genotype D isolates were considered as subgenotype D2 and D3, respectively since they clustered with subgenotype D2 and D3 from the other countries. The *PreS1* mutations at position 28 (A/S, T, K in D1 and A/T in D2 and D3) as well as position 85 (S/A, T in D1 and S/P in D2) were not detected in all genotype D isolates, and could not be used to subgenotype the D isolates. However, *PreS2* mutations at position 39 (V/A in D1, A/V in D2 and V in D3) and at position 42 (I/T,A,I in D1, I in D2 and L/I in D3) and at position 54 (L/M,Q,T,R in D1, L in D2 and L/Q,P in D3) were detected in genotype D isolates and were used to confirm if the isolates that clustered with subgenotype D1, D2 or D3 were indeed the correct subgenotypes (Table 3.2). We found subgenotype D1 to be the most common subgenotype. The three genotype E samples clustered with genotype E samples from Sudan. The single genotype A sample clustered with a subgenotype A1 sample from Sudan. This further indicated a correlation of our samples with others from previous studies in Sudan. We

detected an unusual serotype *ay* occurring in one genotype D isolate (Table 3.3). Genotype A and E isolates had expected serotypes (Table 3.3).

**Table 3.2:** Amino acid residues encoded by the different subgenomic regions of HBV belonging to the subgenotypes of D. Adapted from (Yousif and Kramvis, 2013).

| Region | Amino acids position | Subgenotypes | | |
|---|---|---|---|---|
| | | D1 | D2 | D3 |
| Pre S1 | 28<br>85 | A/S,T,K<br>S/A,T | A/T<br>S/P | A/T<br>S |
| Pre S2 | 39<br>42<br>43<br>54 | **V/A**<br>**I/T,A,L**<br>S/L<br>**L/M,Q,T,R** | **A/V**<br>**I**<br>S/L<br>**L** | **A**<br>**L/I**<br>S<br>**L/Q,P** |

* The amino acids found in the genotype D isolates are highlighted in **bold**.

**Table 3.3:** The serotypes of complete Surface gene sequences (Bell and Kramvis, 2015, Purdy et al., 2007).

| Sequence ID | Serotype | Motif1 | Motif3 | Motif Sequence |
|---|---|---|---|---|
| SDB 1E SAL | ayw4 | RKLGS | ArgLysLeuGlySer | CGAAAACTTGGATCA |
| SDB4D SAL | ayw3 | RKTGT | ArgLysThrGlyThr | AGAAAAACTGGAACC |
| SDB 5D SAL | (ay) | RKSGT | ArgLysSerGlyThr | AGAAAATCTGGAACC |
| SDB 10D SAL | ayw3 | RKTGT | ArgLysThrGlyThr | AGAAAAACTGGAACC |
| SDB 14D SAL | ayw3 | RKTGT | ArgLysThrGlyThr | CGGAAAACTGGAACC |
| SDB15D SAL | adw2 | KKPGT | LysLysProGlyThr | AAAAAACCTGGAACC |
| SDB17D SAL | ayw2 | RKPGT | ArgLysProGlyThr | AGAAAACCTGGAACC |
| SDB19D IBN | ayw2 | RKPGT | ArgLysProGlyThr | AGGAAACCTGGAACC |
| SDB24E IBN | ayw4 | RKLGS | ArgLysLeuGlySer | CGAAAACTTGGATCA |
| SDB27D IBN | ayw2 | RKPGT | ArgLysProGlyThr | AGGAAACCTGGAACC |
| SDB38E IBN | ayw4 | RKLGS | ArgLysLeuGlySer | CGAAAACTTGGATCA |
| SDB 57D ALN | ayw2 | RKPGT | ArgLysProGlyThr | AGAAAACCTGGAACC |
| SDB307A ELT | adw2 | KKPAT | LysLysProAlaThr | AAAAAACCTGCAACA |
| SDB326D ELT | ayw2 | RKPGT | ArgLysProGlyThr | AGAAAACCTGGAACC |
| SDB363D ELT | ayw2 | RKPGT | ArgLysProGlyThr | AGGAAACCTGGAACC |

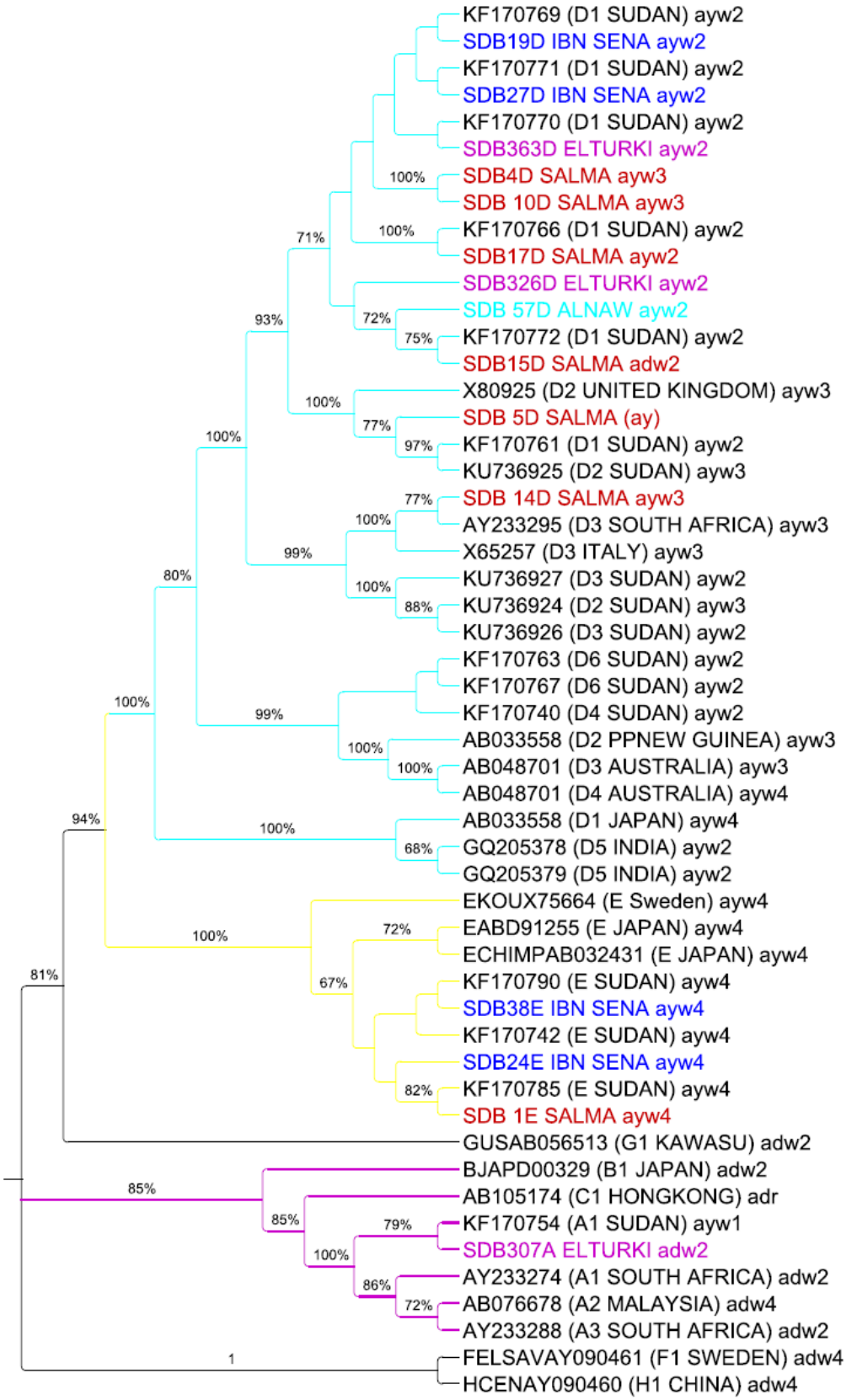

KF170769 (D1 SUDAN) ayw2
SDB19D IBN SENA ayw2
KF170771 (D1 SUDAN) ayw2
SDB27D IBN SENA ayw2
KF170770 (D1 SUDAN) ayw2
SDB363D ELTURKI ayw2
SDB4D SALMA ayw3
SDB 10D SALMA ayw3
KF170766 (D1 SUDAN) ayw2
SDB17D SALMA ayw2
SDB326D ELTURKI ayw2
SDB 57D ALNAW ayw2
KF170772 (D1 SUDAN) ayw2
SDB15D SALMA adw2
X80925 (D2 UNITED KINGDOM) ayw3
SDB 5D SALMA (ay)
KF170761 (D1 SUDAN) ayw2
KU736925 (D2 SUDAN) ayw3
SDB 14D SALMA ayw3
AY233295 (D3 SOUTH AFRICA) ayw3
X65257 (D3 ITALY) ayw3
KU736927 (D3 SUDAN) ayw2
KU736924 (D2 SUDAN) ayw3
KU736926 (D3 SUDAN) ayw2
KF170763 (D6 SUDAN) ayw2
KF170767 (D6 SUDAN) ayw2
KF170740 (D4 SUDAN) ayw2
AB033558 (D2 PPNEW GUINEA) ayw3
AB048701 (D3 AUSTRALIA) ayw3
AB048701 (D4 AUSTRALIA) ayw4
AB033558 (D1 JAPAN) ayw4
GQ205378 (D5 INDIA) ayw2
GQ205379 (D5 INDIA) ayw2
EKOUX75664 (E Sweden) ayw4
EABD91255 (E JAPAN) ayw4
ECHIMPAB032431 (E JAPAN) ayw4
KF170790 (E SUDAN) ayw4
SDB38E IBN SENA ayw4
KF170742 (E SUDAN) ayw4
SDB24E IBN SENA ayw4
KF170785 (E SUDAN) ayw4
SDB 1E SALMA ayw4
GUSAB056513 (G1 KAWASU) adw2
BJAPD00329 (B1 JAPAN) adw2
AB105174 (C1 HONGKONG) adr
KF170754 (A1 SUDAN) ayw1
SDB307A ELTURKI adw2
AY233274 (A1 SOUTH AFRICA) adw2
AB076678 (A2 MALAYSIA) adw4
AY233288 (A3 SOUTH AFRICA) adw2
FELSAVAY090461 (F1 SWEDEN) adw4
HCENAY090460 (H1 CHINA) adw4
100%
100%
71%
100%
93%
72%
75%
100%
77%
97%
100%
77%
100%
99%
80%
100%
88%
100%
99%
100%
100%
94%
100%
68%
100%
72%
67%
81%
82%
85%
85%
79%
100%
86%
72%
1

**Figure 3.4:** Phylogenetic tree using coordinates 2624 to 1240 from *Eco*RI, covering the polymerase region and overlapping complete S (2848 to 835 from the *Eco*RI site). Comparing the fifteen ESRD isolates complete S region with Sudanese references and other references from other countries obtained from GenBank (designated by accession numbers), established using neighbour-joining (Mega 7.2). Bootstrap statistical analysis was performed using 1000 data set and the numbers on the nodes indicate the percentage of occurrences. The accession numbers that start with KF, were obtained from (Yousif et al., 2013). Each centre on the tree is highlighted in different colours. Salma (highlighted in red), Ibn Sena (highlighted in dark blue), Alnaw (highlighted in light blue) and Elturki (highlighted in pink). Genotype Ds branches are highlighted in light blue. Genotype As branches are highlighted in light yellow. Genotype Es are highlighted in dark blue.

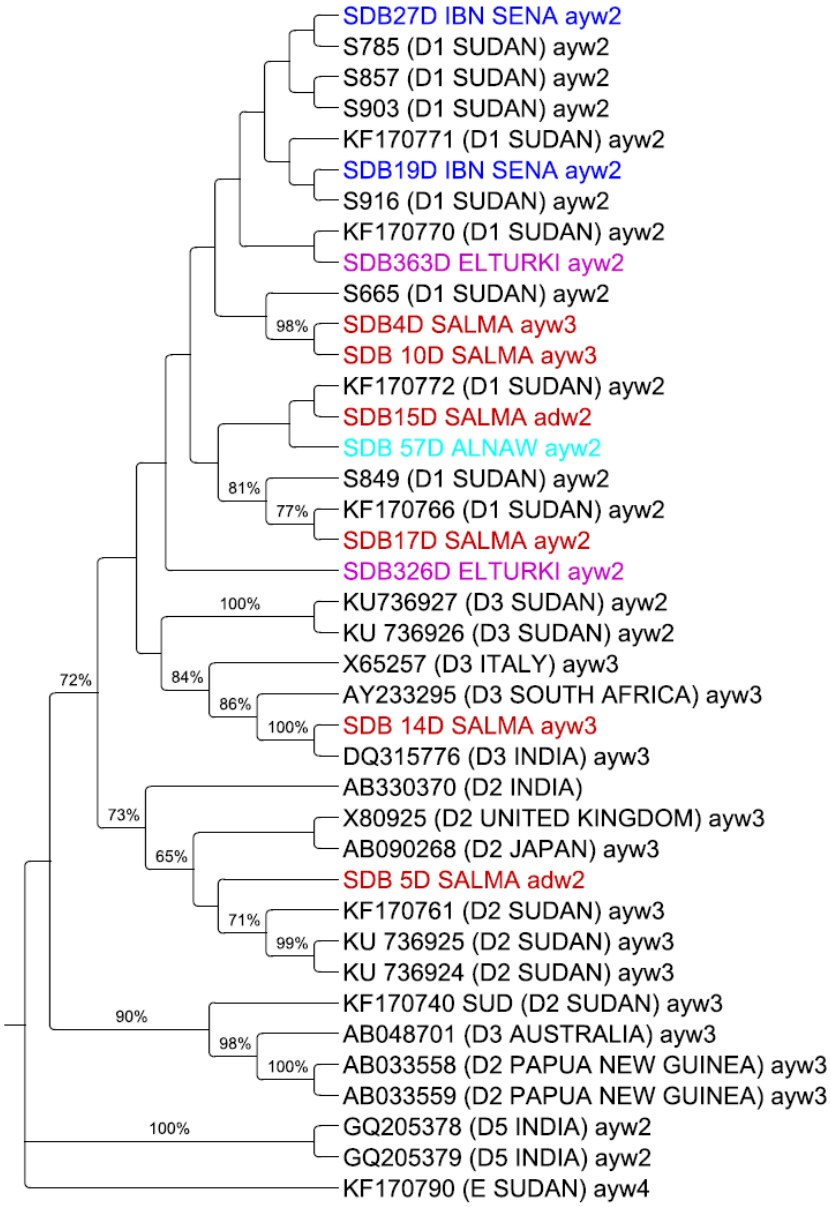



**Figure 3.5:** Phylogenetic tree using coordinates from 2848 to 835 from the *Eco*RI comparing the eleven ESRD Genotype D isolates complete S region with Sudanese references from GenBank (designated by accession numbers), established using neighbour-joining (Mega 7.2). Bootstrap statistical analysis was performed using 1000 data set and the numbers on the nodes indicate the percentage of occurrences. The accession numbers that start with KF, were obtained from (Yousif et al., 2013). The ones that start with S were obtained from (Mahgoub et al., 2011). Each centre on the tree is highlighted in different colours. Salma (highlighted in red), Ibn Sena (highlighted in dark blue), Alnaw (highlighted in light blue) and Elturki (highlighted in pink).

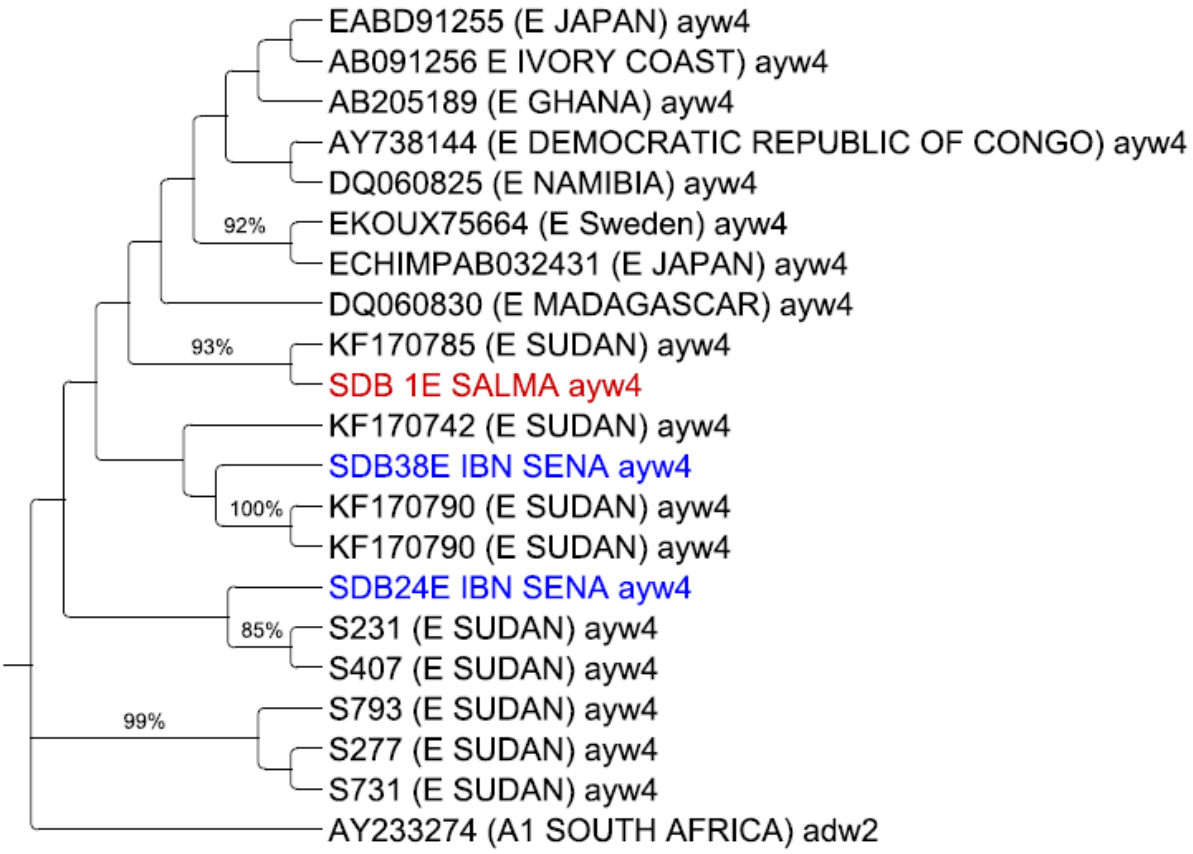



**Figure 3.6:** Phylogenetic tree using coordinates from 2848 to 835 from the *Eco*RI comparing the three ESRD Genotype E isolates complete S region with Sudanese references from GenBank (designated by accession numbers), established using neighbour-joining (Mega 7.2). Bootstrap statistical analysis was performed using 1000 data set and the numbers on the nodes indicate the percentage of occurrences. The accession numbers that start with KF, were obtained from (Yousif et al., 2013). The ones that start with S were obtained from (Mahgoub et al., 2011). Each centre on the tree is highlighted in different colours. Salma in red and Ibn Sena in blue.

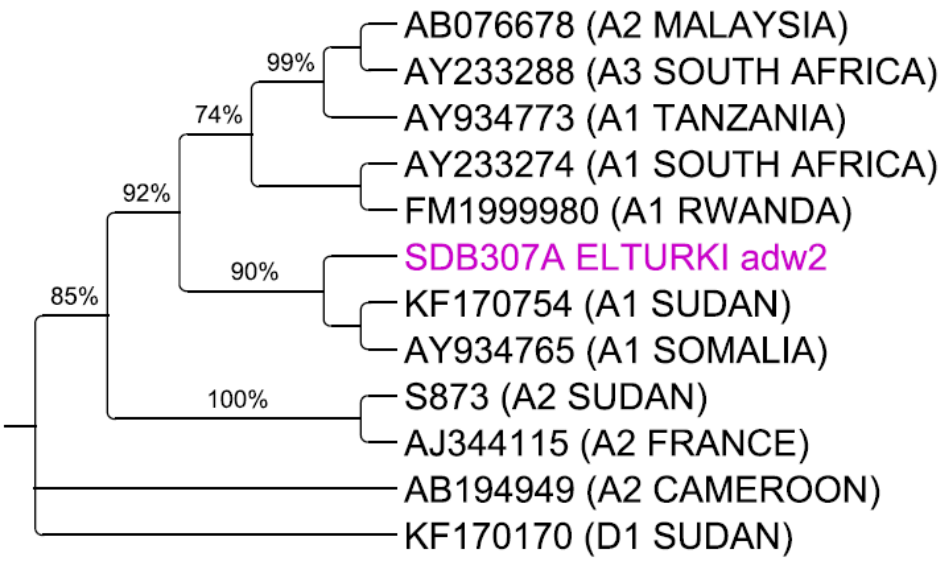



**Figure 3.7:** Phylogenetic tree using coordinates from 2848 to 835 from the *Eco*RI comparing the ESRD Genotype A isolate complete S region with Sudanese references from GenBank (designated by accession numbers), established using neighbour-joining (Mega 7.2). Bootstrap statistical analysis was performed using 1000 data set and the numbers on the nodes indicate the percentage of occurrences. The accession numbers that start with KF, were obtained from (Yousif et al., 2013). The ones that start with S were obtained from (Mahgoub et al., 2011). Elturki is highlighted in pink.

### 3.6 Genotypes distribution in the different treatment centres

The distribution of HBV genotypes between treatment centres is shown in Table 3.4. Genotype D was detected in all treatment centres at a higher frequency than other genotypes. Genotype E was only present in Ibn Sena and Salma centres. Lastly, genotype A was only detected in Elturki centre.

**Table 3.4:** Genotype relation to treatment centres ESRD patients in Sudan.

| Centres | No of isolates genotyped n = 15 | Genotype A n = 1 | Genotype D n = 11 | Genotype E n = 3 |
|---|---|---|---|---|
| Salma | 7 | 0 | 6 [86%] | 1 [14%] |
| Ibn Sena | 4 | 0 | 2 [50%] | 2 [50%] |
| Elturki | 3 | 1 [33%] | 2 [67%] | 0 |
| Alnaw | 1 | 0 | 1 [100%] | 0 |

* Only 15 samples were sequenced in the complete S region.

### 3.7 BCP/PC mutational analysis

All BCP/PC PCR products were sent for sequencing. Both the forward and reverse directions were sequenced and contigs were generated using automatic Contig Generator Tool (Bell and Kramvis, 2015). Twenty seven BCP/PC DNA sequences were analysed for mutations in the region 1750 to 1900 from the *Eco*RI site as described by (Kramvis et al., 2008). Genotype A is characterised by nucleotide C in position 1858 instead of T. This was used to differentiate between genotype A (Figure 3.9) from genotypes D or E isolates (Figure 3.10). Eight isolates were classified as Genotype A (Figure 3.9). These isolates also had 1888A, which differentiates subgenotype A1 from other genotypes (Figure 3.9). Nineteen isolates were classified as genotype D or E (Figure 3.10). In the BCP/PC region of genotype A isolates, mutation C1766T occurred the most at 7/27 (25.93% in the entire study) (Table 3.5a), whilst mutation C1678T was detected the most in genotype D or E isolates at 10/27 (37.04% in the

entire study). In Core region of genotype A isolates, C1950G mutation was detected in 4/27 (14.81% in the entire study) (Table 3.5b), whilst T1934A mutation was detected the most in genotype D or E isolates at 17/27 (62.96% in the entire study) (Table 3.6b). Four isolates; SDB 3, SDB 5, SDB 6, SDB 7, had a 6-nucleotide insertion of sequence "GAACAT", starting at position 1909 (Figure 3.8). This insertion resulted in the duplication of amino acids Methionine and Asparagine. HBeAg results were available for all analysed overt samples. The HBeAg-negativity in six ESRD infected patients could be explained by the presence of either the G1862T or G1896A BCP/PC mutations. The intragroup divergence between these four isolates was lower in terms of mean percentage ratio to stand deviation (0.55%±0.36) compared to 3.13%±1.64 in the other remaining isolates with no insertion (Table 3.6).

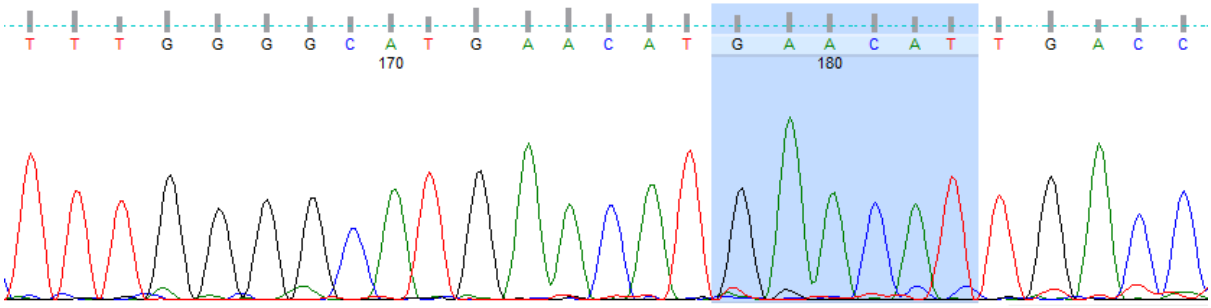



**Figure 3.8:** Chromatogram of a region of the Core of sample (SDB 5), showing the 6-nucleotide insertion of sequence "GAACAT", starting at position 1909.

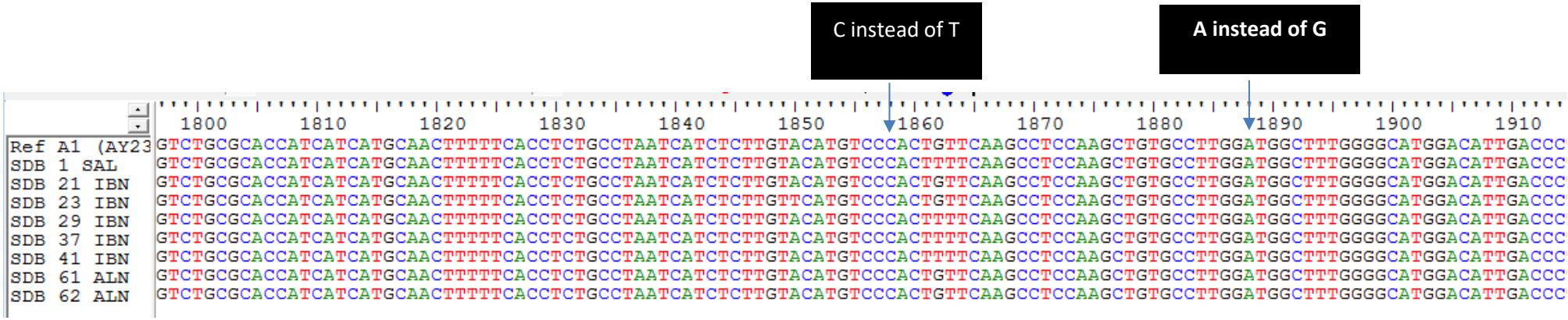



**Figure 3.9:** BCP/PC region analysis showing Genotype A isolates. The reference sequence used was AY233274 (obtained from GenBank, NCBI).

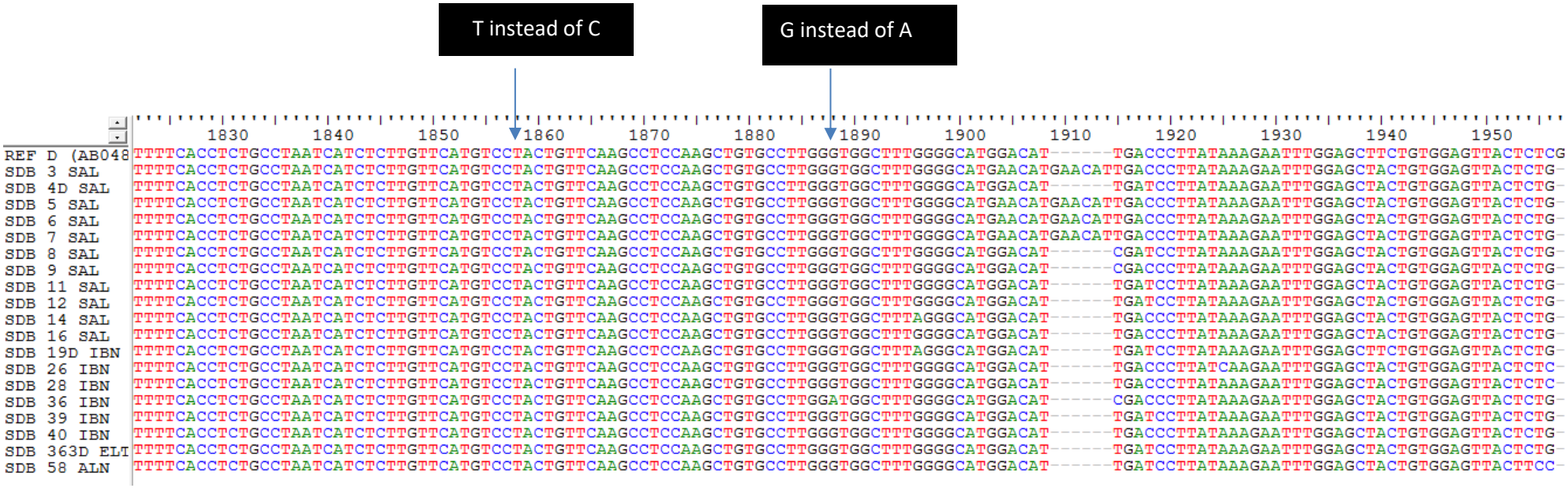



**Figure 3.10:** BCP/PC region analysis showing Genotype D or E isolates. The reference sequence used was AB048701 (obtained from Genbank, NCBI).

**Table 3.5a:** BCP/PC Mutational analysis for Genotype A and D or E isolates**.**

| Mutation/ Genotype | A1662G | C1674T | A1676T | G1677C | C1678T | C1703A | A1727G | A1752C | A1757G | A1762T | G1764A | C1766T | C1773T | A1850T | G1862T | G1888A | G1896A |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Genotype A | 1 | 3 | 1 | 1 | 2 | 3 | 1 | 1 | 1 | 0 | 1 | 7 | 1 | 1 | 4 | 0 | 0 |
| Genotype D or E | 1 | 0 | 6 | 1 | 10 | 0 | 2 | 2 | 4 | 7 | 9 | 0 | 3 | 0 | 0 | 1 | 2 |

* Mutations occurring the most in each genotypes are highlighted in red

**Table 3.5b:** Core Mutational analysis for Genotype A and D or E isolates.

| Mutation/ Genotype | G1904A | T1909C | C1912T | A1919C | T1934A | C1948T | T1949C | C1950G |
|---|---|---|---|---|---|---|---|---|
| Genotype A | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| Genotype D or E | 4 | 3 | 7 | 1 | 17 | 1 | 1 | 14 |

* Mutations occurring the most in each genotypes are highlighted in red.

**Table 3.6:** Intragroup divergence calculations from BCP/PC alignment.

| Samples | Intragroup divergence (mean ± SD) |
|---|---|
| All 27 samples from all 4 centres | 3.63% ± 1.91 |
| 23 samples with no insertion from all 4 centres | 3.13% ± 1.64 |
| 4 samples with insertion from Salma | 0.55% ± 0.36 |
| 8 samples with no insertion from Salma | 2.17 % 1.59 |

### 3.8 Complete Surface gene mutation analysis

Fifteen complete S sequences of HBV from the ESRD from Sudan were analysed for mutations in the *preS1*, *preS2*, HBsAg regions and the polymerase region overlapping the HBsAg.

#### 3.8.1 *PreS1* mutations

One genotype A isolate (SDB 307) was analysed for mutations. In the genotype A isolate the ps1I48V, V90A/T, which occurred together with the, A6S, mutation were detected (Figure 3.11a). Eleven genotype D isolates (SDB 4, SDB 5, SDB 10, SDB 14, SDB 15, SDB 17, SDB 19, SDB 27, SDB 57, SDB 326, SDB 363) were analysed. In Genotype D isolates, ps1N29H, mutation was detected in three isolates (SDB 15, SDB 17, SDB 363) (Figure 3.11d), whilst, ps1L54P, mutation was detected in two isolates (SDB 5, SDB 27) (Figure 3.11d). Three genotype E (SDB 1, SDB 24, SDB 38) isolates were analysed with no unique mutations detected.

### 3.8.2 *PreS2* mutations

The *preS2* mutations detected in genotype A isolates are shown in Figure 3.11b. We detected two different mutations occurring in *preS2* at position 41 of genotype D isolates (Figure 3.11e). At this position, ps2S41P, mutation occurring in three isolates (SDB 5 SDB 14, SDB 326) (Figure 3.11e) and, ps2S41H, mutation occurring in eight isolates (SDB 4, SDB 10, SDB 15, SDB 17, SDB 19, SDB 27, SDB 57, SDB 363) (Figure 3.11e) were detected. Moreover, a four amino acids deletion (twelve nucleotides) in one isolate of genotype E (SDB 1) was detected at amino acids position 18 to 21 (54 to 63 nucleotides) (Figure 3.11g).

### 3.8.3 Complete Surface

Four mutations were found in genotype A (Figure 3.11c). The HBV reactivation marker sS174N was found in one genotype D isolate (SDB 326) (Figure 3.11f).

### 3.8.4 Drug resistance mutations

Drug resistance mutations (rtQ215H) were found in genotype D isolate SDB 14 (from the Salma centre) and SDB 19 (from Ibn Sena). Mutations rtS213P and rtQ215H were found in one genotype E isolate SDB 24 (from Ibn Sena). No drug resistance mutations were found in genotype A isolates.

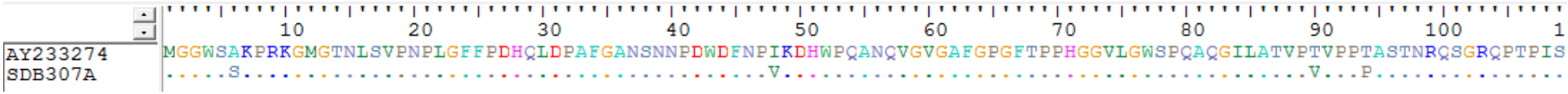



**Figure 3.11a:** *PreS1* alignment of a Genotype A isolate with mutations indicated.

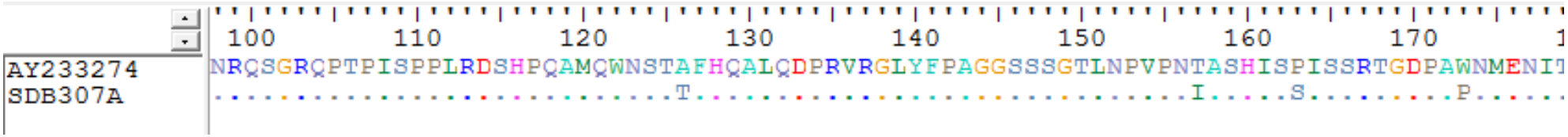



**Figure 3.11b:** *PreS2* alignment of a Genotype A isolate with mutations indicated.

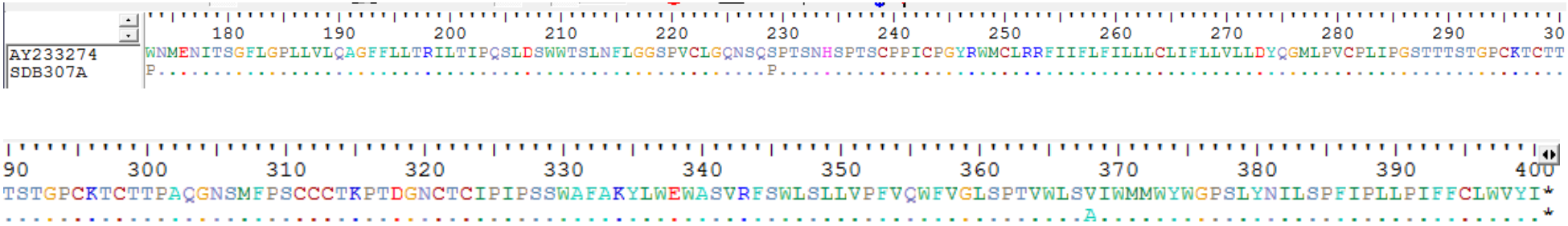



**Figure 3.11c:** Complete S alignment of a Genotype A isolate with mutations indicated.

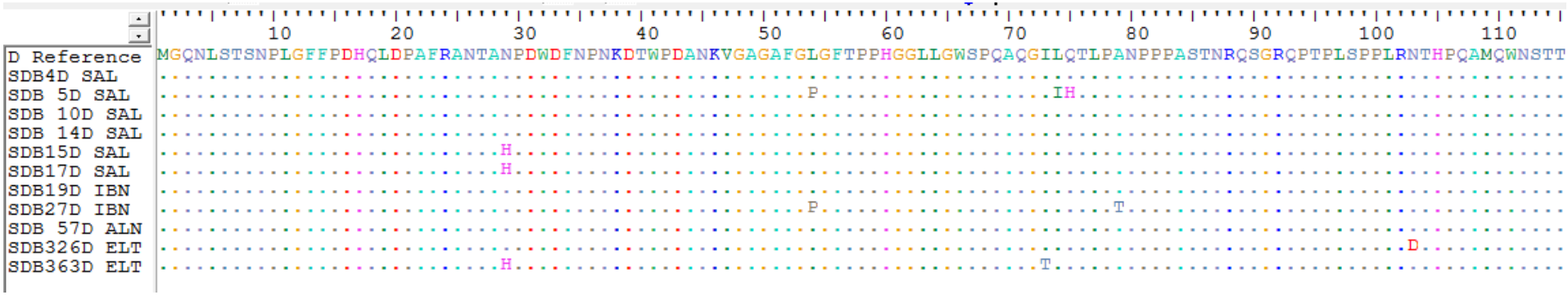



**Figure 3.11d:** *PreS1* alignment of a Genotype D isolates with mutations indicated.

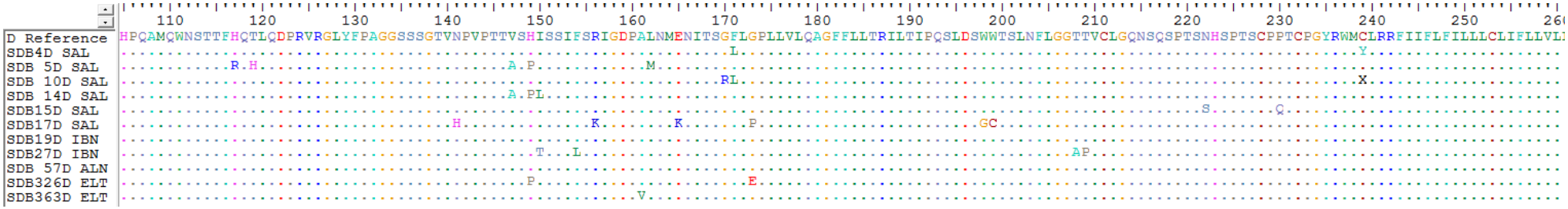



**Figure 3.11e:** *PreS2* alignment of a Genotype D isolate in with mutations indicated.




**Figure 3.11f:** Complete S alignment of a Genotype D isolate with mutations indicated.

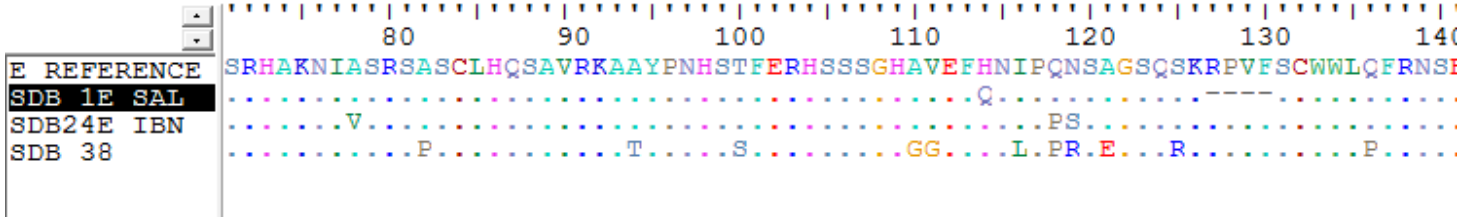



**Figure 3.11g:** *PreS2* alignment of a Genotype E isolate mutations indicated.

## Chapter 4: Discussion

HBV is hyperendemic in Africa, with greater than 8% prevalence. Sub-Saharan Africa lies south of the Sahara Desert, and according to the WHO, has the highest prevalence of HBV (WHO, 2015). Furthermore, in reported non-AIDS cases, HBV/HIV co-infection in sub-Saharan Africa has been shown to cause high mortality and morbidity (Lewden et al., 2005).

Sudan, located in sub-Saharan Africa, is a republic country, which in 2011, was split into two independent countries. The larger, northern region is now termed "Sudan" and the smaller, southern region is known as "South Sudan". The term "Sudan" in this document includes both Sudan and South Sudan. In Sudan, where HBV is hyperendemic, little is known about HBV genotypes and mutations in HBV isolated from ESRD patients, who are undergoing haemodialysis. From 541 ESRD patients undergoing haemodyalysis, 68 were HBV DNA positive and serum samples from these patients were used to molecularly characterise HBV. Of these 68 patients, ten were co-infected with HCV.

### 4.1 Amplification of PCR and Sequencing

In total, 68 HBV DNA positive samples from Sudan were available. However, some samples did not amplify. Thus, 28 overt, 2 occult and 4 HBV/HCV co-infected samples were available for analysis of the BCP/PC region and 23 overt, 4 occult and 3 HBV/HCV co-infected samples for analysis in the complete S region. After various PCR optimisations, the number of positive samples was increased. DNA in samples, which did not amplify, may have become degraded due to storage conditions or the presence of DNA-degrading enzymes (Kawane et al., 2014). The viral loads between overt HBV mono-infected and overt HBV/HCV co-infected were not significantly different ($p>0.05$), with the overt HBV/HCV co-infected samples having a higher viral load. However, only two overt HBV/HCV co-

infected samples were compared to forty six overt HBV mono-infected samples. The viral loads between occult HBV mono-infected and occult HBV/HCV co-infected were significantly different ($p<0.05$), with occult HBV/HCVco-infected samples having a higher viral load. Furthermore, the viral loads between overt HBV mono-infected and occult HBV mono-infected were significantly different ($p<0.05$), as well as the viral loads between overt HBV/HCV co-infected and occult HBV/HCV co-infected ($p<0.05$). The higher viral load in overt samples was in correlation with a higher amplification frequency in the BCP/PC (60.9%) and complete S (50%), respectively. These samples showed a very strong band on the agarose gel when viewed under UV light after PCR. Thus, PCR results correlate with viral loads of the samples/isolates.

### 4.2 HBV Genotyping by BCP/PC region analysis

To identify nucleotide polymorphisms, which differ between genotypes, sequence analysis was undertaken (Bartholomeusz and Schaefer, 2004). Analysis of the BCP/PC region can distinguish between genotype A and non-A isolates (Kramvis et al., 2008). However, it cannot distinguish between genotypes D and E.

The following loci in the BCP/PC region were analysed in 27 of the 34 samples that successfully amplified and sequenced, to identify genotype-specific nucleotides: 1809 to 1812 ("Kozak" sequence), 1858 and 1888. A Kozak sequence of "TCAT" with 1858C and 1888A in eight sequences from this study indicated that these samples belonged to subgenotype A1 (Kramvis et al., 2008). Previous studies in Sudan reported the presence of subgenotype A1 at 7% in liver disease patients and 18.9% in HBV/HIV co-infected patients (Yousif et al., 2013, Yousif et al., 2014). However, another study reported finding only subgenotype A2, rather than A1, in blood donors (Mahgoub et al., 2011). Genotype D or E

characteristics, “GCAC” Kozak sequence and 1858T (Figure 22), were seen in the remaining nineteen samples.

The higher prevalence of genotype D or E isolates (70.4%) compared to genotype A (29.6%) in Sudan is in agreement with previous studies (Yousif et al., 2013, Yousif et al., 2014). As genotype A circulates mainly in southern and eastern Africa (Kramvis and Kew, 2005), a low prevalence is expected in Sudan. Moreover, the higher prevalence of subgenotype A1 in the present study compared to the previous Sudanese studies could be a result of migration in recent years from countries like Kenya, where subgenotype A1 predominates (Ochwoto et al., 2013). The genotype of isolate SDB 36 could not be determined, as it had “GCAC” Kozak, 1858T and 1888A. As such, we deduced it was either genotype D or E (Figure 3.13). The surface region of this sample did not amplify, which made further analysis impossible.

### 4.3 HBV Genotyping by Complete Surface Gene region analysis

The complete surface region of HBV from 15 of the 30 isolates was successfully amplified and sequenced. A neighbour-joining phylogenetic tree was obtained as described earlier and used to determine the genotype of the isolates (Figure 3.6).

All but one of the samples in this study clustered with Sudanese sequences from asymptomatic carriers and liver disease patients, rather than with sequences from blood donors (Mahgoub et al., 2011, Yousif et al., 2013). This might suggest a correlation between clinical manifestation of HBV infection and genotypes in Sudan. The predominance of genotype D in ESRD Sudanese patients in the present study (11 of the 15 sequenced isolates) is in agreement with previous Sudanese studies in liver disease patients and asymptomatic carriers, and HBV/HIV co-infected patients (Mahgoub et al., 2011, Yousif et al., 2013, Yousif et al., 2014), but in contrast to the predominance of genotype E in Sudanese blood

donors (Mahgoub et al., 2011). This further supports the fact that genotype D might be more prevalent in patients suffering from a disease condition in Sudan. Of the remaining four samples, three were genotype E and one was genotype A.

The remaining 15 samples that amplified in the complete S gene region could not be genotyped. In four isolates, the start of the *PreS1* region did not amplify successfully and therefore the genotype could not reliably be determined phylogenetically. In the 11 remaining samples, mixed populations were seen in the complete S gene region, with the BCP/PC results indicating the samples were either genotype D or E. Finally, D/E recombinants have been reported previously from full genomes of HBV in HBV/HIV co-infected Sudanese patients suggesting that these two genotypes can be found in a single individuals (Yousif et al., 2014). By cloning the HBV DNA isolated from the 11 samples with mixed populationes one could determine whether they were co-infected with genotypes D and E. Another alternative would be to use next generation sequencing, which would also determine the quasispecies distribution of the patients with mixed populations.

Genotype D has been classified into six widely distributed subgenotypes: D1 to D6 (Yousif and Kramvis, 2013). Subgenotype D1 is found mainly in the Middle East, subgenotype D2 in Europe and Asia, subgenotype D3 in South Africa, Northern America and Europe, subgenotype D4 in Northern and Central Americas and Australia, subgenotype D5 only in India, and subgenotype D6 in Papua New Guinea, Indonesia, Morocco and Tunisia (Yousif and Kramvis, 2013). Subgenotypes were determined by identifying amino acid markers in genotype D samples. Subgenotype D1 predominated in ESRD individuals in Sudan in the present study (9 of 11 samples), which is in agreement with the previous studies of HBV in Sudan (Mahgoub et al., 2011, Yousif et al., 2013, Yousif et al., 2014). Thus, the predominant

subgenotype of D found in Sudan is D1. This is the subgenotype of D that prevails in the Middle East and the Arabian subcontinent (Yousif and Kramvis, 2013).

### 4.4 Core Region Analysis

A 6 nucleotide insertion, starting at position 1909 in the Core region, was identified in four isolates from ESRD patients (SDB3, SDB5, SDB6, SDB7) from Salma centre. This insertion resulted in amino acids motif: "MNMNIDP" compared to the normal motif of "MDIDP", thus removing aspartic acid (D) and adding Methionine (M) and two Asparagine (N) in the core region. These four isolates with the insertion were classified as either genotype D or E using BCP/PC analysis. Sequence analysis of the complete S gene region of sample SDB5 indicated that it belongs to genotype D. The complete Surface gene region did not amplify in the remaining three samples. Three of the patients from which these isolates were obtained were male with one being a female. All of them were not vaccinated and have been on haemodialysis for more than two years. The lower mean percentage intragroup divergence showed low variation of 0.55% between these four isolates compared to higher nuclear divergence in the remaining isolates with no insertion or other isolates from Salma and the remaining treatment centres (Table 3.7). A BLAST search of these sequences showed a match with a subgenotype A2 sequence from blood donors in Poland (Grabarczyk et al., 2010) and this insertion has been reported previously in genotype A (Kramvis et al., 2005), but, as far as we can determine, has not been reported in genotype D. The low nucleotide divergence between these four samples and this unique insertion from the same treatment centre may implicate nosocomial infection. Three of the patients carrying the isolate with the insert have been undergoing dialysis for longer than 10 years, whereas the fourth patients has been on dialysis for 2 years. The minor variations detected in the remainder of the BCP/PC region (Figure 3.13), may be the result of intra-patient evolution after infection with the founder strain. Further quasispecies analysis, following cloning or next generation

sequencing of the HBV isolates may be able to provide more concrete evidence for the presence or absence of nosocomial infection (Kothari et al., 2017).

### 4.5 HBV Serotype Analysis

HBV serological subtype can be determined from translated complete S region sequence data by examining the amino acids at positions 122, 127, 134, 140, 159 and 160 (Purdy et al., 2007). Six of the eleven genotype D samples showed a serotype of *ayw2*, which is the major genotype D serotype found in Africa (Arankalle et al., 2010, Swenson et al., 2001). The identification of serotype *adw2* in sample SDB15 was interesting, as this serotype is common in genotypes A, B, C, F and G, rather than D. The serotype of isolate SDB5 could only be determined as *ay*. This is as a result of a serine in the third position of the motif, instead of a proline or threonine. The serotypes of the Genotype A and E isolates were typical *adw2* and *ayw*4 respectively.

In summary, genotype D was found to be most prevalent in Sudanese ESRD individuals in this study. The single HBV/HCV co-infected isolate (SDB57) that was sequenced belonged to genotype D, as did most HBV mono-infected samples.

### 4.6 BCP/PC Mutation Analysis

The BCP/PC region of HBV encodes for HBcAg and HBeAg from different mRNAs, the pre-genomic RNA and the precore mRNA, respectively. The BCP 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 fusion protein that is the precursor of HBeAg. Additionally, this region possesses two start codons, namely the HBeAg precursor protein, which is synthesised by translation from the first start codon, and the second start codon, which translates core protein (HBcAg) upon translation from the pgRNA (Yoo et al., 2003). A number of mutations that abolish and/or decrease HBeAg expression have previously been described

(section 1.3.1) (Kramvis et al., 1997, Kramvis et al., 1998). In this study, the BCP/PC region of 27 HBV isolates from ESRD Sudanese patients was analysed.

The BCP/PC region (1750 to 1900 from the *Eco*RI site) was analysed for mutations as described previously (Kramvis et al., 2008). BCP/PC mutations affecting HBeAg expression were found in only six isolates. These mutations can affect expression of HBeAg at the transcriptional, translational and post-translational levels (Chen et al., 2008, Kramvis and Kew, 2007). Other isolates were HBeAg-negative with none of the BCP/PC mutations known to down-regulate the expression of HBeAg. Variations or mutations that are characteristic of subgenotype A1 as well as those that are occurring at different positions such as G1862T were identified. These mutations accounted for the HBeAg negativity in ESRD patients.

The A1762T and G1764A double mutation, was found in five genotype D or E ESRD patients (SDB3, SDB5, SDB6, SDB7 and SDB11), who were not vaccinated. The presence of the double mutation has been shown to down regulate pre-core mRNA at the transcriptional level, thus, decreasing HBeAg expression and increasing pre-genomic RNA packaging (Buckwold et al., 1996). The double mutation can occur in conjunction with T1753C as markers for advanced liver disease (Kramvis and Kew, 1999). However, in our ESRD patients T1753C together with A1762T/G1764A was not detected.

The classic pre-core, G1896A mutation, which completely abolishes HBeAg expression, was found in two genotype D or E ESRD isolates (SDB14 and SDB 19) (Carman, 1997). This mutation changes the TGG codon for tryptophan to a TAG stop codon, leading to the truncation of the HBeAg precursor (Carman et al., 1989). This mutation occurred together with C1858T in both isolates. The occurrence of G1896A and C1858T, is crucial for the

formation of G-C Watson-crick base pair to maintain stability of ε signal. This was in agreement with previous findings, which reported that C1858T only occurs in genotypes B, D, E, G and some strains of C (Kramvis et al., 2005, Locarnini et al., 2003, Lok et al., 1994, Tong et al., 2013). Both of these patients (SDB14 and SDB 19) were not vaccinated against HBV.

HBeAg translation can be terminated by precore start codon mutations at positions 1814, 1815 and 1816 (Gunther et al., 1999). Further mutations at 1817, 1874 and 1897 can also cause HBeAg truncation (Gunther et al., 1999). None of these mutations were detected in our study. Furthermore, the G1862T mutation was found in four HBeAg negative ESRD genotype A isolates (SDB1, SDB29, SDB37 and SDB41). This mutation occurs in the bulge of the ε signal (Kramvis et al., 1997, Kramvis et al., 1998), and results in a valine to phenylalanine mutation in the precore protein. This interferes with post-translational modification of the HBeAg precursor, resulting in intracellular retention and impaired secretion of HBeAg (Chen et al., 2008). G1862T mutation has been detected more frequently in genotype A, particularly in subgenotype A1 (Kramvis et al., 1997), which is in correlation with our findings.

Lastly, the G1888A mutation, which introduces an out-of-frame AUG start codon, was found in one genotype D or E isolates (SDB 39). This mutation introduces 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' (Kimbi et al., 2004). Thus, 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). This mutation was

previously reported to occur most in genotype A isolates and rarely in other genotypes (Kimbi et al., 2004).

### 4.7 Complete Surface Gene region Analysis

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 (Makondo et al., 2012). These mutants are able to infect vaccinated and unvaccinated individuals. Therefore in the context of HBV/HCV coinfection, and especially in the presence of HBsAg-negativity seen in this patient population, it was of great importance that these mutations be defined.

In our study, fifteen isolates were analysed. The *preS1*, *preS2* and complete surface regions were analysed for mutations, including immune/vaccine escape mutants, "a" determinant mutations and drug resistant mutations. A four amino acid deletion (twelve nucleotides) in the *preS2* region of one isolate of Genotype E (SDB 1) was detected (Figure 3.14g). This isolate is HBsAg positive and HBV mono-infected. Deletion mutants in the preS region have been previously found to occur more frequently in HIV co-infected patients (Audsley et al., 2010) and in HCC patients (Kew et al., 2005, Raimondo et al., 1991).

The *preS1* mutation, ps1I48V, which is often detected within the neutralising epitope and hepatocellular binding sites, was detected in one genotype A isolate (SDB307). This mutation has been previously detected in subgenotype A1 isolates in South Africa (Makondo et al., 2012). Furthermore, ps1L54P, which is a *preS1* mutation, was detected in two genotype D isolates (SDB 5 and SDB 27). This mutation has been previously found in HCC patients in Italy (Coppola et al., 2015). Another *preS1* mutation, ps1N103D, was also detected in one genotype D isolate (SDB326). This mutation has been found in non-HCC patients (Coppola

et al., 2015). We further identified the HBV reactivation marker, S174N, which contributes to severity or the reactivation of the virus in occult patients in one genotype D isolate (SDB 326) (Henke-Gendo et al., 2008). Detection of mutations in HBsAg negative participants was limited because of the small number of OBI patients in the present study. Further analysis of such mutations from larger studies is required.

Several mutations, which may lead to Lamivudine, Adefovir and drug resistance, are found in the reverse transcriptase domain of HBV. The most common mutation, which confers resistance to Lamivudine, is rtM204V/I, known as the "YMDD" motif (Ogata et al., 1999) This mutation may occur together with mutations, rtV173L or rtL180M (Ogata et al., 1999). Mutations associated with Adefovir resistance are, rtA181V/T and rtN236T (Tong et al., 2013). None of these mutations were found in our study. The drug resistance mutation, rtQ215H, was detected in one genotype D isolate (SDB19) and one genotype E isolate (SDB24). Both these patients were not vaccinated and had been on haemodialysis for more than one year. Another drug resistance mutation, rtS213P, was detected in one genotype E isolate (SDB24). The patient was not vaccinated and had been on haemodialysis for four years. Thus vaccination might limit the presence of these mutations.

Vaccine escape mutants in the "a" determinant region normally occur under selection pressure after HBV vaccine administration (Torresi, 2002). The most common vaccine escape mutations are, sP120L, sT123N, sT126N, sQ129R, sM133L, sM133T, and sD144A (Carman et al., 1990, Yokosuka and Arai, 2006). HBV immune/vaccine escape mutants, "a" determinant mutations were not detected in our study. This was the case even though some of the ESRD patients that participated in the study were vaccinated. Most HBV immune/vaccine escape mutants, "a" determinant and drug resistance mutations have been reported from

genotype A (Tong et al., 2013). This explains why most were not detected in our study, since genotype D predominated. However, the detection of a few mutations that confer drug resistance indicates that these mutations might be present or they are evolving in the Sudanese population. Hence, more investigations are needed in Sudan.

### 4.8 Treatment centres

Analyses were undertaken on isolates or patients from four centres, namely Salma, Ibn Sena, Elturki and Alnaw. These four centres are geographically separate. Samples from the other five centres (Soba, Elakadeemi, Tropical hospital, Police hospital and Elshurta) did not amplify, and no sequences were available for further analyses. The HIV status of patients enrolled in the study was not available. From the initial patients who enrolled in the study, only 229/541 (42.3%) were vaccinated, compared to 312/541 (57.7%) who were not vaccinated. All the patients from Salma, Ibn Sena, Elturki and Alnaw, who were vaccinated were HBsAg negative. This is a clear indication that vaccination protects against HBV infection.

In the BCP/PC region, we found the A1662G mutation in only two isolates from Alnaw (SDB 58 and SDB61). The double mutation, A1762T/G1764A was only found in five isolates from Salma (SDB 3, SDB 5, SDB6, SDB 7, SDB 11). This mutation results in transcription down-regulation of precore mRNA, decreasing the HBeAg production and increasing the pre-genomic RNA packaging (Hunt et al., 2000).

Genotype D was the most predominant genotype in ESRD Sudanese patients, and at a higher frequency in all four treatment centres. These findings correlate with other studies done in Sudan on liver disease patients and asymptomatic carriers, and HBV/HIV co-infected patients (Mahgoub et al., 2011, Yousif et al., 2013, Yousif et al., 2014). Four patients from a single centre Salma were infected with an HBV with a unique insertion in the core region. The fact

that the nucleotide divergence between the four isolates was relatively low and that there were only minor variations in the remainded of the BCP/PC implicates nosocomial infection. Further quasispecies analysis, following cloning of the HBV isolates may be able to provide more concrete evidence for the presence or absence of nosocomial infection. A relatively high percentage of ESRD patients were HBV DNA positive in the presence or absence of HBsAg. The 14% HBV prelavance in ESRD patients undergoing haemodialysis is in correlation with prevalence of HBV previously reported in Sudan, which ranges between 3.9% to 26% (Mahgoub et al., 2011, McCarthy et al., 1994). The majority of ESRD patients (57.7%) were not vaccinated, which is against the treatment guidelines. The fact that none of the vaccinated patients from the four centres Salma, Ibn Sena, Elturki, Alnaw were PCR-positive fro HBV DNA is evidence that vaccination is important in prevening HBV infection in ESRD patients undergoing haemodialysis. Thus, it is important that vaccination is mandatory for ESRD patients before initiating haemodialysis to prevent nosocomial infection.

Limitations of the Study

- Only a small number of HBV DNA-positive sera were available.
- Not all HBV DNA-positive sera amplified in the BCP/PC and/or S region. This reduced the sample size further.
- Although full length amplification of the HBV DNA genome was attempted it was not successful. This could have allowed us to determine whether some samples that had amplified in the complete S region were genotype D/E recombinants or not.
- Only HBV from 15 out of a total of 68 HBV patient samples were sequenced, representing 22% of samples being genotyped. These samples do not necessarily reflect the entire distribution of genotypes.

- Our collaborators did not provide the requested information about the locations of the dialysis centers.
- The HIV status of the ESRD patients was not determined because of financial constraints.

## Chapter 5: Conclusion

In conclusion, the study showed that genotype D predominates in HBV ESRD infected individuals undergoing haemodialysis at Sudan. Mutations that affect the HBeAg-expression and account for HBeAg negativity were detected in a few patients. Apart from the HBV reactivation marker that was found in one occult HBV affected individual, there were no differences or unique mutations that separated the overt and occult ESRD patients. The low amplification of HBV/HCV co-infected samples made it difficult to determine unique markers between HBV/HCV mono-infected and HBV/HCV co-infected patients. The deletion mutants in the preS region were also detected in ESRD affected patients in Sudan and may play a role in the development of HCC. Drug resistance mutations, which contribute towards reactivation and the development of chronic hepatitis were detected.

With regard to treatment centres, genotype A and E were found circulating only in two centres (Salma and Ibn Sena), with genotype D found in all centres. These findings in this study correlate with those found in two previous studies in Sudan (Yousif et al., 2013, Yousif et al., 2014), in terms of genotype and presence of certain mutations. However, due to the small numbers that were sequenced, it is difficult to deduce much difference in the genotypes between the centres. Four individuals from a single treatment centre were infected with HBV isolates with a unique six nucleotide insertion in the core region, with low nucleotide variation between the four isolates and minor variations in the remainder of the BCP/PC region implicating a possible nosocomial infection. Further quasispecies analysis could provide more conclusive data/results. Lastly, prevention of HBV and ESRD through education and vaccination against HBV is essential in African countries. Therefore, all Sudanese ESRD patients undergoing haemodialysis should be carefully screened for HBV infection, with HBsAg and anti-HBc Ig G antibodies. If possible HBV nucleic acid testing prior to initiation of haemodialysis should be done in patients, as well as treating those HBV

positive with antiretroviral treatment (ART), which should include Tenofovir and Lamivudine (or Emtricitabine) as first line therapy. All haemodialysis patients testing negative for HBV should be vaccinated in order to elicit protective immunity against HBV before initiating haemodialysis and ensuring that they are not infected while under treatment.

## Appendix A

Primers used for PCR

| Region Amplified | Primer name | Primer sequence | Nucleotide position in HBV genome |
|---|---|---|---|
| Complete S region | S (1F) | 5'TCA ATC GCC GCG TCG CAG AAG ATC TCA ATC | nt 2410-2439 |
| | S (1R) | 5'TCC AGA CCX GCT GCG AGC AAA ACA 3' | nt 1314-1291 |
| | S (2F) | 5'AAT GTT AGT ATT CCT TGG ACT CAT AAG GTG GG 3' | nt 245–2482 |
| | S (2R) | 5'AGT TCC GCA GTA TGG ATC GGC AGA GGA 3' | nt 1254 -1286 |
| BCP/PC | BCP (1F) | 5' GCA TGG AGA CCA CCG TGA AC 3' | nt 1606-1625 |
| | BCP (1R) | 5' GGA AAG AAG TCA GAA GGC CAA 3' | nt 1955-1974 |
| | BCP (2F) | 5' CTA AAG AGG ACT CTT GGA CT 3' | nt 1653-1672 |
| | BCP (2R) | 5' GGC AAA AAA AGA GTA ACT A 3' | nt 1940-1959 |
| Short S | P7 (1F) | 5' GTGGTGGACTTCTCTCAATTTTC 3' | nt 253–275 |
| | P8 (1R) | 5' CGGTATAAAGGGACTCACGAT 3' | nt 759–739 |

- Primers were adapted from (Yousif et al 2014)

Primer sequences and PCR conditions used in the study

| Name | Position | Nucleotide sequence | Orientation | PCR round | Denaturation | Annealing | Extension | cycles |
|---|---|---|---|---|---|---|---|---|
| 2410(+) | 2401-2439 | 5'-TCA ATC GCC GCG TCG CAG AAG ATC TCA ATC-3' | Forward | First | 94 °C for 1 min | 66 °C for 1 min | 72 °C for 3 min | 40 |
| 1314(-) | 1314-1291 | 5'-TCC AGA CCK GCT GCG AGC AAA ACA-3' | Reverse | | | | | |
| 2451(+) | 2451-2482 | 5-'AAT GTT AGT ATT CCT TGG ACT CAT AAG GTG GG-3' | Forward | Second | 94 °C for 1 min | 65 °C for 1 min | 72 °C for 3 min | 40 |
| 1280(-) | 1286-1254 | 5-'AGT TCC GCA GTA TGG ATC GGC AGA GGA-3' | Reverse | | | | | |
| 1606(+) | 1606-1625 | 5'-GCA TGG AGA CCA CCG TGA AC-3' | Forward | First | 94 °C for 1 min | 55 °C for 1 min | 72 °C for 2 min | 30 |
| 1974(-) | 1955-1974 | 5'-GGA AAG AAG TCA GAA GGC AA-3' | Reverse | | | | | |
| 1653(+) | 1653-1672 | 5'-CAT AAG AGG ACT CTT GGA CT-3' | Forward | Second | 94 °C for 1 min | 55 °C for 1 min | 72 °C for 2 min | 30 |
| 1959(-) | 1940-1959 | 5'-GGC AAA AAC GAG AGT AAC TC-3' | Reverse | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 255(+) | 253–275 | 5'GTG GTG GAC TTC TCT CAA TTT TC 3' | Forward | First | 94°C for 30 sec | 56°C for 40 sec | 72°C for 1 min | 40 |
| 759(−) | 759–739 | 5' CGG TAT AAA GGG ACT CAC GAT 3' | Reverse | Second | 94°C for 30 sec | 56°C for 40 sec | 72°C for 50 sec | 40 |

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

| Region | Primer name | Sequence | Nucleotide position in the 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 *Eco*RI site

- Primers were adapted from Yousif et al 2014

**Appendix B**

Solutions

10 x TBE buffer PH 8.3

108 g Tris base

55 g Boric acid

9,3 g EDTA

Adjust volume to 1000 ml with distilled water.

Sterilise by autoclaving and store at room temperature.

1% Agarose gel

1 g Agarose

100 ml 1 x TBE buffer

Dissolve agarose in 1 x TBE buffer by heating in a microwave oven for ~4 minutes, swirling occasionally, until all the agarose particles are completely dissolved. Care must be taken that especially the high percentage gels do not boil over.

Cool to ~60 °C.

Add 10µl mg / ml Ethidium Bromide and swirl to mix.

Pour into a prepared casting tray and allow to solidify completely at room temperature.

## Appendix C

R14/49 Mr Khudani Nekhwevha et al

**HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)**

**CLEARANCE CERTIFICATE NO. M170164**

| | |
|---|---|
| **NAME: (Principal Investigator)** | Mr Khudani Nekhwevha et al |
| **DEPARTMENT:** | Internal Medicine |
| **PROJECT TITLE:** | Molecular Characterization and Genotyping of Hepatitis B Virus (HBV) from Sudanese End Stage Renal Disease (ESRD) Patients with Overt and Occult HBV Infection |
| **DATE CONSIDERED:** | Adhoc |
| **DECISION:** | Approved unconditionally |
| **CONDITIONS:** | A Sub-study under Primary Study M150158 Prof A. Kramvis |
| **SUPERVISOR:** | Prof Anna Kramvis |
| **APPROVED BY:** | Professor P Cleaton-Jones, Chairperson, HREC (Medical) |
| **DATE OF APPROVAL:** | 15/02/2017 |

**This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.**

**DECLARATION OF INVESTIGATORS**

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 301, Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in January and will therefore be due in the month of January each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature Date

**PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES**

**Appendix D**: A summary of the viral loads and PCR amplification in ESRD infected patients.

| HBV status | n | Realtime PCR | Average viral loads (log IU/ml) | BCP/PC amplification | Complete S amplification | HCV status |
|---|---|---|---|---|---|---|
| HBsAg positive (Overt) | 46 | 30/46 (65.2%) | 5.7 | 28/46 (60.9%) | 23/46 (50%) | Negative |
| HBsAg negative (Occult) | 12 | 11/12 (92%) | 2.5 | 2/12 (16.7%) | 4/12 (33.3%) | Negative |
| HBV/HCV co-infected (2 overt and 8 occult) | 10 | 10/10 (100%) | 5.1 | 4/10 (40%) | 3/10 (30%) | Positive |

p- value HBV overt vs HBV occult = 0.008889 ($p<0.05$)

HBV occult vs HBV/HCV co-infected = 0.007968 ($p<0.005$)

HBV occult vs (HBV overt+ and HCV+) = 7.95e-07 ($p<0.05$)

HBV occult vs (HBV occult+ and HCV+) = 0.022 ($p<0.05$)

HBV Mono infected vs HBV/HCV co-infected = 0.98 ($p>0.05$)

**Appendix E:** Information about samples

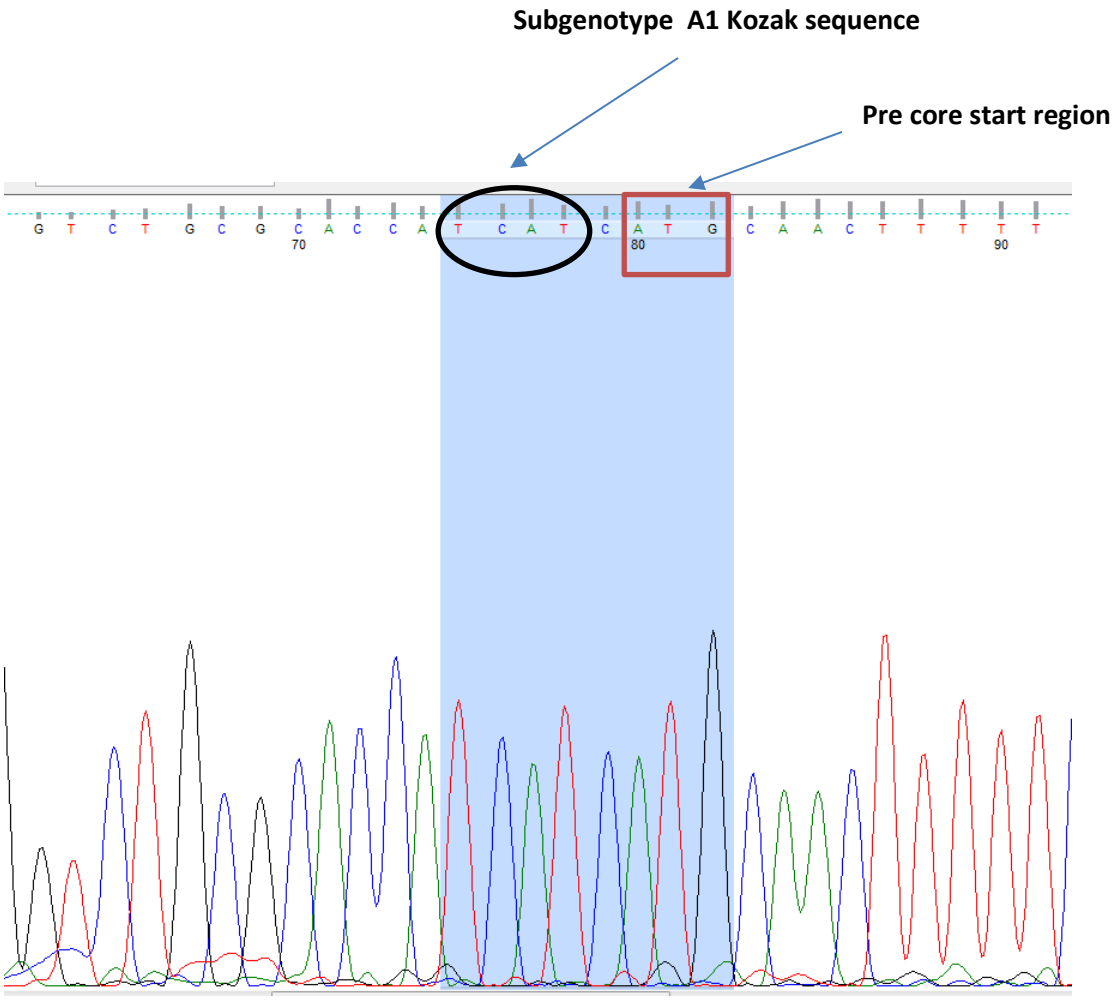
| Dialysis center | HVDRU number | HBs Ag | Vaccination | HBV DNA positive | Sequenced (BCP/PC) | Sequenced (Complete S) |
|---|---|---|---|---|---|---|
| Soba | SDN022 | non reactive | no | Positive | No | No |
| Elakadeemi | SDN187 | non reactive | no | Positive | No | No |
| Elakadeemi | SDN191 | non reactive | yes | Positive | No | No |
| Elakadeemi | SDN197 | non reactive | yes | Positive | No | No |
| Elturki | SDN239 | non reactive | no | Positive | No | No |
| Elturki | SDN244 | non reactive | no | Positive | No | No |
| Elturki | SDN303 | non reactive | yes | Positive | No | No |
| Elturki | SDN305 | non reactive | no | Positive | No | No |
| Elturki | SDN307 | non reactive | yes | Positive | No | Yes |
| Elturki | SDN326 | non reactive | no | Positive | No | No |
| Ibn sena | SDN363 | non reactive | yes | Positive | Yes | Yes |
| tropical hospital | SDN401 | non reactive | yes | Positive | No | No |
| salma | SDB001 | reactive | no | Positive | Yes | Yes |
| salma | SDB002 | reactive | no | Positive | No | No |
| salma | SDB003 | reactive | no | Positive | Yes | No |
| salma | SDB004 | reactive | no | Positive | Yes | Yes |

| | | | | | | |
|---|---|---|---|---|---|---|
| salma | SDB005 | reactive | no | Positive | Yes | Yes |
| salma | SDB006 | reactive | no | Positive | Yes | No |
| salma | SDB007 | reactive | no | Positive | Yes | No |
| salma | SDB008 | reactive | no | Positive | Yes | No |
| salma | SDB009 | reactive | no | Positive | Yes | No |
| salma | SDB010 | reactive | no | Positive | No | Yes |
| salma | SDB011 | reactive | no | Positive | Yes | No |
| salma | SDB012 | reactive | no | Positive | Yes | No |
| salma | SDB013 | reactive | no | Positive | No | No |
| salma | SDB014 | reactive | no | Positive | Yes | No |
| salma | SDB015 | reactive | no | Positive | No | Yes |
| salma | SDB016 | reactive | no | Positive | Yes | No |
| salma | SDB017 | reactive | no | Positive | No | Yes |
| Ibn sena | SDB018 | reactive | no | Positive | No | No |
| Ibn sena | SDB019 | reactive | no | Positive | Yes | No |
| Ibn sena | SDB020 | reactive | no | Positive | No | No |
| Ibn sena | SDB021 | reactive | no | Positive | Yes | No |
| Ibn sena | SDB022 | reactive | no | Positive | No | No |
| Ibn sena | SDB023 | reactive | no | Positive | Yes | No |
| Ibn sena | SDB024 | reactive | no | Positive | No | Yes |
| Ibn sena | SDB025 | reactive | no | Positive | No | No |
| Ibn sena | SDB026 | reactive | no | Positive | Yes | No |
| Ibn sena | SDB027 | reactive | no | Positive | No | Yes |
| Ibn sena | SDB028 | reactive | no | Positive | Yes | No |
| Ibn sena | SDB029 | reactive | no | Positive | Yes | No |
| Ibn sena | SDB030 | reactive | no | Positive | No | No |

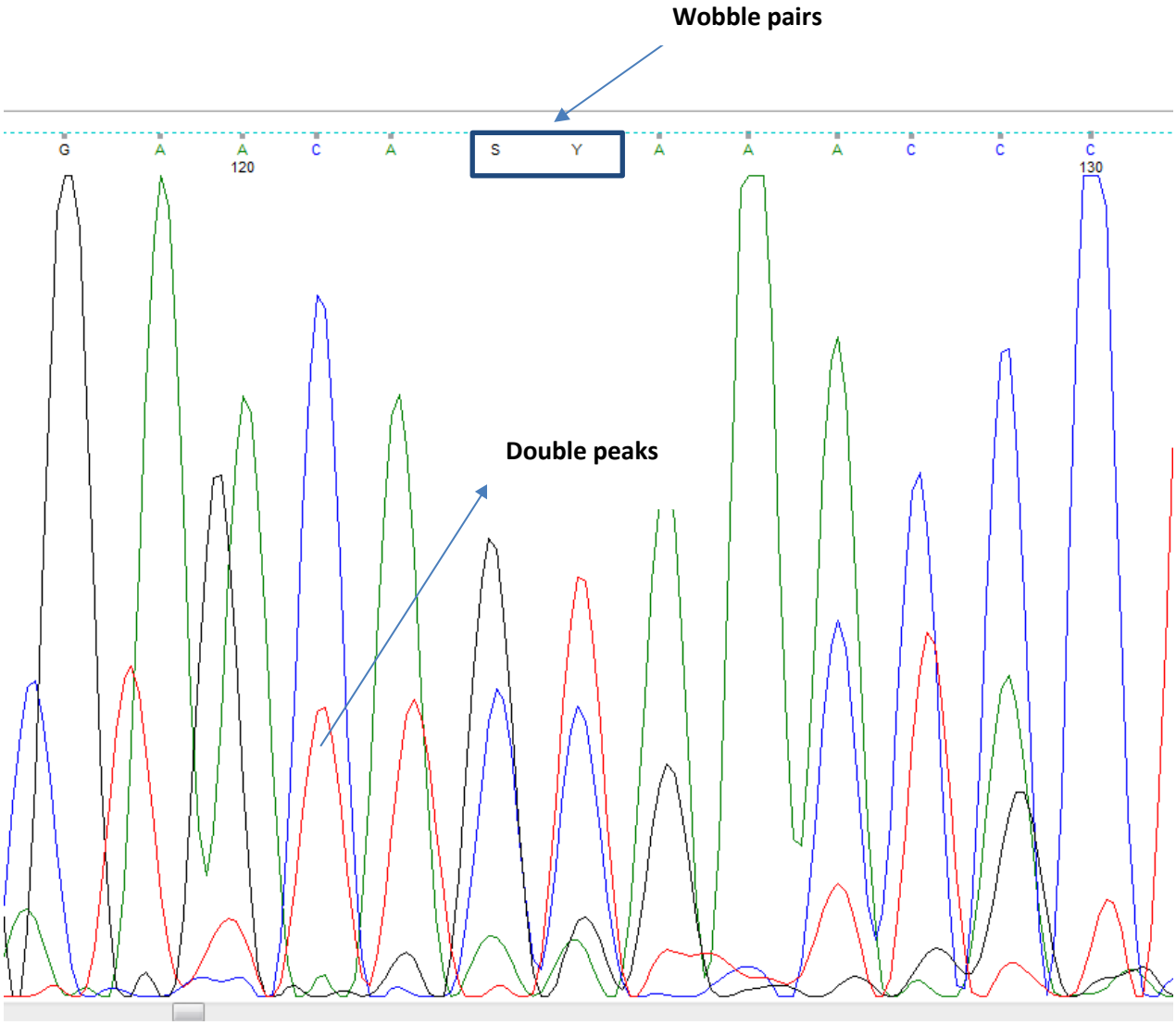
| | | | | | | |
|---|---|---|---|---|---|---|
| Ibn sena | SDB031 | reactive | no | Positive | No | No |
| Ibn sena | SDB032 | reactive | no | Positive | No | No |
| Ibn sena | SDB033 | reactive | no | Positive | No | No |
| Ibn sena | SDB034 | reactive | no | Positive | No | No |
| Ibn sena | SDB035 | reactive | no | Positive | No | No |
| Ibn sena | SDB036 | reactive | no | Positive | Yes | No |
| Ibn sena | SDB037 | reactive | no | Positive | Yes | No |
| Ibn sena | SDB038 | reactive | no | Positive | No | Yes |
| Ibn sena | SDB039 | reactive | no | Positive | Yes | No |
| Ibn sena | SDB040 | reactive | no | Positive | Yes | No |
| Ibn sena | SDB041 | reactive | no | Positive | Yes | No |
| Ibn sena | SDB042 | reactive | no | Positive | No | No |
| Ibn sena | SDB043 | reactive | no | Positive | No | No |
| Ibn sena | SDB044 | reactive | no | Positive | No | No |
| Ibn sena | SDB045 | reactive | no | Positive | No | No |
| Ibn sena | SDB046 | reactive | no | Positive | No | No |
| elshurta | SDB047 | reactive | no | Positive | No | No |
| elshurta | SDB048 | reactive | no | Positive | No | No |
| elshurta | SDB049 | reactive | no | Positive | No | No |
| elshurta | SDB050 | reactive | no | Positive | No | No |
| Salma | SDB066 | reactive | no | Positive | No | No |
| Elakadeemi | SDB116 | reactive | no | Positive | No | No |
| Elakadeemi | SDB215 | reactive | no | Positive | No | No |
| Ibn sena | SDB376 | reactive | no | Positive | No | No |
| Alnaw | SDC057 | reactive | no | Positive | No | Yes |
| Alnaw | SDC058 | reactive | no | Positive | Yes | No |

| | | | | | | |
|---|---|---|---|---|---|---|
| Alnaw | SDC059 | non reactive | no | Positive | No | No |
| Alnaw | SDC060 | non reactive | yes | Positive | No | No |
| Alnaw | SDC061 | non reactive | no | Positive | Yes | No |
| Alnaw | SDC062 | non reactive | yes | Positive | Yes | No |
| Alnaw | SDC064 | non reactive | no | Positive | No | No |
| Alnaw | SDC065 | non reactive | yes | Positive | No | No |
| Alnaw | SDC076 | non reactive | no | Positive | No | No |
| police hospital | SDC090 | non reactive | no | Positive | No | No |

## Appendix F

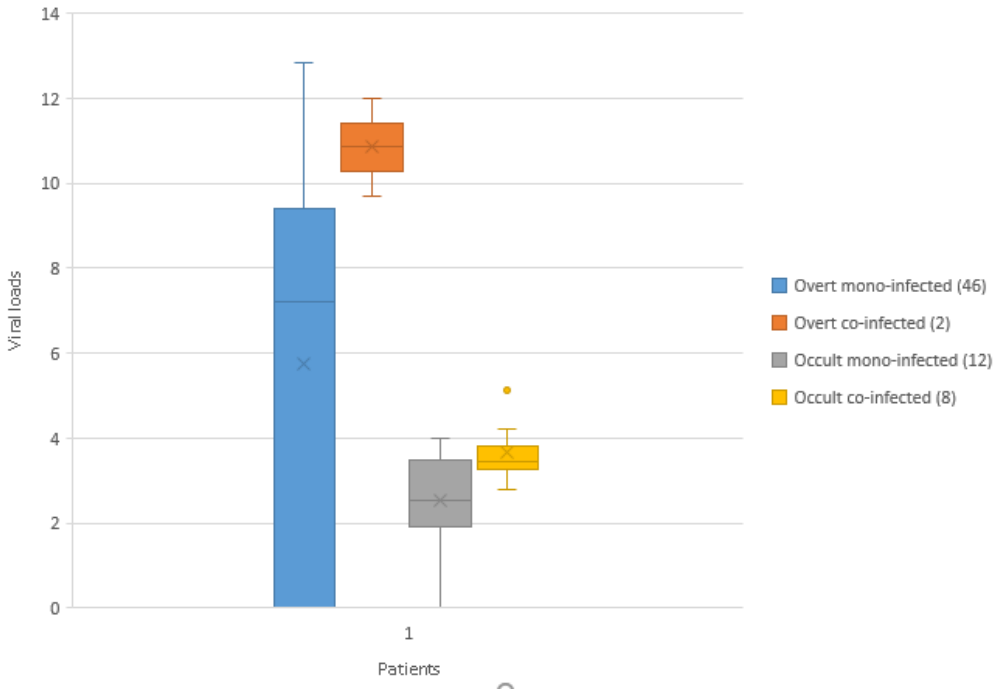



Chromatogram acquired after automated sequencing of the BCP/PC gene of the HBV genome of an HBV isolates from an ESRD patient with overt HBV infection (SDB 41), showing the Kozak sequence (1809 to 1812 from the *Eco*RI cleavage site) preceding the precore start codon of HBV belonging to subgenotype A1.

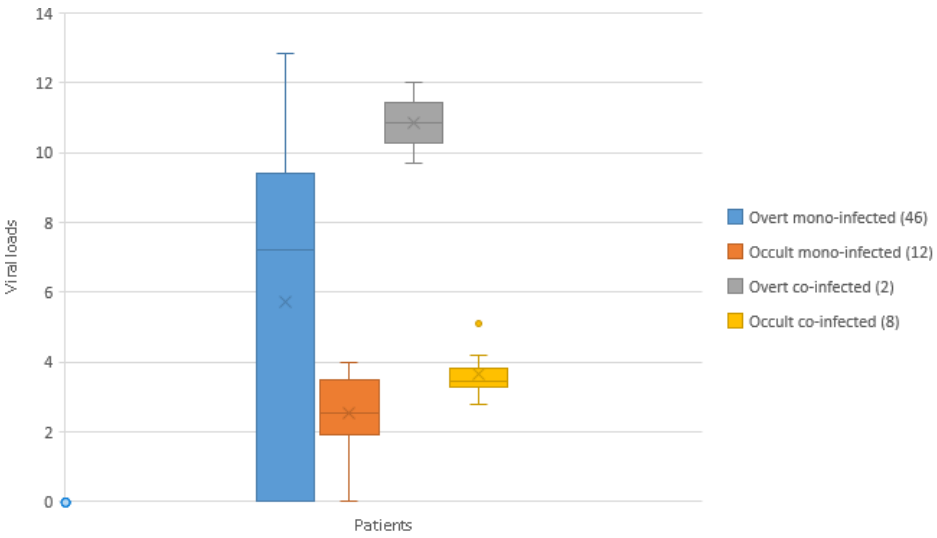



***PreS2*** region chromatogram generated following direct sequencing of the complete surface gene of isolate number SDB35. This shows double peaks at position 123 and 124, indicating detection of mixed genotypes / quasispecies of HBV isolated from a single serum sample. S represents (C, G) mixed bases and Y represents (C, T) mixed bases.

## Appendix G




Box and whiskers for overt HBV mono-infected vs overt HBV/HCV co-infected and occult HBV mono-infected vs occult HBV/HCV co-infected




Box and whisker for overt HBV mono-infected vs occult HBV mono-infected and overt HBV/HCV co-infected and occult HBV/HCV co-infected

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