A STUDY OF THE PREVALENCE OF HEPATITIS B VIRUS INFECTION IN THE INFANTS OF HIV POSITIVE MOTHERS PARTICIPATING IN P1041 IN SOUTH AFRICA

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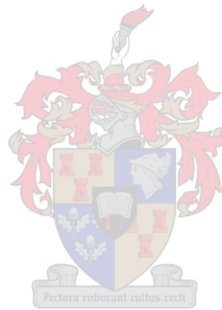
Thesis presented in fulfilment of the requirements for the degree Master of Medical Science (Medical Virology) at the University of Stellenbosch



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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work and that I have not previously in its entirety submitted it for any qualification.

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September 2014

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ABSTRACT

Despite the decreased rate of HBV horizontal transmission in South Africa (SA) due to the HB vaccine, the risk of perinatal transmission remains of concern, especially in HIV/HBV co-infected women. Loss of HBV immune control, resulting in higher HBV replication and thus increasing the risk of transmission is described in HIV/HBV co-infected women. Chronic hepatitis is a well-recognized risk factor for hepatocellular carcinoma (HCC). The presence of specific HBV mutations has been reported in chronic and HCC patients and is used in algorithms for the prediction of HCC in CHB patients in Asia. While these mutations are extensively described in male patients, little is known regarding the antenatal and paediatric populations. This study aimed to determine the prevalence of HBV infection in HIV-exposed infants and to investigate the presence of HCC-related mutations in pregnant women and HIV-exposed children in SA.

Residual samples of infants born to HIV-infected mothers were collected from the P1041 study previously conducted in SA. HBV markers (HBsAg, anti-HBs and anti-HBc) were tested on the Architect (Abbott). HBsAg positive samples were tested for HBV DNA to determine HBV viral loads. HBV strains were characterised by sequencing of the HBsAg gene and genotypes were determined by phylogenetic analysis using HepSEQ (www.hepseq.org.uk). For the HCC-related mutations investigation, samples and data were collected from three HBV-related studies: the NHLS Paediatric Study, an Antenatal Study and the current study. Pre-S, basal core promoter (BCP) and pre-core data was collected from all samples. Multiple alignments were formed and the nucleotide sequences of these extracts were translated into protein sequences. These protein sequences were compared manually to the HBV reference genes to identify HCC-related mutations.

Of 850 HIV-exposed infants tested, three infants were positive for both HBsAg and HBV DNA. Two samples show evidence of past, but cleared HBV infection. Sequence analysis showed that the infants were infected with a subgenotype A1. At follow up, only one infant and mother were able to be traced and contacted. The infant was HIV-infected and had been on an ART regimen, including lamivudine for two years. HBV testing showed that the infant was HBsAg positive and had an undetectable viral load. Core sequence analysis showed clustering between mother and infant sequences. Transmission of mutant HBV previously associated with HCC prompted the question of what the prevalence of mutations in the

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antenatal and paediatric population is. In this investigation of HCC-related mutations study, a higher prevalence of combined pre-S, BCP and pre-core mutations was found in HIV-infected as compared to HIV-uninfected women.

This study shows that vertical transmission is occurring in HIV-exposed infants in SA despite HB vaccination. Data described in this study suggests the importance of HB vaccination closer to the time of birth in SA. Moreover, data on the higher prevalence of HCC-related mutations in HIV-infected pregnant women provide a background for further longitudinal studies to confirm these findings and their implications in SA.

OPSOMMING

As gevolg van die beskikbaarheid van die Hepatitis B virus (HBV) entstof , het horisontale transmissie van die virus drasties in Suid-Afrika (SA) verminder. Ten spyte hiervan, is daar steeds ’n hoë risiko van perinatale transmissie van swanger vroue na hulle babas, dit word veral gesien met MIV/HBV positiewe vroue. Dit is wyd beskryf dat vroue wat mede-besmet is met MIV/HBV gewoonlik beheer verloor oor hulle immuunstelsel, wat lei tot ’n hoër mate van HBV replikasie en dus ’n hoër risiko van virus oordrag. Kroniese hepatitis is wel bekend as ’n hoë risiko faktor vir HCC. Die teenwoordigheid van spesifieke HBV mutasies in kroniese en HCC pasiënte word alreeds in Asië gebruik in sekere algoritmes en formules om infeksie aan te dui en te voorspel. Hierdie mutasies is omvattend beskryf in manlike pasiënte, maar baie min is bekend in voorgeboorte en pediatriese gevalle. In hierdie studie het ons die teenwoordigheid van HCC-verwante mutasies in swanger vroue en MIV-blootgestelde kinders in Suid-Afrika ondersoek.

Monsters is verkry van babas gebore van MIV-positiewe moeders van die P1041 studie wat voorheen in SA gedoen is. Die HBV merkers (HbsAg, teen-HBs en teen-HBc) was op die Architect (Abbott) getoets. HBsAg positiewe monsters was getoets vir HBV DNA om die virale lading te bepaal. Die verskeidenheid HBV stamme was gekarakteriseer deur die virus se nukleïensuur volgordes te bepaal. Die verskillende genotipes is bepaal deur filogenetiese analises te doen met behulp van die HepSEQ (www.hepseq.org.uk) program. Vir die HCC-verwante mutasie studie is monsters en data vergelyk met 3 HBV-verwante studies: die NHLS pediatriese studie, ’n voorgeboorte studie en hierdie spesifieke studie. Voor-S, basale kern promoter en voor-kern data was van alle monsters bekom. ’n Veelvoudige belyning was gedoen met die nukleïensuur volgordes van die verskeie DNA ekstrakte, wat daarna vertaal is in proteïen volgordes. Hierdie proteïenvolgordes translasie was by hand vergelyk met verwysings gene om die relatiewe HCC mutasies te probeer identifiseer.

Van die 850 blootgestelde MIV babas wat getoets is, het 3 positief getoets vir beide HbsAg en HBV DNA. Twee monsters het bewys van verlede , maar vrygestelde HBV infeksie. Data analise bewys dat die babas met subtipe A1 besmet was. Ons kon slegs een moeder en baba

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paar opvolg en kontak vir verdere toetse. Die baba was MIV-positief en was op antiretrovirale behandeling , insluitend lamivudine, vir ten minste 2 jaar. HBV toetse het gewys dat die baba HbsAg positief is en 'n onopspoorbare virale lading gehad het. Kern nukleïensuur volgorde analise het groepering getoon tussen die ma en baba se virus monsters . Die transmissie van die mutante HBV wat geassosieer is met HCC het gelei tot die vraag wat die voorkomssyfer is van hierdie spesifieke mutasies in die voorgeboorte en pediatriese populasies in SA. In hierdie studie het ons 'n hoër gekombineerde voorkomssyfer gevind van die voor-S, basale kern promoter en voor-kern mutasies in MIV-positiewe vroue, in vergelyking met MIV-negatiewe vroue.

Hierdie studie bewys dus dat vertikale transmissie van HBV in blootgestelde MIV babas steeds plaasvind, ten spyte van HBV inenting. Die data wat in hierdie studie beskryf was dui daarop dat die belangrikheid van HBV inenting nader aan die tyd van die geboorte in SA gegee moet word.As gevolg van die hoë voorkomssyfer van HCC-verwante mutasies in swanger vroue, is daar verdere longitudinale studies nodig om hierdie bevindinge en hul implikasies in SA te bevestig.

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To Andre and Alice Tamandjou

‹‹ At the end of the day, the most overwhelming key to a child's success is the positive involvement of parents – Jane D. Hull ››.

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LIST OF ABBREVIATIONS

3TC – Lamivudine

Aa – amino acid

ABI − Applied Biosystems Incorporated

ADV – Adefovir

AFB1 – Aflatoxin B1

ALT – Alanine amino transferase

Anti-HBc − Antibody to hepatitis B core antigen

Anti-HBe − Antibody to hepatitis B e antigen

Anti-HBs − Antibody to hepatitis B surface antigen

ART – Antiretroviral therapy

BBVU – Blood Borne Viruses Unit

BCP – Basal Core Promoter

bp − base pair

ccc – covalently closed circular

CMIA – Chemiluminescent microparticle immunoassay

CHB – Chronic Hepatitis B

Ct – Cycle threshold

D_L – Limit of detection

DNA – Deoxyribonucleic Acid

dNTPs – deoxynucleoside triphosphate

DR – Direct repeat

Eco R1 − *Escherichia coli* restriction enzyme 1

EPI – Expanded Programme on Immunization

FCS – Fetal calf serum

HAART – Highly Active Antiretroviral Therapy

HBcAg – Hepatitis B core antigen

HBeAg − Hepatitis B e antigen

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HBIg – Hepatitis B immunoglobulin

HBsAg − Hepatitis B surface antigen

HBV − Hepatitis B Virus

HBx – Hepatitis B X protein

HCC – Hepatocellular carcinoma

HCV – Hepatitis C virus

HIV − Human Immunodeficiency Virus

IFN – Interferon

IgM – Immunoglobulin M

IU − International Unit

kb − Kilo base

LHBs − Large hepatitis B surface protein

MHBs – Middle hepatitis B surface protein

mCMV − Murine Cytomegalovirus

mL – millilitres

mM – millimole

MTCT – Mother-to-child transmission

mRNA− Messenger RNA

n/a − not applicable

nt - nucleotide

NC – negative control

NHP − Normal Human Plasma

NTC − No-Template Control

OBI – Occult hepatitis B infection

OD − Optical Density

ORF − Open Reading Frame

PgRNA – pregenomic RNA

pmol – picomole

PHE – Public Health England

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P – Polymerase

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

Pol – Polymerase

Pre-S – Pre Surface

PRF – Poliomyelitis Research Foundation

qPCR – quantitative PCR

RCF – Relative centrifugal force

RLU – Relative Light Unit

RNA – Ribonucleic acid

RNAse – Ribonuclease

RPM – Revolution per minute

S – Surface

SA – South Africa

S/CO – Signal-to-cut-off

SHBs – Small hepatitis B surface protein

S/N – Signal-to-noise

SSA – Sub-Saharan Africa

Surf – Surface

TAE – Tris Acetate EDTA

Taq – Thermus aquaticus

TMB – 3,3', 5,5'-tetramethylbenzidine

μL – microliters

WHO – World Health Organization

YMDD – tyrosine-methionine-aspartate-aspartate

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CHAPTER 1: INTRODUCTION

Hepatitis B virus (HBV) infection is a major global public health problem and remains one of the most important causes of cirrhosis and hepatocellular carcinoma (HCC) internationally. HBV-related HCC accounts for approximately 55% of global HCC cases and around 80% of Sub-Saharan Africa cases (SSA) **(Kew, 2010)**. According to the World Health Organisation (WHO), the virus is responsible for around 2 billion infections worldwide with 250 million chronic carriers, despite the availability of a safe and effective vaccine for more than 20 years **(Bertoletti & Gehring, 2013)**. The prevalence of chronic hepatitis B (CHB) varies from regions: a low rate (0.1 – 2%) in the USA and Western Europe, an intermediate rate (2 – 8%) in Mediterranean countries and Japan and a high rate (8 – 20%) in Southeast Asia and SSA regions where infections are the most common **(Liaw & Chu, 2009)**. HBV-infected children are most at risk to develop CHB, putting them at high risk of developing the complications of chronic infection.

Rationale of the study

Prior the implementation of the Hepatitis B (HB) vaccine in South Africa (SA) in 1995, studies had reported high prevalence of Hepatitis B surface (HBs) antigenemia in the population, with a rate ranging from 1% to 20% in children of a young age. One study from the early 70s reported a 54% (34/63) hepatitis B surface antigen (HBsAg) prevalence among children of 14 years old or younger and a 65% (55/84) prevalence among adults aged between 15 and 70 years old **(Kew *et al.,* 1974)**. Prozesky *et al*. compared different age groups when describing the epidemiology of HBV in children. They found the highest prevalence of HBsAg among 3 to 5 years old children (10/85(11.8%)) and the lowest prevalence in aged less than 6 months old (1/103 (1%)) **(Prozesky *et al.,* 1983)**. In 1988, Abdool Karim *et al*. reported 51 infants less than a year old who tested negative for HBsAg, but 136 infants aged less than two years old had an HBsAg seroprevalence of 1.5% and HBeAg prevalence of 0.7% **(Abdool Karim *et al.,* 1988)**. These studies showed that the highest rate of HBV infection was found among children older than a year, indicating that horizontal transmission was the major mode of HBV transmission. These observations provided a background for the implementation of the HB vaccine in the local Expanded Programme on Immunization (EPI) in 1995. The first dose of the vaccine is delivered at six

weeks of age rather than within the 24-hours of birth as is recommended by the WHO. Although the rate of mono-infection decreased, an increased rate of infection in HIV-infected individuals varying from regions was observed **(National Department of Health, 2009)**. HIV/HBV co-infection is associated with higher HBV viral loads, which increases the risk of HBV mother to child transmission (MTCT) **(Burnett *et al.,* 2005)**. Paganelli *et al*. explained that HIV could induce immune suppression in HIV/HBV co-infected patients, resulting in a delay in the seroconversion to anti-HBe, reactivation of HBe antigenemia and seroreversion to HBsAg positivity **(Paganelli, Stephenne & Sokal, 2012)**. The prevalence of HBV carriage in HIV infected individuals in SA was reported to range between 3% and 22% in adults **(Mphahlele *et al.,* 2006; Firnhaber *et al.,* 2008; Hoffmann *et al.,* 2008; Lukhwareni *et al.,* 2009)**. However, little is known regarding the prevalence of HBV in children in the HIV era. This study will thus be reporting on the epidemiology of HBV among HIV-exposed children.

The Mother to Child Transmission Prevention program (PMTCT) guidelines recommended a routine HIV testing for all pregnant women with an unknown HIV status and a CD4 count test if found HIV positive. At the time of recruitment for this study, women with CD4 counts less or equal to 200cells/mm^3 were started immediately on antiretroviral therapy (ART) including stavudine (d4T), lamivudine (3TC) and nevirapine (NVP) **(National Department of Health, 2008)**. Antenatal screening for HBV is not routinely practiced. Whilst Burnett *et al*. reported HBsAg prevalence rates of 7.4% to 8.3% in pregnant women in Limpopo Province, SA **(Burnett *et al.,* 2005)**, recent data from an antenatal Western Province cohort showed an HBsAg prevalence of 2.9% to 3.4% **(Andersson *et al.,* 2013)**. Epidemiological data showed that approximately 30% of women attending antenatal clinics are now living with HIV **(National Department of Health, 2009)** hence with a high risk of perinatal transmission. 3TC is active against both the reverse transcriptase (RT) enzyme of HIV and of HBV. However, prolonged used of the drug causes a high rate of HBV antiviral resistance **(Yuen *et al.,* 2009)**. Due to the compact organisation of HBV, mutations selected in the presence of 3TC which arise in the viral polymerase (*pol* gene) will alter the overlapping surface (HBsAg) gene and this, in the face of a failing immune system, may lead to the selection of mutated viruses potentially resistant to both vaccine and nucleoside antiviral drugs **(Toressi, 2002)**. It remains unknown whether the potentially increased infectivity of maternal HBV due to HIV could result in perinatal transmission and whether the transmitted viruses are wild type or drug driven/immune escape mutant viruses. This study has

investigated whether either antenatal screening for HBV or an alteration of immunization schedules are now needed to prevent perinatal transmission and whether the nature of any transmitted viruses is such that they may escape from vaccine control because of the altered HBsAg antigenicity.

The introduction of ART has brought about a decreased mortality and morbidity rate in the HIV-infected population globally. However, an increase in liver-related diseases mortality and morbidity is observed in HIV/HBV co-infected individuals. These individuals present with increased liver fibrosis brought about by hepatic flares caused by the increased levels of HBV replication **(Puoti *et al.,* 2006)**. The latter is recognized as an increasing risk factor for HCC **(Dwevedi *et al.,* 2011)**. Furthermore, specific HBV mutations such as basal core promoter (BCP), pre-core and pre-S mutations, are well recognized as risk factors for HCC in Asia thus the suggestion that the detection of these mutations may help in the early identification of HBV chronic patients at high risk of developing liver malignancy **(Chen *et al.,* 2008)**. These mutations have been previously described in both chronically infected HBV and HCC patients in SA **(Baptista *et al.,* 1999; Mayaphi *et al.,* 2013)**. An epidemiology study on antenatal women in the Western Cape Province revealed a high presence of some of these HCC-related mutations, among HIV-infected as compared to HIV-uninfected women **(Andersson *et al.,* 2013)**. This suggested that HIV could be an important risk factor in the development of these HBV mutations. Considering the risk of peripartum HBV transmission in HIV co-infected women, we were wondering if these mutations are transmitted to HIV-exposed children. Additionally, given the association of these mutations to HCC, it would be important to assess if they could be used as markers of prediction of HCC development in HBV-infected children and pregnant women.

Chapter 2 describes the organization of the HB viral genome and its diversity. A detailed report on the epidemiology of HBV infection, HIV/HBV co-infection and HBV-related HCC in SA are also presented.

Chapter 3 describes the prevalence of HBV in a large cohort of HIV-exposed infants. The reasons for doing the study included (1) the scarcity of studies on HBV in HIV-exposed and infected children, (2) the high prevalence of HBV in HIV-infected women with loss of HBV immune control, (3) the drug-resistance caused by 3TC in HIV-infected individuals raised the importance of describing the epidemiology of HBV infection in children born of HIV-infected mothers. The primary aim was to describe infant HBV epidemiology and to

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investigate the mode of transmission of HBV in SA in the era of HIV. In this chapter, the sample population and the methodology used to answer our research question are elaborated on. Results on the prevalence of HBV infection in our cohort are also presented.

Having identified important HBV mutations in the mother-to-child transmission study described in chapter 3, coupled with a previous study conducted in the Division of Medical Virology which found a high prevalence of HCC-related mutations (BCP and pre-core mutations) in HIV-infected as compared to HIV-uninfected pregnant women **(Maponga, TG, MSc thesis, Stellenbosch University, 2012),** we investigated the prevalence of these mutations in HIV-exposed children and pregnant women. This is described in chapter 4.

In chapter 5, the significance of these findings and the impact these results could have in addressing the problems of HBV in SA and perhaps beyond are discussed.

The concluding remarks from this work are found in chapter 6.

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CHAPTER 2: LITERATURE REVIEW

HBV was discovered in 1965 in Australia, by a scientist named Blumberg and his colleagues. They discovered a “new” antigen in leukaemia sera from transfused patients. This newly discovered antigen was called the “Australian antigen”. After being associated with type B hepatitis, this new antigen was named Hepatitis B surface antigen **(Blumberg & Alter, 1965)**. From the *Hepadnaviridae* family (*hepato*tropic DNA viruses) and further grouped in the *Orthohepadnavirus* genus. HBV was found to infect only mammals and be highly infectious to humans. Other members of this virus family have been found to infect mammals and birds but are not infectious to humans. These are the Woodchuck Hepatitis Virus (WHV) and Beechey Ground Squirrel Hepatitis Virus (GSHV) affecting mammals and the Avian Virus Pekin Duck Hepatitis B Virus (DHBV). As the name of the family suggests, these viruses target the liver with viral replication occurring predominantly in hepatocytes **(Collier & Oxford, 2006)**.

2.1 Hepatitis B structure and genomic organization

Morphologically, HBV under an electron microscope (EM) appears as three distinct particles of different size and shape of which only one is the virus itself. It is a 42 nm particle, also referred to as the Dane particle. This is, an icosahedral nucleocapsid core surrounded by the hepatitis B surface proteins (HBsAg) embedded in a lipid bilayer and contains the following: the DNA genome, a DNA-dependent DNA polymerase, the hepatitis B core antigen (HBcAg) and the hepatitis B e antigen (HBeAg). Associated with the Dane particle, are tubular and spherical particles of 20-22 nm in diameter, which make up the excess HBsAg.

As shown on Figure 2.1, HBV is encoded by a circular double-stranded DNA genome of about 3200 base pairs with one strand shorter than the other one by about 700 nucleotides, and associated with a DNA polymerase enzyme similar to the retroviral reverse transcriptase (RT) enzyme found in retroviruses. The complete strand has been identified as the “minus strand” and the incomplete one is the “plus strand” **(Collier & Oxford, 2006)**. The 5’ends of both strands are repeated by an eleven nucleotides (nt) motif named DR1 and DR2 respectively involved in the switching of templates during viral DNA synthesis. This genome codes for four overlapping ORFs (S, C, P and X) with the polymerase gene overlapping the

surface gene, situated on the incomplete or “plus” strand. These genes encode for seven essential viral structural and functional proteins named the small, middle and large surface proteins (HBsAg), the pre-core (HBeAg) and core (HBcAg) proteins, the polymerase enzyme and the X protein (HBx) respectively **(Chen & Chen, 2011)**. Additional acting elements being enhancers, promoters, polyadenylation and replication signals involve in the initiation and termination of both genomic replication and translation are found within these ORFs.

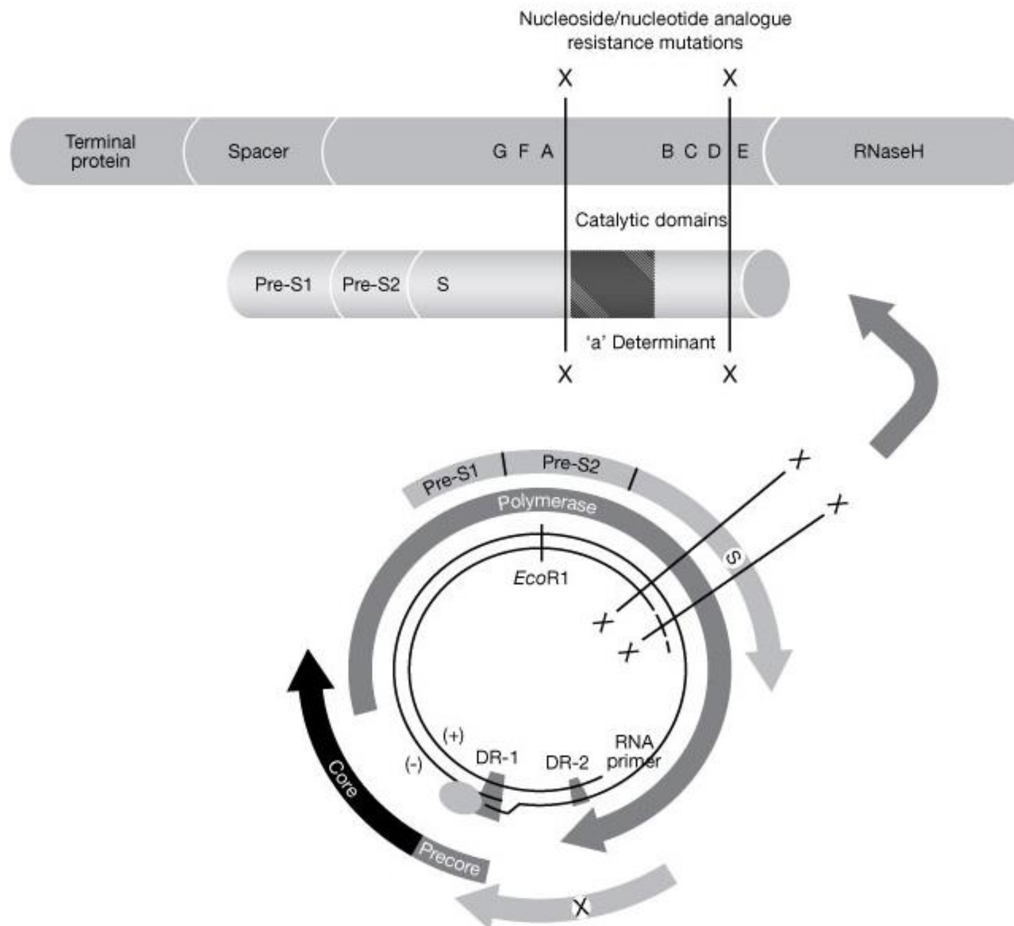

Figure 2. 1: Schematic representation of the HBV genome and its transcripts. The genome of 3.2 kbp of length is made of two DNA strands: the complete minus (-) strand and the incomplete plus (+) strand. The latter is made up of four overlapping genes: S, C, P and X with P being the longest gene and X the smallest gene. At the 5’end of the (-) and (+) stands are found two repeat motifs of 11 nucleotides each named DR1 and DR2 respectively (**Locarnini & Zoulim, 2010 Reproduced with permission**).

The S or surface gene

The surface gene is divided into three domains - pre-S1, pre-S2 and S - encoding for the small (S), middle (M) and large (L) surface glycoproteins or HBsAgs. The latter are

translated from three different start codons (AUG) situated at the beginning of each region of the complete S ORF; hence they possess a common C-terminus but different N-terminus.

Unlike the middle (M) and large (L) surface glycoproteins, the small (S) surface protein is found abundantly in the complete virion. The gene coding for this protein is of 226 base pairs in length and is translated from the third of the three-in frame initiation codons, situated at the beginning of the S domain. An antigenic determinant called group specific antigen or '*a'* is attached to the protein, forming a region of the S gene. HBV vaccination aims for the formation of anti-*a* antibodies to conferred protective immunity **(Harrison, 2008)**. As represented on Figure 2.1, there is overlapping between the surface gene and the polymerase gene. Consequently, mutations generated through viral replication or prophylaxis in the polymerase gene can affect the nucleotide sequence of the S domain on the surface gene. Similarly, changes occurring in the surface gene could affect the nucleotide sequence of the polymerase gene **(Torresi, 2002; Locarnini, 2003)**.

The M protein is translated from two of the three-in frame initiation codons situated in the pre-S2 and S regions and thus, has an additional 55 amino acids (aa) added to 226 aa at its N-terminus.

Complete translation of the surface reading frame (pre-S1 + pre-S2 + S) results in the formation of the large surface protein (L) with an additional 125 base pairs, the pre-S1 domain. A region within this domain is thought to be involved in the attachment of the virus to its receptor on the hepatocytes **(Harrison, Dusheiko & Zuckerman, 2009)**.

The C or core gene

This gene contains two domains – the pre-core and the core – with two in-frame initiation start sites (AUG) at the beginning of each domain. Thus translation of this gene yields two different proteins. The core protein (HBcAg), serological marker of the presence of an HBV infection, is the end result of translation of the second initiation codon situated in the core domain of the C ORF. Translation of the upstream initiation codon in the pre-core domain results in the synthesis of a precursor polypeptide and a signal sequence. The latter directs further processing of the formed polypeptide in the endoplasmic reticulum (ER) at its C-terminus. Secretion of the HBeAg, serological marker of an active viral replication, follows. HBeAg has been thought to be an immune tolerogen hence its role in facilitating persistent HBV infection **(Lee, 1997)**. Moreover, HBeAg has been demonstrated to be able to cross the

placenta during pregnancy, increasing the risk of transmission of HBV infection in the foetus and inducing a T-cell tolerance in the newborn against HBeAg **(Wang & Zhu, 2000)**.

The P or polymerase gene

The polymerase gene codes for the viral polymerase enzyme. The ORF is divided into three functional domains: at the N-terminus is the protein terminal (TP), followed by the reverse transcriptase (RT) domain, and the RNaseH domain at the N-terminus. A spacer domain links the TP domain and the RT domain of the gene.

- The TP domain primes the initiation of the minus-strand synthesis during viral replication via a tyrosine residue used as a primer for reverse transcription. Hence it is covalently bound at the 5'end of the minus strand during reverse transcription.
- The RT domain encodes the RT enzyme involved in the reverse transcription process during viral replication. This domain is further divided into seven conserved subdomains named A through G **(Kim, Lee & Ryu, 2009)**.
- The RNaseH domain codes for the RNaseH enzyme. Reverse transcription involves formation of the minus DNA strand using an mRNA as a template. At the end of the process, the mRNA is degraded from the RNA-DNA hybrid by the RNaseH enzyme **(Harrison, Dusheiko & Zuckerman, 2009)**.

The X gene

This gene encodes a 154 aa long protein termed the X protein. The term "X" was associated to this protein because of its unknown function at the time it was discovered. Today the protein is considered to be a transactivator of viral replication and is critical to a number of cellular functions such as signal transduction of cytoplasmic pathways, cell apoptosis and regulatory effects on the cell. The protein is also thought to have a role in HBV-induced liver carcinogenesis **(Bouchard & Schneider, 2004)**.

2.2 Hepatitis B virus replication

Figure 2.2 illustrates the HBV replication cycle, characterized by reverse transcription. It starts with the binding of the viral particle to receptors found on the lipoid plasma membrane of hepatocytes. Binding is thought to be mediated by a segment of the pre-S1 region

(**Neurath *et al.*, 1986**). Following membrane fusion, the viral core is released in the cytosol where uncoating occurs. The genome then translocates into the nucleus of the liver cell, and is converted into a covalently closed circular form (cccDNA) forming a "minichromosome". Formation of the cccDNA involves completion of the incomplete (+) strand, repair of the (-) strand and ligation of the 5' ends of the two complete strands through superhelical turns. The formed cccDNA is used as template for the formation of four polyadenylated 3' co-terminal RNAs of different length (0.7kb, 2.1kb, 2.4kb and 3.5 kb), initiated by four viral promoters and two enhancers (Enh1 and Enh2) (**Harrison, Dusheiko & Zuckerman, 2009**). Transcription of the core promoter results in the 3.5 kb RNA. The latter comprises the pre-core RNA and the pregenomic RNA (pgRNA) whose translation yield HBeAg, HBcAg and the polymerase respectively. The 2.4 kb RNA is transcribed from the surface promoter 1 (SP1), encoding the L surface protein. The pre-S1, encoding for the M and S surface proteins, is transcribed into the 2.1 kb RNA, and the 0.7 kb RNA is transcribed from the X promoter which encodes the X protein (**Nassal & Schaller, 1993**).

The pgRNA is used as template for reverse transcription by the viral polymerase enzyme for synthesis of the (-) DNA strand. The N-terminal domain of the polymerase, made of a tyrosine residue covalently attached through phosphodiester bonds to a dGTP residue, acts as a primer for reverse transcription (**Karayiannis & Thomas, 2008**). Binding of the viral polymerase to a bulge on the pgRNA termed epsilon (ε), a packaging signal, initiates encapsulation of the complex with the viral products produced from translation of the viral mRNAs, forming an immature core particle. Subsequent events of replication occur in the nucleocapsid. This process is followed by the translocation of the polymerase-primer complex to a complementary DR1 sequence on the 3' end of the template and then the pgRNA is reverse transcribed while simultaneously degraded by the RNaseH enzyme (**Nassal & Schaller, 1993; Harrison, 2008**). The completed (-) DNA strand is left at the DR1 position with a capped oligoribonucleotide (the not degraded 5'end of the template) (**Loeb, Hirsch & Ganem, 1991**). The latter translocates to the 5' end of the (-) strand and hybridizes with a copy of DR1 being DR2, priming synthesis of the (+) DNA strand. As the (+) strand is being synthesized, the genome is folded in a circular configuration, allowed by the short (eight nucleotides) terminal redundancy of the (-) strand (**Harrison, Dusheiko & Zuckerman, 2009**).

As mentioned above, viral replication occurs in the nucleocapsid with the entry of deoxynucleotide triphosphates (dNTPs) through its pores. Some cores will not be completed

while others will be. The incomplete ones, containing the mature HBV genome, will be transported back into the nucleus for further cccDNA synthesis **(Ganem & Prince, 2004)**. The complete cores get finalised before completion of the (+) strand synthesis, causing a lack of dNTPs for the polymerase; hence the characteristic incomplete (+) strand of the HBV genome. The mature cores then bud through the internal membrane already embedded with the surface proteins, forming mature viral particles delivered out of the cell via exocytosis (**Harrison, 2008; Karayiannis & Thomas, 2008**).

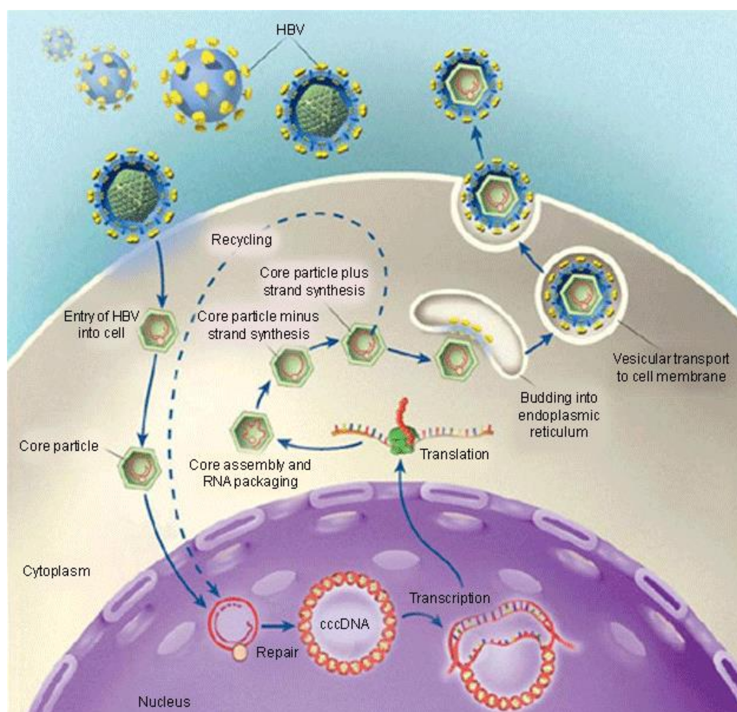

Figure 2. 2: HBV life cycle. HBV targets the hepatocytes to establish infection. The viral particle binds to unknown receptor on the surface of the hepatocytes. Membrane fusion is followed by entry of the nucleocapsid containing the viral genome in the cytosol. The nucleocapsid is uncoated, releasing the viral genome which translocates into the nucleus. The viral genome is converted into cccDNA through repair of the (-) DNA strand and completion of the (+) DNA strand. The cccDNA is used as template for the formation of four viral mRNAs of different lengths and whose transcription leads to the formation of the viral proteins. The longest RNA formed from the transcription of cccDNA, called pgRNA, is used as template for reverse transcription for the formation of the (-) DNA strand. The polymerase binds to a packaging signal (ε) on the pgRNA template, resulting in RNA packaging and core assembly. This is followed with synthesis of the (-) DNA strand using the N-terminal domain of the polymerase and concomitant hydrolysis of the RNA template. The newly synthesized (-) DNA strand is then used as template for (+) DNA strand synthesis. Some core particles are transported back into the nucleus for further formation of cccDNA. Other cores bearing the complete genome will be finalised in the endoplasmic reticulum and transported to the internal membrane embedded with HBsAg, forming a complete viral particle ready for exocytosis out of the cell **(Ganem & Prince, 2004 Reproduced with permission, Copyright Massachusetts Medical Society)**.

2.3 Molecular HBV diversity distribution and clinical significance

HBV has been classified into eight major HBV genotypes, named A to H. Classification is done based on a divergence of >8% or more in the entire genome and 4 % in the S gene

(Kramvis, Kew & Francois, 2005). Recently, two additional genotypes named I and J have been described based on these divergence criteria. However, they are not well characterized yet **(Huy *et al.,* 2008; Tatematsu *et al.,* 2009)**. Genotypes are further subdivided into subtypes or subgenotypes named from 1, 2, 3 etc., with a 4 – 8% difference in their sequences. These genotypes have been well described and distributed globally (Figure 2.3).

Genotype A is subdivided into 7 subgenotypes (A1 to A7) and is responsible for most HBV infections in Cameroon. The subgenotype A1 is mostly found in SSA but has also been reported in Asia and America **(Lin & Kao, 2011)** and was described in SA for the first time in 1997 **(Bowyer *et al.,* 1997)**. Unlike A1, the subgenotype A2 is mainly found in Northern Europe. However, the strain has also been detected in SA, suggesting a possible introduction of the virus subgenotype in Europe by Europeans travelling to SA **(Kramvis & Kew, 2007)**. The subgenotype A3 was found to be restricted to Western Africa with an origin from Cameroon **(Lin & Kao, 2011)**. Subgenotype A4 and A5 were reported in Mali, Gambia and the Southeast part of Nigeria respectively **(Olinger *et al.,* 2006)** but were also described to be present in SSA **(Kimbi, Kramvis & Kew, 2004)**. Subgenotype A6 was characterized as a mixture of three strains originating from African-Belgian individuals originating from Congo and Rwanda **(Pourkarim *et al.,* 2010)**. A new subgenotype, referred as A7, has been so far described in only Cameroon and Rwanda. However, this new subgenotype has yet to be fully characterized **(Hübschen *et al.,* 2011)**. Genotype B is divided into two groups: the "pure" group originating from Japan (also called Ba) and the "recombinant" group from Asia (also called Bj). The genotype was further classified into 6 subgenotypes, named B1 to B6. Subgenotypes B2 – B5, whose sequences consists of recombination of a part of the core region of genotype C added to the core ORF of genotype B, form the "recombinant" group. These subgenotypes dominate East Asia. The "pure" group is made up of subgenotypes B1 and B6, found in Japan and the Artic respectively. Another HBV genotype common in Asia is genotype C. The latter includes subgenotypes C1 – C5, found in East and Southeast Asia **(Lin & Kao, 2011)**. Genotype D is subdivided into 5 subgenotypes and is widespread in Africa, India, and Europe and is the predominant genotype in Mediterranean regions. Genotype E is constrained in West Africa and spans the region going from Mali to Namibia referred to as the "genotype E crescent" **(Kramvis & Kew, 2007)**. Genotype F is subdivided into subgenotypes F1, F2, F3 and F4. Genotype G, unlike the others, needs to be in the presence of another genotype to establish an infection. Few reports of this genotype are available from France and the United States **(Stuyver *et al.,* 2000)**. Genotype H, is

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genetically quite close to genotype F because of the little divergence of their sequences (less than 8%), and is predominant in Central America **(McMahon, 2009a)**. In 2008, a new HBV variant was isolated for the first time in Vietnam. This new variant was suggested to be genotype I. This genotype, originating from Southern China, was shown to be the end result of recombination between genotypes A, C and G **(Huy *et al.,* 2008; Fang *et al.,* 2011)**. The tenth genotype, Genotype J, was discovered in a Japanese man. Phylogenetic analysis of the sequence showed a close clustering with gibbon and orangutan genotypes and the human genotype C **(Tatematsu *et al.,* 2009)**.

HBV diversity has been shown to have an influence on disease progression and in the pattern of transmission of the virus. Genotype C was observed to be predominant in regions of the world where perinatal transmission predominates **(Livingston *et al.,* 2007; McMahon, 2009a)**. Also, HBeAg seroconversion was demonstrated to occur at a later age in individuals infected with HBV genotype C as compared to the other genotypes, hence the increase risk of developing HCC **(Chan *et al.,* 2004)**, liver fibrosis and cirrhosis (LC) **(Chen *et al.,* 2004)**. Genotype A is associated with high replication of HBV DNA thus high viral load, facilitating horizontal transmission in adults. Subtype A1 is linked to high rate of HCC in SSA as compared to subtype A2 which play a role in the development of HCC in older people. Genotype B is related to acute and fulminant hepatitis with high viral load. However HBeAg seroconversion occurs at a younger age in subtype B1 or Bj than in subtype Ba. Genotype D is found in HBeAg- negative chronic infection and, subtype D3 is responsible of occult infection. Very little is known about disease progression related to the other genotypes **(Cao, 2009; McMahon, 2009a)**. Table 2.1 shows a summary of the liver disease progression related to HBV genotypes.

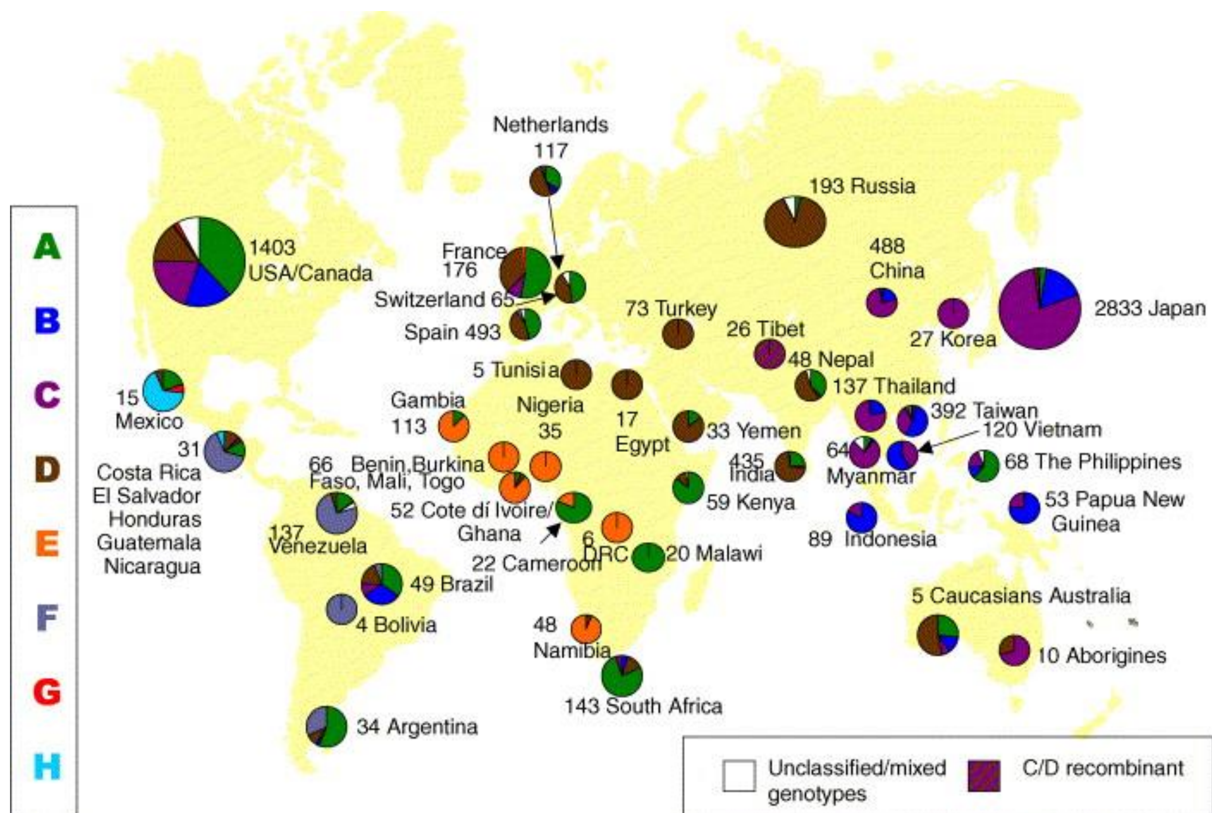

Figure 2. 3: Worldwide geographical distribution of HBV genotypes. This distribution shows a mixture of genotypes in SSA with a higher prevalence of genotype E in Namibia and genotype A in SA. The Northern part of Africa is dominated by genotype D, and as the literature suggests, genotype E is predominant in West Africa. Recombinants are shown to be mostly occurring in Asia (**Kramvis, Kew & François, 2005 Reproduced with permission**).

Recombination between HBV genotypes has been described in some regions where more than one genotype is circulating. It can happen either through co-infection or superinfection of an individual with more than one strain. Time at which the process occurs during replication is unknown but it has been thought to happen during template switches for the synthesis of the (+) and (-) strands **(Kramvis, Kew & François, 2005)**. A/D recombinants were described in SSA **(Owiredu, Kramvis & Kew, 2001)** and B/C recombinants are popular in Asia **(Bowyer & Sim, 2000)**. This mechanism could result in immune escape of the virus or change in the viral replication but its impact on the disease outcome remains uncertain (**Owiredu, Kramvis & Kew, 2001**).

Table 2. 1: Liver disease progression associated with HBV genotypes (**Modified Lin & Kao, 2011 Reproduced with permission**).

Genotype	**C**	**B**	**A**	**D**	**E – J**
Characteristics					
Modes of transmission	Perinatal/ Vertical	Perinatal/ Vertical	Horizontal	Horizontal/Perinatal	Horizontal
Tendency of chronicity	Higher	Lower	Higher	Lower	ND
Positivity of HBeAg	Higher	Lower	Higher	Lower	ND
HBeAg seroconversion	Late	Earlier	Earlier	Late	ND
HBsAg seroclearance	Less	More	More	Less	ND
Histologic activity	Higher	Lower	Lower	Higher	ND
Response to interferon alpha	Lower	Higher	Higher	Lower	Lower in Genotype G
Response to nucleos(t)ide analogues	ND	No significance difference between genotype A to D			
Virologic characteristics					
Serum HBV DNA levels	Higher	Lower	ND	ND	ND
Frequency of pre-core A1896 mutation	Lower	Higher	Lower	Higher	ND
Frequency of basal core promoter T1762/A1764 mutation	Higher	Lower	Higher	Lower	ND
Frequency of pre-S deletion mutation	Higher	Lower	ND	ND	ND

ND = No Available data

2.4 HBV-associated mutations

The HBV genome evolves at a rate of 1.4-3.2 x 10^{-5} nucleotide substitution per site per year, owing to the viral error-prone reverse transcriptase used during reverse transcription as a mechanism of evolution for the virus. Mutations in overlapping genes tend to affect proteins in both genes, and the removal of an epitope of a protein due to a mutation can result in either

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the addition of an epitope to the other protein or harming the protein functionality (**Maman *et al.,* 2011**). This leads to the circulation of a population of genetically different, but related viruses called quasispecies. Although these mutations could be harmful to the viral particles, some could also be beneficial to them by either enhancing replication or easing immune escape (**Karayiannis & Thomas, 2008**).

2.4.1 Mutations in the core gene

Two types of mutations have been described in the pre-core/core gene which affects HBeAg expression. The first functionally characterized was the pre-core stop mutation G1896A at nucleotide (nt) 1896 (or codon 28: TGG), located in the encapsidation signal (ε) structure of the pre-core. This mutation, as the name suggests, causes a stop codon in the pre-core gene (TGG to TAG; TAG: stop codon) hence blocking HBeAg production (**Locarnini, 2004**). The ε structure is critical during viral replication and is stabilized by the base paring between nt G1896 and nt 1858 forming a T – A. The occurrence of the G1896A mutation depends on the base present at nt 1858. Genotypes B, D E, G and some C and F strains have a T1858 mutation enabling the stabilization of the ε structure by the stop codon mutant. However, genotypes A, H and some C and F strains have a C1858 instead hence the rare occurrence of the stop codon mutant. The presence or absence of C or T at nt 1858 explains the difference in prevalence of this mutation geographically (**Kramvis & Kew, 2005; Tong, Wands &Wen, 2013**). The second mutation, rather common in chronic carriers, affects the BCP at nt 1762 and 1764. This mutation, referred to as the double BCP mutation A1762T/G1764A, leads to a 70% decrease in the HBeAg production and the increase of viral replication (**Jammeh *et al.,* 2008**). Other mutations such as T1753C, C1766T and T1768A occurring in the BCP regions have also been associated to increased viral replication and decreased HBeAg expression (**Quarleri, 2014**).

2.4.2 Mutations in the X gene

Due to the overlapping between the pre-core domain of the Core gene from nt 1742 to 1802 and the X gene seen on Figure 2.1, mutations occurring in the X gene could have an impact on the pre-core, specifically on the BCP and Enhancer II regions and vice versa. This explains the double mutation K130M/V131I on the X gene caused by the double BCP A1762T/G1764A mutation. Moreover, any deletions or insertions in the BCP could cause a shift of the X gene, hence producing truncated X proteins (**Locarnini, 2004**).

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2.4.3 Mutations in the surface gene

Numerous mutations occurring in the surface gene have been described. These mutations are considerably clinically important in a context of HBV prevention (through vaccination) and of diagnosis (HBsAg serology testing) **(Hunt *et al.,* 2000)**. Pre-S mutants form the largest group of mutations occurring in the surface/envelope region of the HBV genome. They are more frequent among genotypes B and C than other genotypes and range from deletions, insertions, point mutations to genetic recombination **(Kramvis & Kew, 2005)**. The pre-S1 and pre-S2 contain several epitopes for T and B cells demonstrating their importance for the interaction between the immune system. Mutations in the pre-S region have been found to appear in late stage CHB patients, and also in fulminant hepatitis and HCC patients **(Shen & Yan, 2014)**. As HBV chronicity persists, a progression of pre-S deletions is observed leading to an accumulation of two types of large HBV surface antigens (LHBs) being S1-LHBs and S2-LHBs and viral particles in the hepatocytes. Patients exhibiting pre-S mutations and core mutations had higher incidences of HCC than those without or with single mutations **(Chen *et al.,* 2006; Qu *et al.,* 2013)**.

2.4.4 Mutations in the polymerase gene

This gene is the main target of HBV treatment and contains the error prone RT enzyme, the cause of mutations occurring in this region of the genome. Because of the overlap of the P gene with the S gene, mutations in the RT domain could potentially result into changes in the coding sequence of the HBsAg region **(Cento *et al.,* 2013)**. These mutants have the ability to escape serological diagnosis and evade vaccine protection. Polymerase mutants have been identified in patients on ART such as 3TC, adefovir (ADV) and entecavir **(Locarnini, 2004)**. The most common mutation reported from 3TC therapy is referred to as the tyrosine-methionine-aspartate or YMDD mutation. The latter may cause changes in the S ORF leading to the formation of mutants HBs proteins **(Clements *et al.,* 2010)**. The mutant HBs antigens failed to be recognized by immune anti-HBs antibodies hence maintaining the replication of the virus. This phenomenon could also be seen through HB vaccination. The latter is based on the induction of antibodies against the antigenic epitope found on the HBsAg protein called the “a” determinant. However in some cases, the immune pressure brought about by these anti-HBs proteins causes the selection of immune HBV escape mutants. The most common mutation causing this evasion is the substitution of a glycine to an arginine at

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position 145 (sG145R) **(Cooreman , Leroux-Roels & Paulij, 2001; Sheldon & Soriano, 2008)**.

2.5 Natural history and pathogenesis of the virus

As the name "hepatotropic" suggests, HBV targets the liver and results in either a transient or chronic infection. A transient infection can cause serious illness and terminates with acute or fulminant hepatitis. Developing chronicity is highly dependent on the age at which primary infection occurred. A chronic infection can produce serious problems with a 25% risk of primary liver cancer or HCC and a 2-3% risk of liver cirrhosis annually **(McMahon, 2009b)**.

2.5.1 Acute hepatitis

Acute HBV infection can be symptomatic or asymptomatic depending on the age at which infection occurs. The infection is characterized by complete recovery at the end of its course with formation of anti-HBs antibodies, lowering the risk of developing liver cirrhosis or HCC **(Shepard *et al.*, 2006)**. Acute hepatitis is not common in neonates unless born from HBeAg-negative mothers. These infants develop fulminant hepatitis characterized by liver failure and a strong immune response against HBV which leads to rapid recovery (**Liaw & Chu, 2009**). However symptomatic acute hepatitis occurs in about 10% of young children aged between one to five years, in most cases of infections in younger children, adolescents and adults. Incubation period is around three to six months before onset of symptoms which include nausea, vomiting, abdominal pain, anorexia, malaise, jaundice and changes in stool and urine colour **(Chang, 2007)**. Diagnostic of an acute infection involves serological testing for HBsAg, total anti-HBc antibodies and immunoglobulin M antibodies to HBcAg (IgM anti-HBc). The first marker to appear in the blood is HBsAg, followed by IgM anti-HBc two weeks later. Figure 2.4 shows that as the disease progresses, HBsAg and IgM anti-HBc become undetectable in the blood, leaving only anti-HBc (total) and anti-HBs antibodies. Anti-HBc (total) antibodies persist for life whereas anti-HBs antibodies tend to wane with time. Anti-HBs antibodies are also considered as markers of previous HBV active immunization, inducing protective immunity against HBV **(Lee, 1997; Liaw & Chu, 2009)**.

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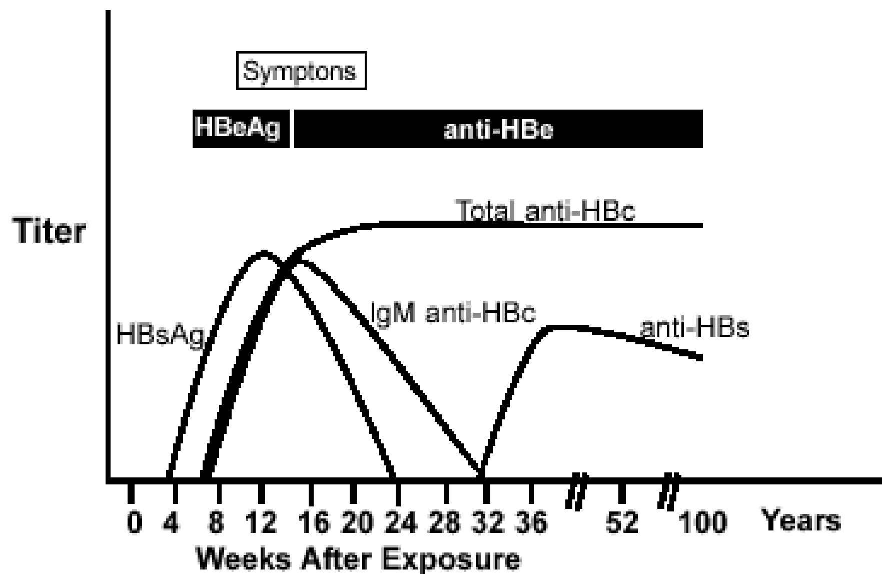

Figure 2. 4: Serological changes in acute HBV infection. The first serological marker released in the circulation is HBsAg between 0-3 weeks after infection followed by IgM anti-HBc and total anti-HBc antibodies which persist for life. The production of anti-HBs antibodies from week 32 marks the point at which resolution of the infection starts **(Paar, 2001 Reproduced with permission).**

2.5.2 Chronic hepatitis

Chronic hepatitis infection (CHB) is confirmed with the presence of HBsAg for more than six months. It affects around 90% of infants born from both HBeAg and HBsAg-positive mothers **(Beasley *et al.,* 1977),** 25-30% of children infected between one to five years, and 5-10% of adults unless immunocompromised **(McMahon *et al.,* 1985)**. Chronicity may persist for life and leads to severe hepatic damage such as liver cirrhosis or HCC. In countries with common childhood infection, there is high seroprevalence (80-85%) of HBsAg and HBeAg in children below the age of 15 years than in adults carriers **(Harrison, Dusheiko & Zuckerman, 2009)**. Developing chronicity is inversely proportional to the age at which infection was acquired. CHB is divided into four phases illustrated on Figure 2.5: (1) the immune tolerant phase, (2) the immune clearance phase, (3) the non-replicative phase, and (4) the reactivation phase which may be seen in some patients **(Karayiannis &Thomas, 2008)**.

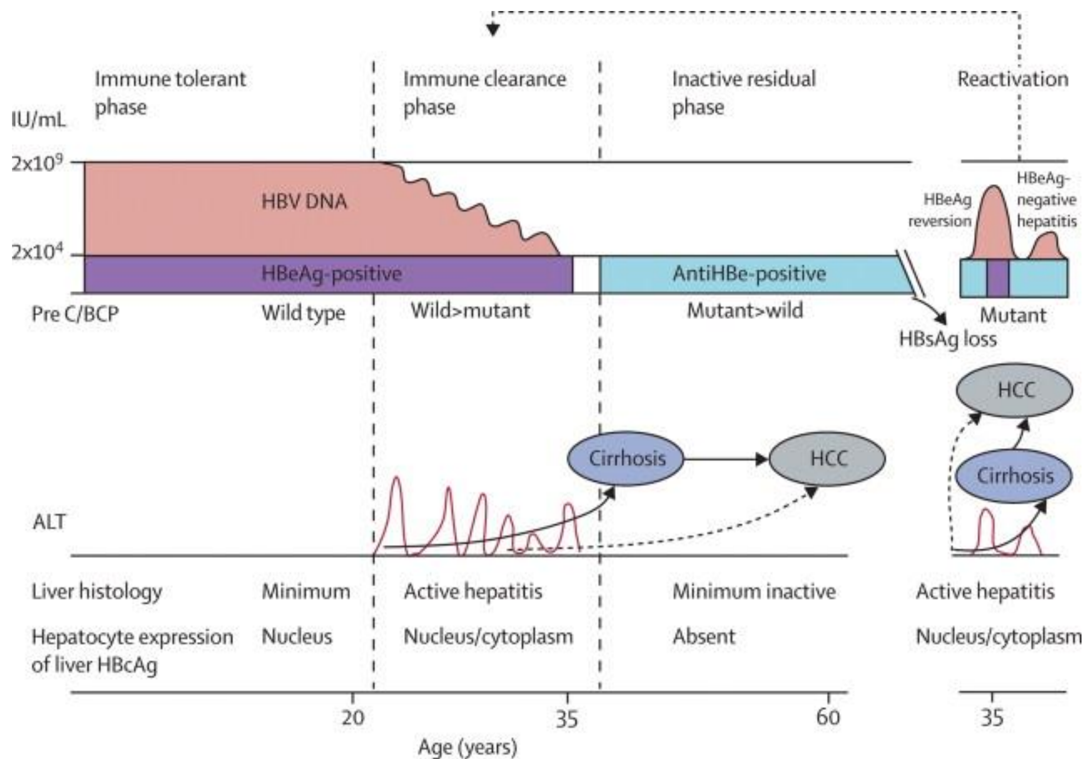

Figure 2. 5: Natural course of chronic HBV infection. This diagram shows the course of a chronic HBV infection with time. A chronic HBV infection is divided into four phases being the (1) the immune tolerant phase characterized with high HBV DNA and HBeAg levels, normal ALT levels and asymptomatic; (2) the immune clearance phase is characterized by a decrease of HBV DNA, HBeAg levels and ALT flares causing acute liver damage which may lead to cirrhosis, (3) the inactive residual or non-replicative phase characterized with complete HBeAg seroconversion and undetectable levels of HBV DNA levels. At this stage liver cirrhosis could develop and lead to HCC and accumulation of pre-core HBV mutants occur, and (4) the reactivation phase characterized with HBeAg seroreversion due to either HBeAg- negative hepatitis which developed from the accumulation of pre-core mutations or immunosuppression. Here the ALT flares and active hepatitis occur again **(Liaw & Chu, 2009, Reproduced with permission).**

Immune tolerant phase

This phase is characterized by a seropositive status of all HBV serological markers with high viral load ($2x10^6$ to $2x10^7$ IU/mL) levels, normal alanine aminotransferase (ALT) levels and liver histology with no apparent symptoms. CHB patients can remain in this phase for two to three decades if infected perinatally or during childhood, but for two to four weeks if the infection is acquired during adulthood **(Wright, 2006; Chen & Chen, 2011)**. There is no evidence of immune response against HBV, explained by the HBeAg-specific helper T lymphocytes tolerance induced by the maternal transplacental transfer of HBeAg during pregnancy. Hence the tolerogenic function attributed to HBeAg. The immune system fails to recognize HBeAg as a foreign particle, leading to high viral replication and high viral load. However in case of mutations in the pre-core or core promoter, HBeAg production is

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decreased, reducing immunotolerance and leading to an increase in the ability of the immune system to react against the infected hepatocytes (**Paganelli, Stephenne & Sokal, 2012**). This event could be a participant of the transition from the immune tolerant phase to the immune clearance phase.

Immune clearance phase

Here HBeAg seroconversion is accompanied by an increase of ALT values exceeding 1000 IU/L, and a decrease in HBV DNA levels below 2000 IU/mL. The stronger the immune response, the higher the ALT flares. The higher the ALT levels, the higher the liver damage - resulting in liver cirrhosis and/or fibrosis. However there is insufficient data on the risk of liver disease progression in children (**Nebbia, Peppa & Maini, 2012**).

Seroconversion to anti-HBe depends on many factors such as age, HBV genotype, maternal HBsAg status and the geography. Children born from HBeAg positive mothers tend to have a lower rate of HBeAg seroconversion (<2% annually in children less than 3 years of age and 4-5% after 3 years) **(Jonas, 2013)** than older children or those born from HBeAg-negative mothers. Also, individuals infected with genotype B tend to seroconvert to anti-HBe earlier (less than 20 years of age) than genotype C carriers (over 40 years of age) **(Chu, Hussain & Lok, 2002; Livingston *et al.,* 2007**). In relation to geography, there is a higher HBeAg seroconversion rate observed in the Euro-Mediterranean region and Africa as compared to Southeast Asia annually **(Ott *et al.,* 2012**). Little data is available concerning this issue in SSA.

Spontaneous clearance of HBsAg also occurs but at a very low rate depending on the age of infection. Rate of incidence is estimated to be 0.4% to 2% if infection occurred in adulthood and 0.1% to 0.8% if infection occurred via MTCT or during childhood **(Huo *et al.,* 1998**).

Non-replicative phase

As the word “non-replicative” suggests, there is absence of HBV replication. Complete HBeAg seroconversion, normalization of ALT levels, very low level of HBV DNA (undetectable with hybridization methods) and improvement of liver histology are observed at this stage of the infection. The patient is said to be an HBeAg- negative inactive carrier. Although HBV replication is absent, permanent liver damage accompanied by development of cirrhosis and/or HCC may occur in childhood **(Liaw and Chu, 2009**). Most CHB carriers

could remain in this phase indefinitely. However, a small proportion could serorevert to HBeAg or could develop HBeAg-negative active hepatitis **(Chang, 2007)**. The latter results from a pre-core mutation, also called G → A mutation, leading to a lack of HBeAg formation. Loss of the tolerogen HBeAg activates the immune system leading to HBV reactivation or to the immune clearance phase of the disease.

Reactivation phase

This phase is characterized by reactivation of HBV viral replication leading to seroreversion to HBeAg. This phase is mostly seen in patients undergoing chemotherapy, on immunosuppressive treatment, and HIV-immunosuppressed. As the immune system weakens, it loses its control over the low rate of viral replication. This results in an increase of ALT levels, HBV DNA levels higher than 20,000 IU/mL and a high risk of developing liver cirrhosis, fibrosis and/or HCC **(McMahon, 2009b)**.

2.5.3 Occult infection

Considered as the fifth phase of CHB by some, occult HBV infection (OBI) is defined by the presence of HBV DNA in the liver in individuals with a negative HBsAg and a positive anti-HBc (total) antibodies status. However in some cases all HBV serological markers are absent. This distinguishes between seropositive and seronegative OBI respectively **(Conjeevaram &Lok, 2001)**. The presence of the HBV genome in the liver could be explained by: the integration of the viral genome in the form of cccDNA in the genome of the host hepatic cells, immunosuppression, mutations in the S gene infection caused by a HBV unable to synthesize the S antigen, insensitive serological assays in the detection of HBsAg immunological markers, presence of immune complexes hiding HBsAg **(Ramezani *et al.,* 2011)**. However in most OBI cases, the virus shows mutations in its genome's sequence but has full ability of replication in the host **(Hu, 2002; Raimondo *et al.*, 2013)**. OBI has been implicated clinically in scenarios such as the occurrence of transfusion-transmitted HBV hepatitis through blood transfusions or liver transplantation **(Chazouilleres *et al.*, 1994)**, development of chronic liver disease and fulminant hepatitis **(Cacciola *et al.*, 1999)** and was presented to play a role in the development of HCC **(Pollicino, Saitta & Raimondo, 2011)**.

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2.6 HBV-related hepatocellular carcinoma

Liver cancer is one of the leading causes of cancer deaths in Africa. As mentioned earlier, early childhood infections is the main risk factor of chronic HBV. Chronic HBV infection is the leading cause of HCC in SSA. The association between CHB and the development of HCC was first established in 1975 **(Blumberg *et al.,* 1975)**. The geographical prevalence of the tumour is determined by the geographical distribution of HBV. Thus, the highest incidence of the tumour is found in highly HBV endemic regions such as Asia and SSA. In low endemic regions, where the HBV carriage is low, factors other than HBV are the main risk factors. A range of risk factors have been associated to the increased risk of malignancy in HBV chronic patients. These are host genetic, viral and environmental factors.

Host genetic factors

The important aspects concern gender and age. In regard to gender, African and Asian studies have shown that males are at greater risk of malignant transformation as compared to females **(Beasley *et al.,* 1981; Beasley *et al.,* 1984; Kew *et al.,* 1983)**. While HCC is common among old individuals with an increasing risk as the age increases in most populations and in Taiwanese **(Beasley *et al.,* 1984)**, black Africans show a highest prevalence of HCC in patients younger than 30 years old and a decreasing prevalence in older than 60 years old **(Kew *et al.,* 1983)**.

Although mostly common in adults and uncommon in children (0.5% - 2% of paediatric malignancies worldwide), liver tumours have been previously reported in the South African paediatric population **(Moore *et al.,* 1997; Moore *et al.,* 2004)**. The latest audit from Moore and colleagues reported an increase in the epidemiology of HBV-related HCC among children in Southern Africa, which differs from the rest of the world **(Moore *et al.,* 2008)**. The increased prevalence of liver malignant formation may be caused by perinatal HBV infection, an underestimated mode of transmission in SA.

Viral factors

These concern characteristics of the virus such as viral load, genomic mutations and genotypes. A high HBV viral load is synonymous with active HBV replication. Studies have demonstrated that patients with high HBV viral load and HBeAg positive are exposed to a quicker progression to liver cancer **(Tang *et al.,* 2004; Yang *et al.,* 2002; Yu *et al.,* 2005)**. Yu *et al*. also described that a high viral load is associated with genotype C. Thus genotype C

carriers are at a greater risk of malignancy than genotype B carriers **(Yu *et al.,* 2005)**. In Southern Africa, genotype A has been reported to present a 4.5-fold increased risk of HCC as compared to genotype D carriers **(Kew *et al.,* 2005)**.

Over time and due to immune pressure, HBV has developed mechanisms to escape immune recognition through mutations in specific regions of its genome. These are BCP, pre-core **(Baptista *et al.,* 1999)** and pre-S mutations **(Shen & Yan, 2014)** which are well recognized risk factors for the development of HCC in Asia. Prospective studies have observed an on-going combination of pre-S deletions in the 3'end of the pre-S1 and 5'end of the pre-S2 domains in chronic patients who eventually developed liver cancer **(Chen *et al.,* 2006; Qu *et al.,* 2013)**. The pre-S1 and pre-S2 domains contain several epitopes for T and B cells thus their important role in the interaction with the immune system **(Ferrari *et al.,* 1992)**. The accumulation of pre-S deletions leads to the accumulation of two types of ground glass hepatocytes (GGHs) containing two different types of mutant LHBs **(Wang *et al.,* 2003)**. The accumulation of these LHBs in the endoplasmic reticulum (ER) causes a cascade of ER stress signalling pathways which leads to oxidative DNA damage **(Hsieh *et al.,* 2004)**. The latter may cause genomic instability resulting in mutation of liver cells thus liver malignancy formation **(Wang *et al.,* 2006)**. In addition to pre-S mutations/deletions, the double BCP mutation A1762T/G1764A is also commonly found in CHB and HCC patients. This mutation leads to a decreased HBeAg production and increased in viral replication, according to transfection studies, **(Parekh *et al.,* 2003; Liang *et al.,* 2013)** and causes a double mutation in the overlapping X gene at position 130 and 131. These combined mutations on the X gene have been shown to induce an increase activity of NF-κB activity through unknown pathways **(Lee *et al.,* 2011)**. The NF-κB protein is an important gene transcriptor involved in many features of cell regulation including oncogenic transformation, regulating immune activation and apoptosis. HBeAg, a tolerogen, acts as an inhibitor of immune clearance of infected hepatocytes. The loss of this tolerogen, brought by either of the two above mentioned mutants, leads to the recognition of HBV-infected hepatocytes by the $CD8^+$ cytotoxic T cells therefore causing hepatocytes apoptosis hence liver damage **(Hunt *et al.,* 2000)**. Liver damage could trigger wound healing responses that in the long run promote the development of hepatic fibrosis and HCC **(Luedde and Schwabe, 2011)**. An increase in NF-κB proteins brought about by the upregulation could result in increased inflammation or insufficient protection from cell death **(Luedde and Schwabe, 2011)**. Other BCP mutations such as T1753C and the double C1766T/ T1768A have also been associated to the formation of liver

tumours **(Qu *et al.,* 2011)**. Although these mutations are independent risk factors for HCC, Qu *et al.* observed that the risk factor is greater when these mutations are combined than single **(Qu *et al.,* 2013)**.

Environmental factors

Repeated dietary exposure to the mycotoxin produced by fungi of the *Aspergillus* species (*flavus* and *parasiticus*) named aflatoxin B1 (AFB1) has been reported as an important cause of HCC in HBV endemic regions **(Bressac *et al.,* 1991a; Bressac *et al.,* 1991b)**. This toxin is found in food such as peanut, corn and rice stored in moist conditions contaminated by the fungus. A product of metabolization of AFB1, AFB1-*exo*-8, 9-epoxide, has the ability to bind to DNA causing impairment of DNA, RNA and protein synthesis. The toxin is also thought to cause a mutation at serine 249 (guanine to thymine transversions), also known as the R249S point mutation, in the tumour-suppressor p53 gene. This mutation has been described in a majority of HCC patients who are HBV-infected **(Bressac *et al.,* 1991b)** but has not yet been found in non-HBV related HCC cases.

2.7 HBV epidemiology in Sub-Saharan Africa

HBV and HIV are major public health problems with HIV causing the highest number of infectious diseases deaths worldwide and viral hepatitis the second most common cause of deaths from infectious diseases. SSA carries a high burden of both these diseases. The two viruses share similar routes of transmission and were shown to cause co-infection in about 5 - 15% of the worldwide population, with a high association rate in highly endemic regions such as SSA. The rate of co-infection depends on many other factors such as the geographic areas and their differences in CHB prevalence, the route of transmission and the age at which infection is contracted.

About two-thirds of the worldwide 33 million HIV infections are found in SSA **(Hogan *et al.,* 2012)**. HBV is the main cause of chronic liver disease ranging from minor disorders to cirrhosis and liver cancer **(Botha *et al.,* 1984; Kew, 2012)**. Since HBV is able to remain viable on dry surfaces for extended periods of time, the virus is considered to be more infectious than HIV **(Alter, 2006)**. Prevalence of hepatitis B infection, illustrated on Figure 2.6, is defined according to the percentage of the population seropositive for HBsAg. The highest prevalence of HBV is shown to be found in intermediate and highly endemic (4 – 8%

HBsAg seroprevalence and >8% HBsAg seroprevalence respectively, according to the WHO) areas such as Southern Africa and West Africa respectively (**Modi & Feld, 2007**).

HBV studies conducted in SSA have shown a higher prevalence of the virus in HIV-infected individuals than in HIV-uninfected ones**,** with a prevalence of 1% to 19% co-infection in children (**Mutwa *et al.,* 2013**). SSA countries such as Senegal (**Diop-Ndiaye *et al.,* 2008),** Nigeria (**Adewole *et al.,* 2009**), Ghana (**Geretti *et al.,* 2010**) and, Zambia (**Kapembwa *et al.,* 2011)** reported HBV- HIV co-infection rates of 16.5%, 11.5%, 16.7% and 9.9% respectively in adults. In the North of Namibia, Brandt *et al*. revealed an 8.7 % occurrence of HIV/HBV co-infection in 1057 chronic hepatitis children (>18 years old) in the immune-tolerant phase of the disease (**Brandt *et al.,* 2012**). A recent study in the North of Tanzania also showed a 9.7% prevalence of HBV in 157 HIV-infected children (**Muro *et al.,* 2013**).

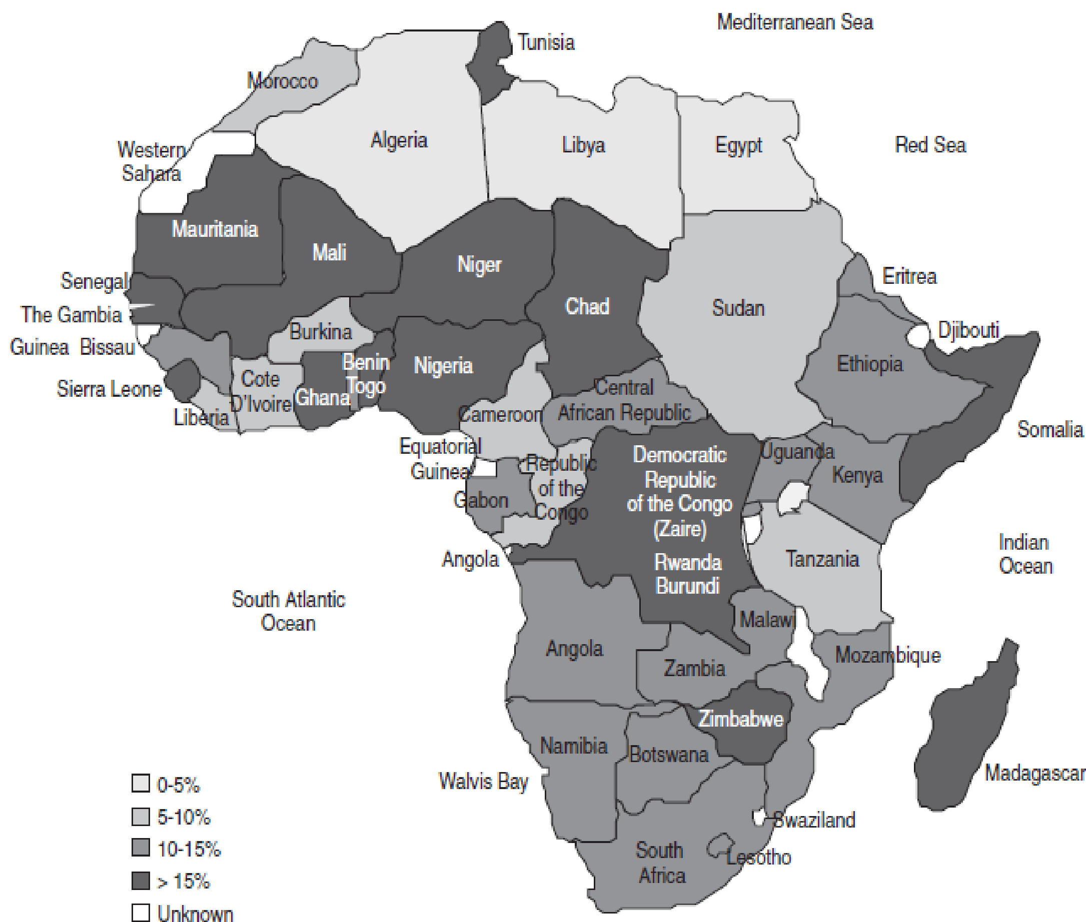

Figure 2. 6: Hepatitis B virus prevalence in Africa. Sub-Saharan Africa harbours the highest number of hepatitis B virus infections compared to the Northern part of Africa (**Modi & Feld, 2007 Reproduced with permission**).

2.7.1 Epidemiology in South Africa

In Johannesburg, Firnhaber *et al*. confirmed a high prevalence of HBV in HIV-positive persons with a total HIV/HBV co-infection rate of 5% **(Firnhaber *et al.,* 2008)**. Two other serosurveys conducted in the rural areas of SA had the same results but with a higher rate of co-infection being around 16.2 % and 17%, respectively **(Mphahlele *et al.,* 2006; Hoffmann *et al.,* 2008)**.The prevalence of hepatitis B in the South African obstetrics population is also of concern. Pre-HBV immunization data have described a low HBV prevalence (1.21%) in pregnant women **(Guidozzi *et al.,* 1993)**. A decade later, Burnett *et al*. reported antenatal HBsAg prevalence rates of 7.4% to 8.3% in the Limpopo province of SA. Recent data from an antenatal Western Province cohort described a hepatitis infection rate of 2.9% whilst of 3.4% among HIV/HBV co-infected pregnant women with high HBeAg prevalence and loss of immune control of HBV **(Andersson *et al.,* 2013)**. This determines the risk of transmission of hepatitis to infants.

Prior to the introduction of the HB vaccine, a 10.4% HBsAg prevalence rate had been described among children, with high HBsAg positivity rate of 8.1% and 8.9% in children aged 0 – 6 and 7 – 12 months, respectively **(Vardas *et al.,* 1999)**. Post immunization data described considerate decrease of the HBV infection rate among children **(Hino *et al.,* 2001; Tsebe *et al.,* 2001).** A recent analysis of pre- versus post-HBV immunization reported (1) increased immunity to HBV infection from 13% to 57%, decreasing as age increased, and (2) a decrease of HBV chronic carriage from 4.2% to 1.4% **(Amponsah-Dacosta *et al.,* 2014).** Although much is known on hepatitis in mono-infected children, data on HBsAg prevalence rate among HBV-HIV co-infected children in SA are sparse.

2.7.2 HBV occult infection in HIV-infected patients

Occult infection prevalence varies in different geographical regions, depending on the prevalence of HBV in the area. The frequency has been shown to be quite high in SA. Firnhaber *et al*. found a prevalence of 88.4% of OBI in 43 individuals positive for HIV and anti-HBc **(Firnhaber *et al.,* 2009)**. The same year, Lukhwareni *et al*. detected HBV DNA in 34 of 148 HBsAg negative HIV-positive patients **(Lukhwareni *et al.,* 2009)**. Although OBI seems to be common in HIV/HBV co-infected patients, insights in the natural history of the disease and risk of developing liver disease in HIV/HBV co-infection are unknown. However, patients with occult HIV/HBV infection tend to respond equally well to HAART as those HIV/HBV co-infected with HBsAg positivity **(Chadwick *et al.,* 2013)**.

2.8 Hepatitis B virus transmission routes

HBV has the ability to survive for extended periods of time on dry surfaces thus its high infectivity. The viral protein HBsAg is found in high quantities in the blood and other body fluids such as saliva, sperm, menstrual and vaginal discharge, breast milk and serous exudates. Contact with any of these fluids from an HBsAg positive individual leads to transmission of the virus. Two main routes of transmission of the virus have been established: vertical and horizontal transmission **(Harrison, Dusheiko & Zuckerman, 2009)**.

Vertical or perinatal transmission

Also referred as MTCT, vertical transmission is the main factor of HBV chronicity development in infants (**Ott, Stevens & Wiersma, 2012**), especially in Asia **(Stevens *et al.,* 1975)**, and the main HBV mode of transmission in high endemic areas (>8% HBsAg seroprevalence). Child-bearing women positive for both HBeAg and HBsAg with a high viral load have a 90% rate of transmission compared to a 25% rate of transmission for HBeAg negative mothers and a 12% transmission rate for HBeAg negative and anti-HBe positive mothers **(Umar *et al.,* 2013)**. HIV co-infection could also be a risk factor for MTCT because co-infected mothers show higher levels of HBeAg and HBV DNA as compared to HBV mono-infected mothers **(Modi & Feld, 2007)**. Vertical transmission can occur through three different patterns: (1) intrauterine, (2) intrapartum, and (3) postpartum **(Pol, Corouge & Fontaine, 2011)**.

(1) <u>In utero or intrauterine transmission:</u> As mentioned above, HBeAg was demonstrated to be able to cross the placenta during pregnancy and infect the foetus. Evidence of an in utero transmission is shown by the detection of HBsAg in a newborn 24 hours after delivery. Studies have been contradictory on whether this type of transmission is related to the infiltration of maternal blood into the placenta and not to HBV replication, maternal HBsAg titre or viral load **(Ohto *et al.,* 1987)**; or if not the other way around **(Zhang, Han and Yue, 1998)**. Further researches performed on the Asian population on this issue showed that the infection occurs through a cellular transfer process, from the placental decidual cells to the villous capillary endothelial cells reaching the foetal circulation through a Fc gamma receptor III- mediated entry of HBsAg – anti-HBs immune complexes mechanism into the placenta; hence the decrease in viral load from the maternal side to the foetal side **(Xu *et al.,* 2001)**. However, this mechanism has not yet been reported in any other continent than Asia **(Xu *et***

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***al.*, 2002**). Studies have also presented high maternal HBV DNA titres to be strongly associated with in utero transmission (**Pande *et al.*, 2008; Wiseman *et al.*, 2009**).

(2) Intrapartum transmission was shown to be associated with the duration (>9 hours) of the first stage of labour (**Wong, Lee & Ip, 1980**). Transmission of maternal HBsAg can also occur during delivery from the mixture of maternal and foetal blood due to placental leakage or instrumentation-induced trauma (**Umar *et al.*, 2013**).

(3) Postpartum: On this pattern of transmission, very little convincing evidence is available. HBsAg and HBV DNA have been detected in breast milk and in infant's gut born from HBeAg mothers. However, many studies have not shown breastfeeding as a risk factor for HBV MTCT transmission (**Chen *et al.*, 2013**).

Horizontal transmission

This is the common HBV mode of transmission in intermediate and low endemic areas (**Alter, 2006**). However, it was found to be the predominant mode of transmission in the highly endemic area SSA, where childhood infection is common (**Botha *et al.*, 1984**). Horizontal transmission may occur either during childhood or in adulthood. Although the mechanism behind the spread of the virus is not well understood, a few risk factors, including the use of unsterile needles during ritual scarification by traditional healers (**Kew, Reis & Macnab, 1973**), contact of bodily fluids such as saliva and blood through skin abrasions, (**Kiire, 1996**), weeping sores (**Mphahlele *et al.*, 2002**) and human bites (**Hui *et al.*, 2005**) have been associated with this mode of transmission.

In childhood, transmission may occur postpartum, through the usage of infected medical instruments in hospitals often happening in resource-limited settings, virus spread among siblings and parents referred as intra household transmission and multiple intramuscular injections (**Kourtis *et al.*, 2012**). Infection in adults occurs often through risky behaviours such as unprotected sexual activity, having more than one partner, and injection drug use; but could also happen from person-to person of a household via infectious body fluids. Transplantation and blood transfusions are other routes through which infection could be contracted. These two are being eradicated in developed countries through the implementation of testing donors for HBsAg or HBV viral nucleic acid but is still seen in developing countries (**Alter, 2006**).

2.9 Prevention and management of Hepatitis B virus infection in children

2.9.1 HBV immunization in children

Preventive measures implemented by public health organizations and governments have been established to reduce the incidence of this disease. Since infants are most likely to develop chronic HBV infection as compared to adults, ensuring children are protected is a cost effective way to prevent on-going infection.

Passive immunization

Passive immunization was introduced in 1974 and involves the preparation of hepatitis B immunoglobulin (HBIG) using pools of plasma with high titres of anti-HBs proteins. The latter form immune complexes with HBsAg and bring about neutralization of the viral particles. HBIG provides passive protection only in case of acute exposure to HBV but isn't used as pre-exposure prophylaxis because of its high cost and short period efficiency **(Harrison, Dusheiko & Zuckerman, 2009)**. This method has been proven to reduce HBV intrauterine infection in pregnant women **(Li *et al.,* 2004)** and to reduce the chance of HBV infection in newborns born of HBeAg and HBV DNA positive mothers. However, this prophylactic measure should be coupled with active immunization to ensure long term protection **(Lee *et al.,* 2006a; Zuckerman, 2007)**. Passive immunization is also used as a preventive measure for reinfection in liver transplant patients **(Shouval & Samuel, 2000)**, in post exposure prophylaxis in those who are non-responsive to the vaccine and after sexual exposure to the virus.

Active HBV immunization

An effective and safe vaccine, the first vaccine to prevent cancer, was developed and has been available for nearly 30 years. It was recommended by the WHO to be part of the EPI of every country by 1997. By 2007, 71 (89%) of the 193 states member of the WHO had introduced a hepatitis B vaccination program **(Chen, 2009)**. The vaccine was designed to reduce the incidence rate of infections and subsequent eradication of the virus. It was launched for the first time in Taiwan in 1984 **(Hsu *et al.,* 1988)** and then followed the establishment of HBV immunization programs in most of the countries with an HBsAg prevalence >8%. Even though mass immunization of children was shown to be quite effective in controlling the HBV epidemic in many regions of the world **(Luo, Li & Ruan,**

2012; Ni *et al.,* 2012), coverage is still not optimal in many countries where HBV prevalence is high.

First generation HBV vaccine was made from plasma-derived HBsAg purified from chronic carriers, but was replaced with the second generation recombinant vaccine made of HBsAg expressed in yeast **(Chen, 2009)**. The latter lacked the pre-S epitopes and thus only contained the small or major S protein. The recombinant third generation vaccines were improved by inserting the pre-S epitopes (pre-S1 and pre-S2) in addition to the S domain, which expressed the complete HBsAg protein. These vaccines although appearing to be more effective are not widely available. This vaccine was developed based on evidence suggesting that the pre-S epitopes could (1) enhance the HBs antibodies response and the cellular immune response, and (2) also prevent the docking of the viral particles to their receptors on hepatocytes **(Harrison, Dusheiko & Zuckerman, 2009)**. Guidelines were produced by the WHO on how to administer the vaccine. According to the EPI, newborn infants should receive their first dose within 24 hours of birth regardless of the maternal HBV or HIV status followed by two additional doses at one and six months of age. In case the child is born from an HBsAg positive mother, the vaccine should be combined with HBIG, inoculated at different sites. This measure is of great importance in areas of high HBV endemicity where HBV is transmitted from mother-to-child. This strategy, low of cost, was implemented for the reason that screening mothers for HBsAg was highly costly and developing countries could not afford such expenses. The need for booster doses in the routine immunization program has not yet been established **(Broderick & Jonas, 2004; WHO, 2010)**. To the high-risk populations such as young children or adolescents, individuals requiring blood transfusions or organ transplantation, dialysis patients, immunocompromised patients, health care workers or any individual exposed to blood products at his/her work place, injecting drug users, individuals with multiple sexual partners and HIV infected individuals, administration of the vaccine was also recommended **(Harrison, Dusheiko & Zuckerman, 2009; WHO, 2010)**. Though efficient in infants born from HBV mono-infected mothers, recent data demonstrated the low effectiveness and response of HBV immunization in infants born from HIV/HBV co-infected mothers **(Mutwa *et al.,* 2013)**.

In SSA, infant HBV vaccine is given six weeks postnatal rather than within the 24 hours of delivery **(Kiire, 1996; Mutwa *et al.,* 2013)**. Also, it was shown in SA that infants born of HIV mothers had less probability of receiving childhood vaccinations **(Ndirangu *et al.,***

2009). More data acquired from other studies on this topic led to the suggestion that HIV maternal status was associated with children's immunization status. Unfortunately, this leaves the HIV-exposed infant with a high viral load vulnerable to acquire an HBV infection with high chances of becoming a chronic carrier **(Healy, Gupta & Melvin, 2013)**.

2.9.2 HBV drug treatment

Hepatitis B viral replication is crucial in the progression of the hepatic disease; hence suppression of the virus to achieve HBeAg seroconversion, genome clearance and improvement of liver injury are the main objectives of antiviral agents. Long term goals include reducing the chances of developing liver cirrhosis and HCC and subsequent survival extension of the patients **(Puoti *et al.,* 2002)**. However, currently available ARTs do not achieve complete eradication of the cccDNA incorporated into the human genome in chronic infections **(Thio, 2009)**. As mentioned above, most cases of acute infection are resolved and patients do not need treatment. However this is not the case for chronic carriers and HIV/HBV co-infected patients who do not clear the infection. Therefore, the need of treatment for these groups becomes crucial to control the disease. To date, little data on the treatment of HBV in co-infected children are available but ARTs, summarized in Table 2.2, are available and recommendations on how to monitor CHB children have been established (See Figure 2.7). These ARTs include the interferon based therapy (interferon alpha) and the nucleos(t)ides analogues drugs lamivudine, adefovir dipivoxil, entecavir, telbivudine, and tenofovir **(Healy, Gupta and Melvin, 2013)**.

Interferon-based therapy

This includes interferon alpha-2b (IFNα-2b) and pegylated interferon, both shown to be effective in treating chronic patients. Yet, only IFNα-2b has been approved to be used in infants. Early seroconversion to anti-HBe and long-term seroconversion in patients with elevated ALT levels above baseline are observed in infants treated with IFNα-2b **(Kurbegov & Sokol, 2009; Paganelli, Stephenne & Sokal, 2012)**.

Using IFN over nucleos(t)ide drugs has the advantage of avoiding emergence of resistance **(Chang, 2007)**. However, this treatment is not without disadvantages. Patients on IFN treatment tend to develop undesired effects such as flu-like symptoms, fatigue, anorexia, weight loss, hair loss, hyperthyroidism, depression, and thrombocytopenia. However these side-effects are less severe in children than in adults **(Liaw & Chu, 2009)**.

Tenofovir

This therapy was recently approved for usage in the treatment of HBV mono-infected children older than 18 years of age and of HIV/HBV co-infected children older than 2 years of age with concerns of renal and bone toxicity if used at long-term in younger children. Like 3TC and entecavir, tenofovir (TNF) is not recommended to be used as monotherapy in HIV/HBV co-infected individuals to avoid emergence of resistance strains but has significant activity against 3TC-resistant strains **(Paganelli, Stephenne & Sokal, 2012; Healy, Gupta & Melvin, 2013)** hence it can be used as a rescue option in 3TC and ADV-resistant patients **(Van Bömmel *et al.,* 2006)**.

Lamivudine

Lamivudine (3TC), a product of 2, 3-dideoxy-3 –thiacytidine, is a nucleoside analogue with antiviral effects on both HBV and HIV and a safety profile in children. The drug competes for cytosine during viral DNA synthesis resulting in suppression of viral replication. The first published data on the effects of 3TC on chronic children showed a significant decrease of HBV DNA levels associated with HBeAg seroconversion after a year of treatment in patients with ALT levels at least two times above the baseline, but only 2% had an HBsAg seroconversion **(Jonas *et al.,* 2002)**. To children, a dosage of 3mg/kg per day with a maximum dose of 100 mg per day is recommended **(Lok & McMahon, 2007)**.

Although efficient, long term 3TC monotherapy have been associated with the occurrence of HBV mutations, often called YMDD motif mutations, leading to a decrease in virological response. 3TC-induced mutations happen to be occurring in the catalytic domain of the P gene, which, because of its overlap with the S gene result in changes in the ‘a’ determinant of the HBsAg protein **(Lok *et al.,* 2000; Harrison, Dusheiko & Zuckerman, 2009)**. This causes the formation of drug resistant strains which may have the potential to induce vaccine escape and nucleos(t)ide antiviral drug resistance **(Clements *et al.*, 2010)**. Incidence of YMDD mutations was found to increase with the duration of the therapy (19% after 12 months of treatment, 49% at 24 months and 70% after 48 months) **(Liaw *et al.,* 1999; Locarnini, 2004; Sokal *et al.,* 2006)**.

Adefovir dipivoxil

Antiviral effect of this purine analogue compound involves direct incorporation in competition with the substrate deoxyadenosine triphosphate (dATP) to cause chain

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termination to bring about the inhibition of both the viral RT enzyme and DNA polymerase **(Harrison, Dusheiko & Zuckerman, 2009)**. No report of drug-resistance was shown to arise from this therapy in children but resistance is reported in adult patients after five years of therapy. Nephrotoxicity is known as the main side effect of this drug if used at high doses **(Paganelli, Stephenne & Sokal, 2012)**. Studies have demonstrated that therapy with ADV is more efficient as compared to 3TC.

The first study working on the effect of ADV in chronic HBV children had its results reported in 2008. It demonstrated that children with chronic HBV and positive for HBeAg aged between 12 and 17 years showed significant virological response (HBV DNA <1000 copies/mL, seroconversion to anti-HBe, and normalization of ALT levels) as compared to children younger than 12 years **(Jonas *et al.,* 2008)**. ADV was then approved as an antiviral therapy for children from 12 years of age **(Paganelli, Stephenne & Sokal, 2012)**.

Entecavir

Entecavir, a carbocyclic analogue of 2-deoxyguanosine, was proven to be more efficient than both 3TC and ADV in decreasing HBV DNA levels and HBeAg seroconversion **(Lai *et al.,* 2006; Leung *et al.,* 2009)**. It is prescribed in children older than 16 years of age **(Paganelli, Stephenne & Sokal, 2012)**. Furthermore, entecavir possesses antiviral effects against HIV but because of its possibility to induce resistance; this therapy should not be used alone in HIV/HBV co-infected patients **(Healy, Gupta & Melvin, 2013)**.

The drug targets three specific events occurring during HBV replication: DNA polymerase priming, negative strand reverse transcription and synthesis of the HBV DNA (+)strand **(Harrison, Dusheiko & Zuckerman, 2009)**.

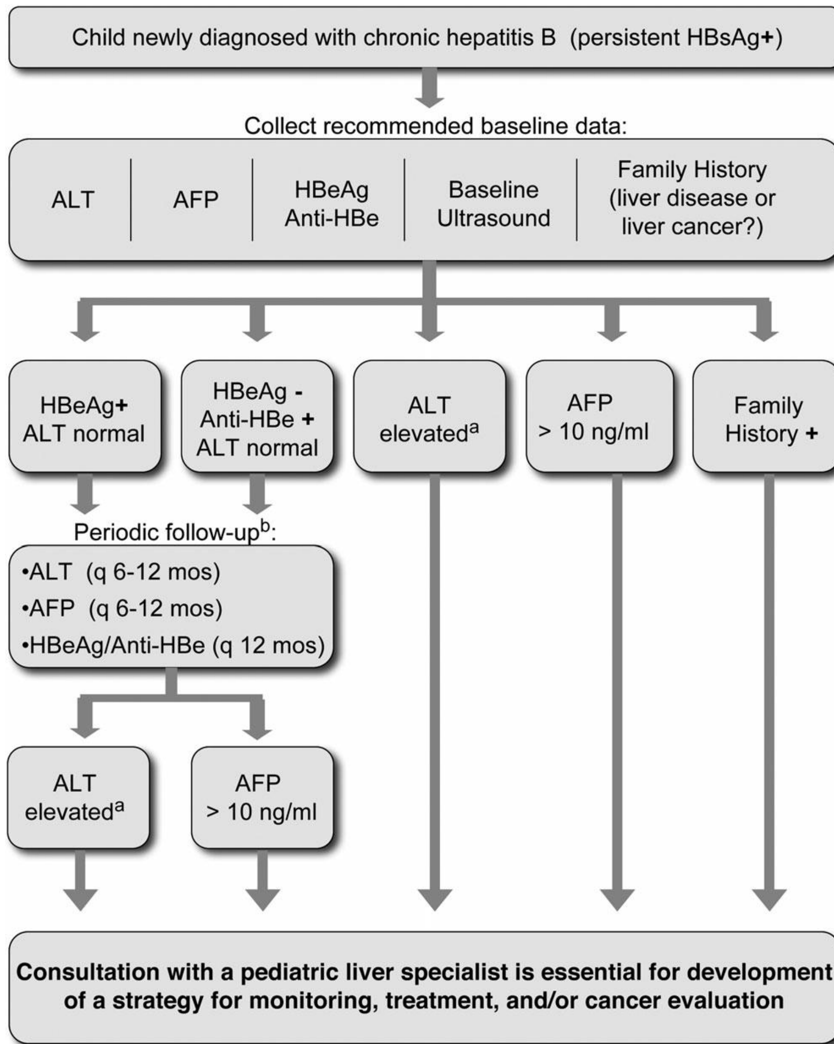

Figure 2. 7: Recommendations in the monitoring of chronic hepatitis B children. AFP: alpha-fetoprotein; ALT: alanine aminotransferase; [a]ALT level and white blood cell/platelet (WBC/Plt) count are generally included in the baseline evaluation as part of a hepatic function panel and complete blood count, respectively. [b]The ALT level should be considered elevated if greater than the testing laboratory ULN or _40 IU/L, whichever is lower (**Jonas, 2013 Reproduced with permission**).

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Table 2. 2: Antiviral therapies of chronic HBV in children **(Healy, Gupta & Melvin, 2013)**

Generic Name	Trade Name	US FDA labelled indications and ages	HIV-1 activity†	HBV resistance mutations
IFN-α-2B	Intron A®	HBV: ≥1 year	No	
Lamivudine	Epivir® Epivir–HBV®	HIV: birth and older HBV: ≥2 years	Yes	M204I/V L180M A181T/V V173L
Adefovir	Hepsera®	HBV: ≥12 years	No‡	N236T A181T/V
Telbivudine	Tyzeka®	HBV: ≥16 years	No	M204I A181T/V
Tenofovir	Viread®	HIV: ≥2 years HBV: ≥18 years	Yes	A194T§ A181T/V N236T
Entecavir	Baraclude®	HBV: ≥16 years	Yes¶	M204I/V# L180M T184I/A/G/L S202I/G I169T
IFN-α-2b	Intron A®	HBV: ≥1 year	No	
PegIFN-α-2a	Pegasys®	HBV: ≥18 years	No	

†Agent also has activity against HIV-1 and can lead to development of HIV resistance mutations if used as monotherapy in an HIV/HBV-coinfected individual. Additional antiretrovirals should always be included in a treatment regimen. ‡While adefovir does have minimal HIV-1 activity, development of HIV-1 resistance mutations has not been demonstrated. §Tenofovir mutations have not been clearly associated with decreased anti-HBV efficacy. ¶Entecavir does not have sufficient antiretroviral activity to be used for HIV-1 therapy. #Presence of several mutations is necessary to decrease efficacy for entecavir.

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CHAPTER 3: EPIDEMIOLOGY OF HEPATITIS B VIRUS INFECTION IN HIV-EXPOSED INFANTS

This chapter aims to describe the prevalence of active HBV infection in a cohort of children born of HIV-infected women. Although horizontal transmission is considered the common mode of HBV transmission in children in SSA, this chapter also reports on the rate of vertical transmission in HBV-infected children.

3.1 Introduction

The implementation of the HB vaccine in SA was based on the finding that horizontal transmission was the main HBV route of transmission in SA. Following the implementation of the HB vaccine, comparative studies have shown a decrease in the prevalence of HBV infection as evidenced by the decrease in chronic HBV carriage from 4.2% to 1.4% as described by Amponsah-Dacosta *et al.* **(Amponsah-Dacosta *et al.,* 2014)** and the increased immunity to the infection **(Hino *et al.,* 2001; Tsebe *et al.,* 2001)**. Its prevalence in HIV-infected adults has been extensively explored but little is known concerning the HIV-infected paediatric population.

A relatively high prevalence of HBsAg in HIV-infected pregnant women has been described in SA (**Burnett *et al.,* 2007; Andersson *et al.,* 2013; Thumbiran *et al.,* 2014**). A proportion of these women presented with high HBV viral load and HBeAg positivity hence were at high risk of transmitting the HB infection to their babies (**Andersson *et al.,* 2012; Hoffmann *et al.,* 2014**). HIV, on its own, has an impact on the natural history of HBV through a delay in HBeAg seroconversion. Additionally, the retrovirus was reported to reduce vaccine efficacy in babies born from HIV-infected women (**Zuccotti *et al.,* 1994; Arrazola *et al.,* 1995**). This is, thus, of concern in SA where active immunization is given post 6-weeks of birth. The infant is left in that perinatal period with a high risk of infection through vertical transmission.

Few studies have looked at the contribution of MTCT to the chronic HBV carriage in infants born of HIV-infected women. Therefore, in the current study, a large population of infants born of HIV-infected women in SA was investigated for HBV infection. The primary aim

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was to describe the prevalence of HBV infection in these HIV-exposed infants and the route of transmission of infection.

3.2 Materials and Methods

3.2.1 Ethical aspects

The study received ethical approval from the Health Research Ethics Committee (HREC) of the Faculty of Health Sciences, Stellenbosch University. The ethics reference number is: N10/04/115.

3.2.2 Sample and data collection

3.2.2.1 Study population

This a retrospective cohort study making use of a repository of banked samples from the P1041 study, part of International Maternal Pediatric Adolescent AIDS Clinical Trials Group (IMPAACT). P1041 was a multi-centred, Phase II-III randomized, double blind, placebo controlled trial to determine the efficacy of isoniazid (INH) in preventing tuberculosis (TB) disease and latent TB infection among South African infants with perinatal exposure to HIV. The enrolment of participants took place during the period of 13 December 2004 to 26 June 2006. Data collection on the infants took place at study entry (approximately 12 weeks from birth), and together with blood sampling at ~25 weeks of age (hence designated "Week 12 on study") and ~60 weeks of age (hence designated "Week 48 on study").

A total of 1354 infants born of HIV-infected mothers in Johannesburg, Durban and Cape Town, SA were enrolled for the PACTG P1041 study. Among these infants, 548 were HIV-infected and 806 were HIV-uninfected. Nine hundred and ninety six infants had plasma samples of at least 0.1 ml at "Week 48". Among the 916 infants with "Week 48" plasma samples, there were 388 infants with "baseline" samples ("Week 0" or "Week 12") from all study sites.

All plasma and serum samples available at "Week 48" were tested in order to determine the overall prevalence of HBV in P1041. This was done in order to ensure all cases infected by "week 48" were identified in the most cost effective way. All samples that tested positive for HBsAg and anti-HBc (total) were identified and contacted for follow up through the health

facilities where sample collection was performed. Blood samples were collected from the children and their mothers to identify whether the infection was of maternal origin. Samples collected at point of entry of the study i.e. 12 weeks of age of the infant and at "Week 12" i.e. 24 weeks of age of the infant were requested, using the patient ID number (PID), from the laboratories where study samples were stored in the cities of origin. The minimum volume requirement was 0.1 ml for both sample types.

3.2.2.2 Data collection

Data collection and blood sampling were performed together on infants at study entry. Blood sampling was performed at ~25 weeks of age designated "Week 12" on study and ~60 weeks of age designated "Week 48" on study.

3.2.3 Serology procedures

Samples collected at ~60 weeks of age ("Week 48" on study) were tested for HBV serological markers using the ARCHITECT system (Abbott Diagnostics, Delkenheim, Germany).

3.2.3.1 Validation of the ARCHITECT assays

The samples were of a low volume; therefore validation of low volume samples was required. The ARCHITECT i2000SR required a minimum volume of 400µL to execute the required serology assays. Samples were diluted to the required volume to complete the assays. The aims of the validation were (1) to determine the limit of detection and, (2) determine the appropriate diluent for the dilution of the samples. A total number of 37 HBsAg positive samples, previously tested neat on the AxSYM platform were retrieved from the NHLS diagnostic laboratory situated at the Division of Medical Virology. These samples were diluted to 1:10 with normal human plasma (NHP). The latter was received from the Western Cape Province Blood Transfusion Service (WPBTS) and known to be HBV, HIV and HCV negative on serological and molecular assays. The neat and diluted samples were tested on the ARCHITECT for HBV markers including HBsAg, anti-HBs and anti-HBc (total) in duplicates on two different days. The dilutions and neat samples were tested on ARCHITECT and results were compared to each other.

Following individual testing, pooled sample testing was performed. A series of samples with different HBsAg profiles were screened. (1)Three samples (STY5017776, STY5018481 and

STY5051715) with HBsAg S/N > 200 on AxSYM and anti-HBc (total) S/CO >10 on ARCHIECT were pooled with 200µL of each sample. Two separate 10-fold serial dilutions from 1:10 to 1:1,000,000 of the pooled specimen using NHP and 50% fetal bovine serum/ phosphate buffered saline (FCS/PBS) were performed. Additionally, a 1:10 dilution of each of the NHP serial dilutions was made in 50% FCS/PBS. The dilutions and neat pools were tested on the AxSYM and ARCHITECT for HBsAg, anti-HBs and anti-HBc (total). (2) Additionally, a sample with a known HBsAg titre of 431,000 IU/ml (quantification done at the Public Health England (PHE)) was also diluted with NHP and 50%FCS/PBS at a range of 1:10 to 1:10,000,000 and tested on both the AxSYM and ARCHITECT. (3) One sample (sample 4) with anti-HBs titre >1,000mIU/mL on the ARCHITECT was diluted from 1:10 to 1:10,000 using NHP and 50%FCS/PBS. Furthermore, 1:10 dilutions of each of the same NHP dilutions were made using 50% FCS/PBS as the diluent. These dilutions, summarized in Table 3.1, were tested on the ARCHITECT.

Table 3. 1: Summary of sample dilutions

	Pooled sample			Anti-HBs> 1000mIU/mL		
	NHP	50% FCS/PBS	10% of NHP dil in 50% FCS/PBS	NHP	50% FCS/PBS	10% of NHP dil in 50% FCS/PBS
Dilution	1:10	1:10	1:10	1:10	1:10	1:10
	1:100	1:100	1:100	1:100	1:100	1:100
	1:1,000	1:1,000	1:1,000	1:1,000	1:1,000	1:1,000
	1:10,000	1:10,000	1:10,000	1:10,000	1:10,000	1:10,000
	1:100,000	1:100,000	1:100,000	1:100,000	1:100,000	1:100,000
	1:1,000,000	1:1,000,000	1:1,000,000	1:1,000,000	1:1,000,000	1:1,000,000
	1:10,000,000	1:10,000,000	1:10,000,000	1:10,000,000	1:10,000,000	
	1:100,000,000	1:100,000,000	1:100,000,000	1:100,000,000	1:100,000,000	

3.2.3.2 Validation of the ARCHITECT results

Validation of the HBsAg assay using diluted samples

As previously mentioned in Section 3.2.3.1, 37 samples were retrieved from the NHLS diagnostic laboratory and were diluted in NHP for the testing of HBV markers being HBsAg, anti-HBs, anti-HBc (total) on the ARCHITECT. The neat samples were tested in parallel for comparison. Table 3.2 shows a summary of the results.

Of these 37 neat samples, 11 samples were positive for HBsAg, 6 were negative and 21 samples were low positive on the AxSYM platform. Out of the 11 HBsAg positive

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specimens, 9 were found positive and 2 negative when tested neat and at 1:10 dilution during both runs on the ARCHITECT. The 2 samples had the lowest positive S/N values as compared to the 9 other positive samples on the AxSYM. Only 14 of the 21 low positive samples tested positive on the ARCHITECT. Eight of those 14 positives specimens were re-tested and were repeatedly positive. The remaining 6 could not be re-tested due to low sample volume.

The 21 low positive samples detected on the AxSYM were neutralized to confirm HBsAg positivity. As represented on Table 3.3, 13 of these samples were confirmed as true HBsAg positive and 7 were negative, indicative of likely false positive results in these samples. One sample had an inconclusive result.

Table 3. 2: ARCHITECT validation of HBsAg testing on 1:10 diluted samples

	Run 1						Run 2			
Lab Number	AxSYM (S/N)		Architect (neat) (S/CO)		Architect 10x dilution (S/CO)		Architect (neat) (S/CO)		Architect 10x dilution (S/CO)	
STY4768186	2.26	LP	1.54	POS	0.47	NEG	ins	ins	ins	ins
STY4809997	4.35	LP	0.36	NEG	0.28	NEG	0.25	NEG	0.38	NEG
STY4827077	4.05	LP	10.39	POS	1.99	POS	ins	ins	ins	ins
STY4857857	2.54	LP	1.23	POS	0.35	NEG	ins	ins	ins	ins
STY4870304	5.28	LP	12.77	POS	2.10	POS	ins	ins	ins	ins
STY4898134	3.03	LP	0.23	NEG	0.36	NEG	ins	ins	ins	ins
STY4904507	2.99	LP	4.34	POS	0.93	NEG	ins	ins	ins	ins
STY5017776	270.9	POS	5234.05	POS	5674.02	POS	5293.96	POS	4749.55	POS
STY5018481	258.36	POS	4537.47	POS	4439.07	POS	4838	POS	4996.25	POS
STY5023527	19.65	POS	0.23	NEG	0.26	NEG	0.25	NEG	0.2	NEG
STY5023934	NA	POS	29.83	POS	5.48	POS	27.55	POS	5.43	POS
STY5028983	15.12	POS	0.28	NEG	0.19	NEG	0.22	NEG	0.23	NEG
STY5030810	28.1	POS	88.90	POS	13.49	POS	90.61	POS	14.1	POS
STY5051715	246.09	POS	4792.26	POS	1603.23	POS	4880.53	POS	1733.07	POS
STY5051766	86.8	POS	554.25	POS	87.16	POS	554.25	POS	87.16	POS
STY5056302	83.05	POS	499.35	POS	72.27	POS	643.09	POS	93.1	POS
STY5085522	88.79	POS	432.48	POS	72.15	POS	374.03	POS	58.55	POS
STY5020925	NA	POS	21.57	POS	3.78	POS	27.08	POS	4.21	POS
NHP	NA	NEG	0.27	NEG	NA	NA	0.3	NEG	NA	NA
STV0544934	3.02	LP	3.56	POS	0.82	NEG	3.35	POS	0.81	NEG
STV0543722	3.38	LP	3.79	POS	0.74	NEG	3.64	POS	0.78	NEG
STV0543515	2.24	LP	0.22	NEG	0.25	NEG	0.26	NEG	0.21	NEG
STV0551517	6.81	LP	15.82	POS	2.73	POS	66.56	POS	2.84	POS
STV0540956	5.77	LP	5.11	POS	0.86	NEG	NA	NA	0.84	NEG
STV0549516	2.3	LP	0.24	NEG	0.22	NEG	0.38	NEG	0.55	NEG
STV0546706	3.81	LP	0.59	NEG	0.27	NEG	0.65	NEG	0.28	NEG
STV0547150	4.02	LP	6.10	POS	0.79	NEG	5.49	POS	0.79	NEG
STV0549277	5.95	LP	11.51	POS	1.11	POS	8.91	POS	1.08	POS
STV0556621	2.24	LP	0.29	NEG	0.24	NEG	0.3	NEG	0.24	NEG
STV0556546	3.57	LP	80.07	POS	7.18	POS	78.47	POS	7.3	POS
STV0556462	8.13	LP	0.24	NEG	0.19	NEG	0.2	NEG	0.24	NEG
STV0554000	3.18	LP	10.72	POS	1.25	POS	10.93	POS	1.16	POS
STV0566338	4.39	LP	4.21	POS	0.62	NEG	3.94	POS	0.61	NEG

LP: Low positive
POS: Positive
NEG: Negative
Ins: Insufficient
NA: Not Available

Table 3. 3: Neutralization assay of low HBsAg positive samples

			Neutralization (neat sample)		
Lab number	AxSYM (S/N)		NHP OD	Anti-S OD	Result
STY4768186	2.26	LP	0.070	0.060	NEG
STY4809997	4.35	LP	0.053	0.054	NEG
STY4827077	4.05	LP	0.278	0.059	POS
STY4857857	2.54	LP	0.116	0.059	POS
STY4870304	5.28	LP	0.320	0.072	POS
STY4898134	3.03	LP	0.052	0.057	NEG
STY4904507	2.99	LP	0.263	0.111	POS
STV0544934	3.02	LP	0.326	0.066	POS
STV0543722	3.38	LP	0.415	0.170	POS
STV0543515	2.24	LP	0.053	0.056	NEG
STV0551517	6.81	LP	1.422	0.285	POS
STV0540956	5.77	LP	0.235	0.094	POS
STV0549516	2.3	LP	0.115	0.089	POS
STV0546706	3.81	LP	0.056	0.054	NEG
STV0547150	4.02	LP	0.164	0.061	POS
STV0549277	5.95	LP	0.189	0.066	POS
STV0556621	2.24	LP	0.054	0.057	NEG
STV0556546	3.57	LP	1.411	0.091	POS
STV0556462	8.13	LP	0.054	0.056	NEG
STV0554000	3.18	LP	0.231	0.060	POS
STV0566338	4.39	LP	0.169	0.072	POS

NHP: Normal Human Plasma
OD: Optical density
LP: Low positive

Further testing on diluted samples was performed using (1) three pooled samples and, (2) a sample with known HBsAg titre. Tables 3.4 and 3.5 show the results obtained from those assays. (1) On the ARCHITECT, HBsAg positivity was detected to a dilution of 1:100,000 with 50% FCS/PBS. However, positivity was lost at a dilution of 1:10,000 with NHP and 10% of NHP diluted in 50% FCS/PBS. On the other hand, HBsAg positivity is not lost until a dilution 1:1,000,000 for all three dilutions on the AxSYM. At further dilutions (1:10,000,000 and 1:100,000,000), the system gave an error code for unknown reasons. These results are graphically confirmed on Figure 3.1. The latter also shows the cut off value of both platforms ARCHITECT and AxSYM being 1 and 2, respectively. All points situated above those cut off values are considered positive and the ones below are considered negative. The graph representing results on the ARCHITECT shows no difference in the behaviour of the results obtained from diluting the pool sample with either NHP or 50% FCS/PBS. However the 10% NHP diluted in 50% FCS/PBS dilution affected the results adversely hence was not an adequate method of dilution for the samples. On the other hand, a difference is observed

between the dilution with NHP and 50% FCS/PBS on the AxSYM graph and result with the 10% NHP dilution in 50% FCS/PBS is similar to what is observed on the ARCHITECT. (2) The sample with known HBsAg titre appears to be still detected as positive until diluted out to 1:10,000,000 dilution with both NHP and 50% FCS/PBS, the highest S/CO values being obtained with NHP. This observation is graphically depicted on Figure 3.2.

Table 3. 4: HBsAg testing on Architect (S/CO values shown)

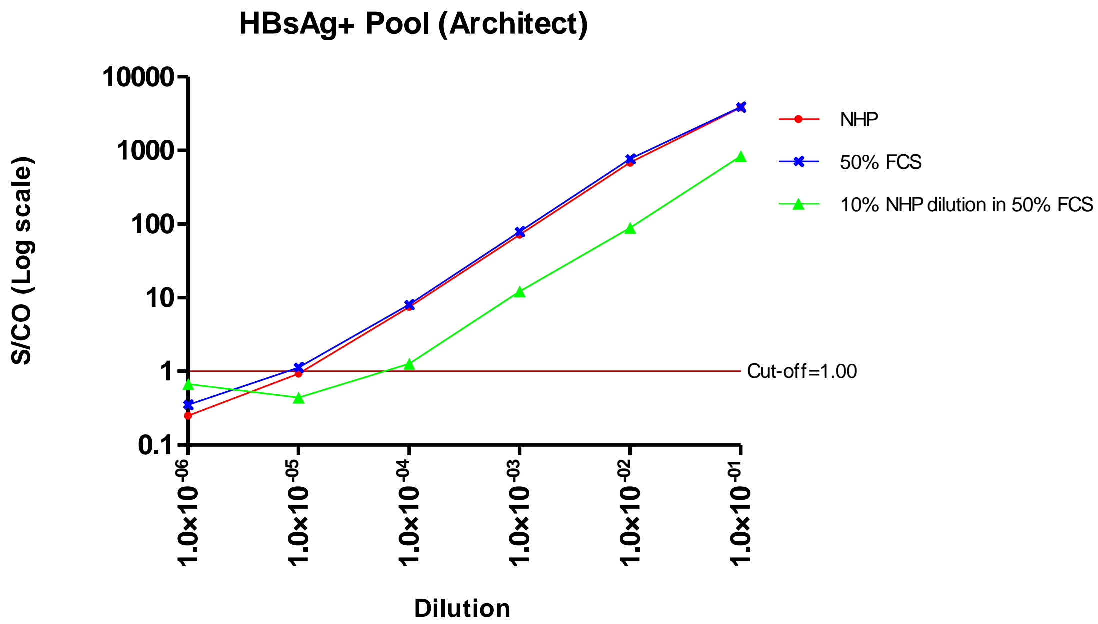
	High HBsAg+ (431 000 IU/ml)		Pooled sample		
Dilution	50% FCS/PBS	NHP	NHP	50% FCS/PBS	10% of NHP dil in 50% FCS/PBS
1:10	3349.00	2894.62	3839.81	3892.48	840.56
1:100	6612.18	6505.56	681.89	766.56	88.48
1:1,000	5350.76	6332.22	71.80	78.86	12.14
1:10,000	1118.02	1946.28	7.49	8.09	1.27
1:100,000	121.17	239.54	0.93	1.13	0.44
1:1,000,000	10.74	23.21	0.25	0.35	0.67
1:10,000,000	2.50	2.52	N/A	N/A	N/A
1:100,000,000	0.53	0.47	N/A	N/A	N/A

Neat high HBsAg+: 590.43 S/CO
Neat Pool: 5740.72 S/CO
Dil: Diluted

Table 3. 5: HBsAg testing on the AxSYM (S/N values shown)

	High HBsAg sample (431 000 IU/ml)		Pooled sample		
Dilution	50% FCS/PBS	NHP	NHP	50% FCS/PBS	10% of NHP dil in 50% FCS/PBS
1:10	301.45	433.45	245.24	211.13	123.56
1:100	427.13	569.06	130.66	118.81	24.73
1:1,000	363.53	459.24	24.61	26.40	6.31
1:10,000	237.98	265.83	10.29	4.08	1.98
1:100,000	81.48	73.81	1.15	1.76	1.22
1:1,000,000	60.02	9.21	1.34	1.27	1.27
1:10,000,000	8.91	2.09	N/A	N/A	N/A
1:100,000,000	1.98	1.34	N/A	N/A	N/A

Neat high HBsAg+: insufficient
Neat pool: 279.25 S/CO
Dil: Diluted



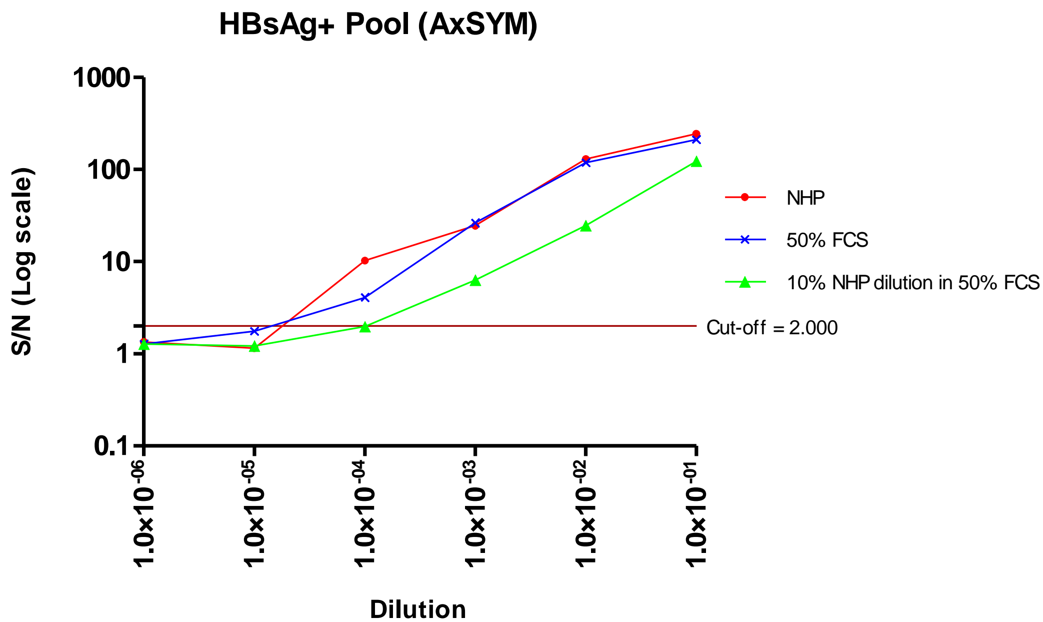
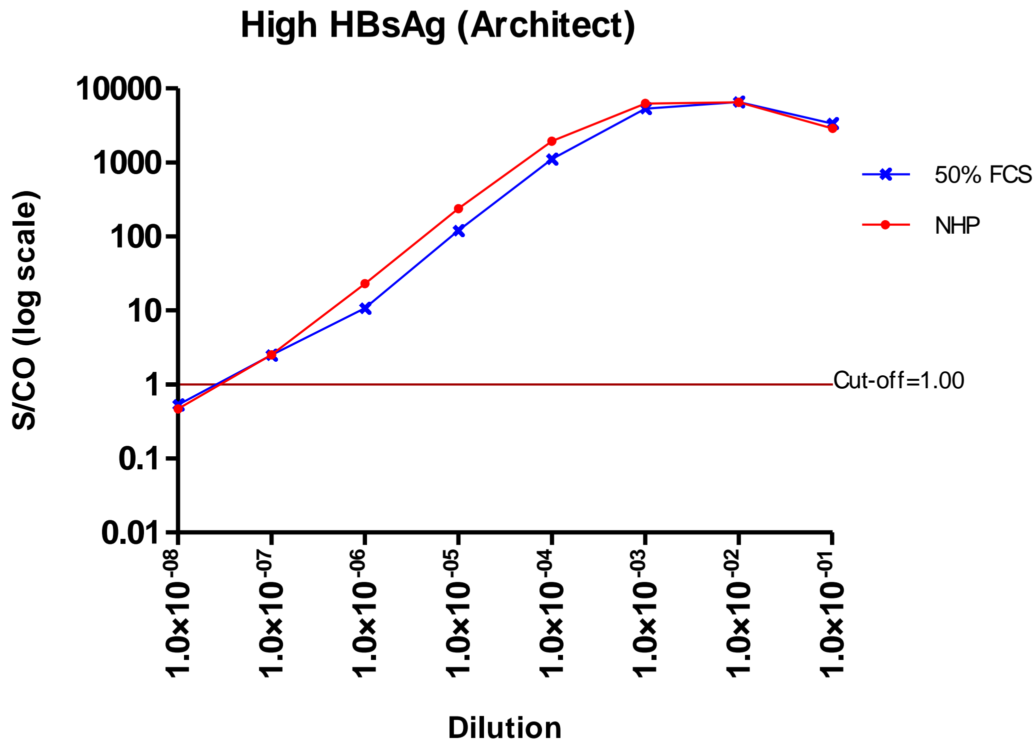


Figure 3. 1: Plot of HBsAg positive pool on the ARCHITECT and AxSYM. The X axis represents the S/CO and S/N readings respectively and the Y axis represent the dilutions. All points situated above the cut-off line are considered as positive whereas points situated below are considered as negative. The three samples that were pooled had HBsAg S/N>200 on the AxSYM.



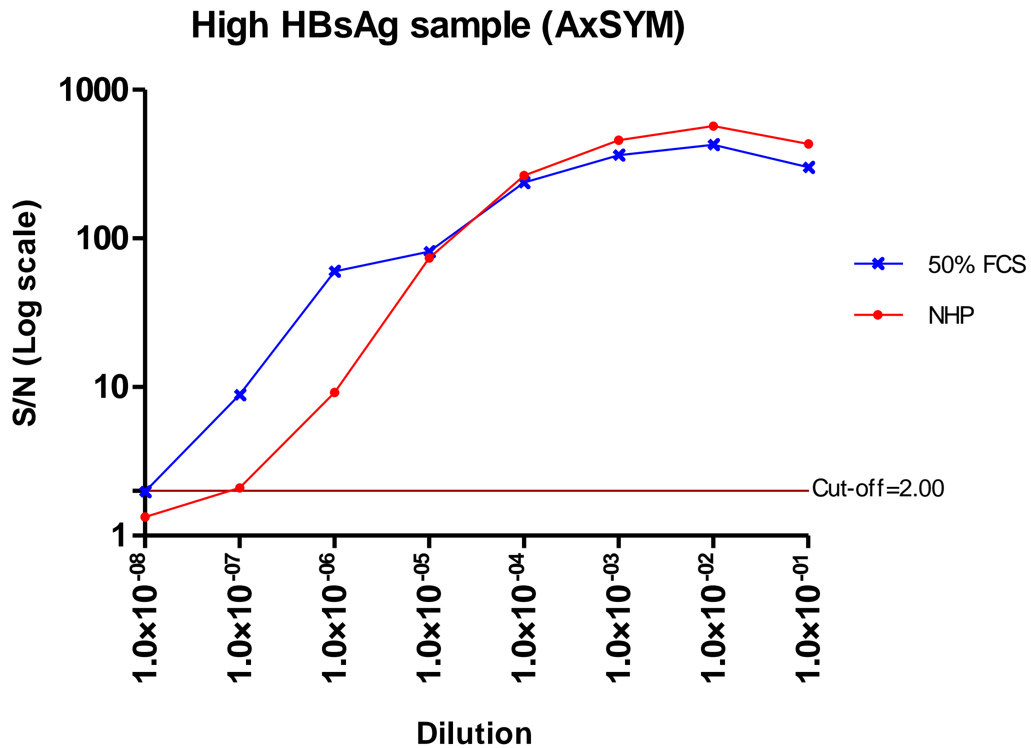


Figure 3. 2: Plot of high HBsAg positive sample on the ARCHITECT and AxSYM. Sample quantified at 431,000 IU/ml at PHE. The X axis represents the S/CO and S/N readings respectively and the Y axis represent the dilutions. All points situated above the cut-off line are considered as positive whereas points situated below are considered as negative.

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Validation of the Anti-HBs assay using diluted samples

The samples used for the HBsAg screening were also used for the anti-HBs assay. As Table 3.6 below illustrates, 5 neat samples were found anti-HBs positive on the ARCHITECT. Of these 5 samples, 4 had low S/CO values as compared to 1 sample with a high S/CO value. The latter was also found anti-HBs positive at a 1:10 dilution. Three of the four samples with low S/CO values were insufficient for testing at the 1:10 dilution and the other sample was found anti-HBs negative when diluted to a 1:10 concentration. Same results were observed on the second run. NHP tested negative for this marker on both platforms, confirming the true results of these diluted samples.

Table 3.7 and Figure 3.3 illustrate detailed results obtained on the dilutions of Sample 4 with anti-HBs>1.000mIU/mL on the ARCHITECT. Table 3.7 shows that at dilution 1:1,000,000, quantification is still possible but positivity is detected up to 1:100 with NHP. With 50% FCS/PBS, the same observations were made although the values obtained with NHP are more accurate. With the 10% NHP diluted in 50% FCS/PBS, anti-HBs positivity is lost after the 1:10 dilution. Beyond that point, the sample appeared anti-HBs negative. These observations are confirmed on Figure 3.3 showing the cut off value of the assay and the behaviour of the assays using diluted samples. The graph also shows no linearity with the data plotted.

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Table 3. 6: ARCHITECT validation of anti-HBs testing on 1:10 diluted samples

		Run 1				Run 2			
Lab Number	AxSYM	Architect (neat)		Architect 10x dilution		Architect (neat)		Architect 10x dilution	
STY4768186	NT	17.73	POS	3.61	NEG	ins		ins	
STY4809997	NT	0.00	NEG	0.99	NEG	0.00	NEG	1.40	NEG
STY4827077	NEG	3.05	NEG	1.43	NEG	ins		ins	
STY4857857	NT	22.63	POS	5.51	NEG	ins		ins	
STY4870304	NT	14.27	POS	1.20	NEG	ins		ins	
STY4898134	NEG	0.10	NEG	1.00	NEG	ins		ins	
STY4904507	NEG	0.89	NEG	1.23	NEG	ins		ins	
STY5017776	NEG	0.56	NEG	0.26	NEG	0.41	NEG	8.19	NEG
STY5018481	NT	0.13	NEG	0.23	NEG	0.00	NEG	0.00	NEG
STY5023527	NEG	0.00	NEG	1.17	NEG	0.00	NEG	1.99	NEG
STY5023934	NT	0.00	NEG	1.15	NEG	0.00	NEG	0.85	NEG
STY5028983	NT	0.00	NEG	1.16	NEG	0.00	NEG	0.98	NEG
STY5030810	NEG	0.00	NEG	1.11	NEG	0.00	NEG	0.25	NEG
STY5051715	NT	0.02	NEG	0.69	NEG	0.00	NEG	1.04	NEG
STY5051766	NEG	0.04	NEG	1.14	NEG	ins		ins	
STY5056302	NT	0.00	NEG	1.03	NEG	0.00	NEG	0.48	NEG
STY5085522	NEG	5.78	NEG	2.18	NEG	5.10	NEG	1.18	NEG
STY5020925	NEG	0.00	NEG	1.10	NEG	0.00	NEG	1.24	NEG
NHP	NEG	1.48	NEG	NA	NA	NA	NA	NA	NA
STV0544934	NEG	0.00	NEG	0.96	NEG	0.00	NEG	0.95	NEG
STV0543722	NT	15.47	POS	2.70	NEG	14.31	POS	2.85	NEG
STV0543515	NEG	4.03	NEG	1.31	NEG	4.27	NEG	1.19	NEG
STV0551517	NEG	8.80	NEG	1.92	NEG	9.60	NEG	2.08	NEG
STV0540956	NEG	0.00	NEG	0.97	NEG	0.07	NEG	0.95	NEG
STV0549516	NT	7.15	NEG	1.79	NEG	6.94	NEG	1.70	NEG
STV0546706	NEG	0.00	NEG	0.99	NEG	0.00	NEG	0.93	NEG
STV0547150	NT	1.72	NEG	1.16	NEG	1.65	NEG	1.14	NEG
STV0549277	NEG	0.98	NEG	1.23	NEG	1.01	NEG	1.34	NEG
STV0556621	NEG	5.82	NEG	1.50	NEG	6.48	NEG	1.54	NEG
STV0556546	NEG	0.00	NEG	0.95	NEG	0.00	NEG	0.93	NEG
STV0556462	NT	668.43	POS	60.50	POS	644.84	POS	61.28	POS
STV0554000	NT	0.00	NEG	0.95	NEG	0.00	NEG	1.02	NEG
STV0566338	NT	0.23	NEG	1.02	NEG	0.29	NEG	1.05	NEG

NT: Not tested
POS: Positive
NEG: Negative
Ins: Insufficient
NA: Not Available

Table 3. 7: Anti-HBs testing of Sample 4 on Architect

	Sample 4 (>1000 mIU/ml)		
Dilution	NHP	50% FCS/PBS	10% of NHP in 50% FCS/PBS
1:10	555.91	546.01	49.62
1:100	75.49	66.42	8.53
1:1,000	9.70	7.60	1.98
1:10,000	2.06	0.68	1.09
1:100,000	1.10	0.00	1.10
1:1,000,000	1.08	0.00	0.97

NHP: 1.09
50% FCS/PBS: 0.00

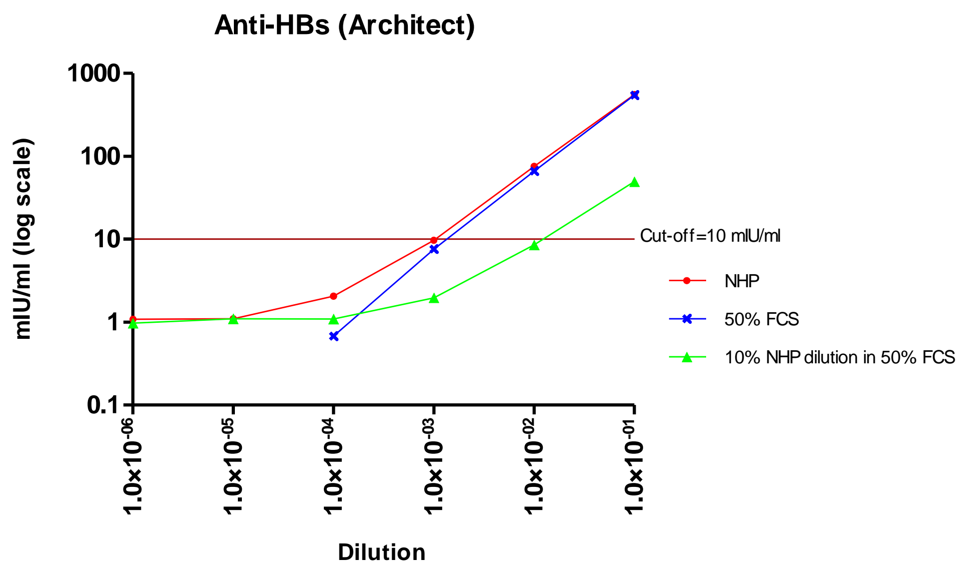

Figure 3. 3: Anti-HBs testing on the ARCHITECT. The neat sample used to make the dilutions had anti-HBs >1,000mIU/mL on the ARCHITECT. The X axis represents anti-HBs titres calculated for each dilution which are represented on the Y axis.

Validation of the anti-HBc (total) assay using diluted samples

Seventeen neat samples which tested positive for anti-HBc (total) on the AxSYM also tested positive on the ARCHITECT and at the 1:10 concentration with the exception of one sample. The latter had low anti-HBc (total) positivity when tested neat.

An additional 13 specimens not tested on the AxSYM, were tested neat on the ARCHITECT. Ten of these samples tested anti-HBc (total) positive and three were anti-HBc (total) negative on the ARCHIECT. Among the 10 anti-HBc (total) positive samples, two had low anti-HBc (total) S/N values as compared to the eight other samples. At a 1:10 dilution, these two

samples tested anti-HBc (total) negative. The other 8 specimens were still positive at the 1:10 dilution. These observations are detailed on Table 3.8 below.

Table 3. 8: ARCHITECT validation of anti-HBc (total) testing on 1:10 diluted samples

		Run 1				Run 2			
Lab number	AxSYM	Architect (neat)		Architect 10x dil		Architect (neat)		Architect 10x dil	
STY4768186	NT	2.50	POS	0.63	NEG	ins		ins	
STY4809997	NT	0.14	NEG	0.29	NEG	0.13	NEG	0.27	NEG
STY4827077	POS	12.72	POS	8.72	POS	ins		ins	
STY4857857	NT	14.07	POS	11.96	POS	ins		ins	
STY4870304	NT	14.27	POS	14.48	POS	ins		ins	
STY4898134	NEG	0.09	NEG	0.29	NEG	ins		ins	
STY4904507	POS	10.73	POS	10.76	POS	ins		ins	
STY5017776	POS	13.79	POS	14.34	POS	13.97	POS	13.28	POS
STY5018481	NT	13.27	POS	14.35	POS	13.90	POS	13.54	POS
*STY5023527	NEG	0.12	NEG	0.29	NEG	0.20	NEG	0.39	NEG
STY5023934	POS	14.41	POS	13.50	POS	13.32	POS	12.54	POS
STY5028983	NT	0.35	NEG	0.36	NEG	0.32	NEG	0.51	NEG
STY5030810	POS	10.69	POS	6.45	POS	10.63	POS	6.11	POS
STY5051715	NT	14.88	POS	14.66	POS	14.31	POS	13.58	POS
STY5051766	POS	12.94	POS	13.18	POS	ins		ins	
STY5056302	NT	11.27	POS	4.10	POS	10.89	POS	3.97	POS
STY5085522	POS	14.56	POS	15.01	POS	13.58	POS	13.97	POS
STY5020925	POS	12.92	POS	13.01	POS	12.62	POS	12.00	POS
NHP	NEG	0.36	NEG	NA	NA	NA	NA	NA	NA
STV0544934	POS	10.48	POS	9.79	POS	10.57	POS	9.81	POS
STV0543722	NT	9.54	POS	8.31	POS	9.82	POS	8.71	POS
STV0543515	POS	2.05	POS	0.43	NEG	1.94	POS	0.42	NEG
STV0551517	POS	10.02	POS	9.82	POS	9.86	POS	9.59	POS
STV0540956	POS	11.71	POS	11.45	POS	11.91	POS	10.80	POS
STV0549516	POS	7.85	POS	1.39	POS	7.87	POS	1.42	POS
STV0546706	POS	9.84	POS	2.58	POS	10.17	POS	2.66	POS
STV0547150	NT	7.50	POS	1.20	POS	7.05	POS	1.19	POS
STV0549277	POS	7.31	POS	1.14	POS	7.28	POS	1.16	POS
STV0556621	POS	10.21	POS	4.15	POS	10.37	POS	4.10	POS
STV0556546	POS	11.83	POS	10.18	POS	12.05	POS	9.96	POS
STV0556462	NT	1.06	NEG	0.35	NEG	1.17	POS	0.34	NEG
STV0554000	NT	3.23	POS	0.59	NEG	3.17	POS	0.62	NEG
STV0566338	NT	12.60	POS	11.92	POS	13.26	POS	12.41	POS

NT: Not tested
POS: Positive
NEG: Negative
Ins: Insufficient
NA: Not Available
Dil: Diluted

The neat pooled sample had an S/CO value of 13.95 on the ARCHITECT and an S/N value of 0.080 on the AxSYM. These two values have two different interpretations given the difference in nature of the assay on each platform. On the AxSYM, the assay is competitive hence all values below the cut off value of 1.00, are considered positive for anti-HBc (total). The opposite is applied on the ARCHITECT on which the assay is not competitive, all values above the cut off value of 1.00 are considered positive.

On ARCHITECT, anti-HBc (total) in the pooled sample was detected up to a dilution of 1:10,000 when using either NHP or 50% FCS/PBS as the diluent. However S/CO values are higher when using NHP. With the 10% of NHP diluted in 50% FCS/PBS, positivity was not detected beyond 1:1,000. The same results are observed on the AxSYM. These are illustrated below on Tables 3.9 and 3.10 and represented graphically on Figure 3.4.

The loss of some sensitivity was not considered to be important, as we wanted to avoid detecting maternal anti-HBc (total) and limit our ability to detect positive anti-HBc (total) in those infants who were exposed to HBV i.e. truly anti-HBc positive.

Table 3. 9: Anti-HBc (total) testing on Architect (S/CO values)

	Anti-HBc (total)+ Pool		
Dilution	NHP	50% FCS/PBS	10% of NHP diluted in 50% FCS/PBS
1:10	13.31	12.51	11.45
1:100	12.65	11.74	9.15
1:1,000	10.16	9.12	2.20
1:10,000	2.40	1.83	0.26
1:100,000	0.50	0.16	0.10
1:1,000,000	0.32	0.02	0.15

Neat pool: 13.95 S/CO

Table 3. 10: Anti-HBc (total) testing on AxSYM (S/N values)

	Anti-HBc (total)+ Pool		
Dilution	NHP	50% FCS/PBS	10% of NHP diluted in 50% FCS/PBS
1:10	0.085	0.076	0.074
1:100	0.092	0.100	0.071
1:1,000	0.094	0.096	0.181
1:10,000	0.208	0.172	1.466
1:100,000	1.070	1.364	1.918
1:1,000,000	1.207	2.091	1.915

Neat pool: 0.080 S/N

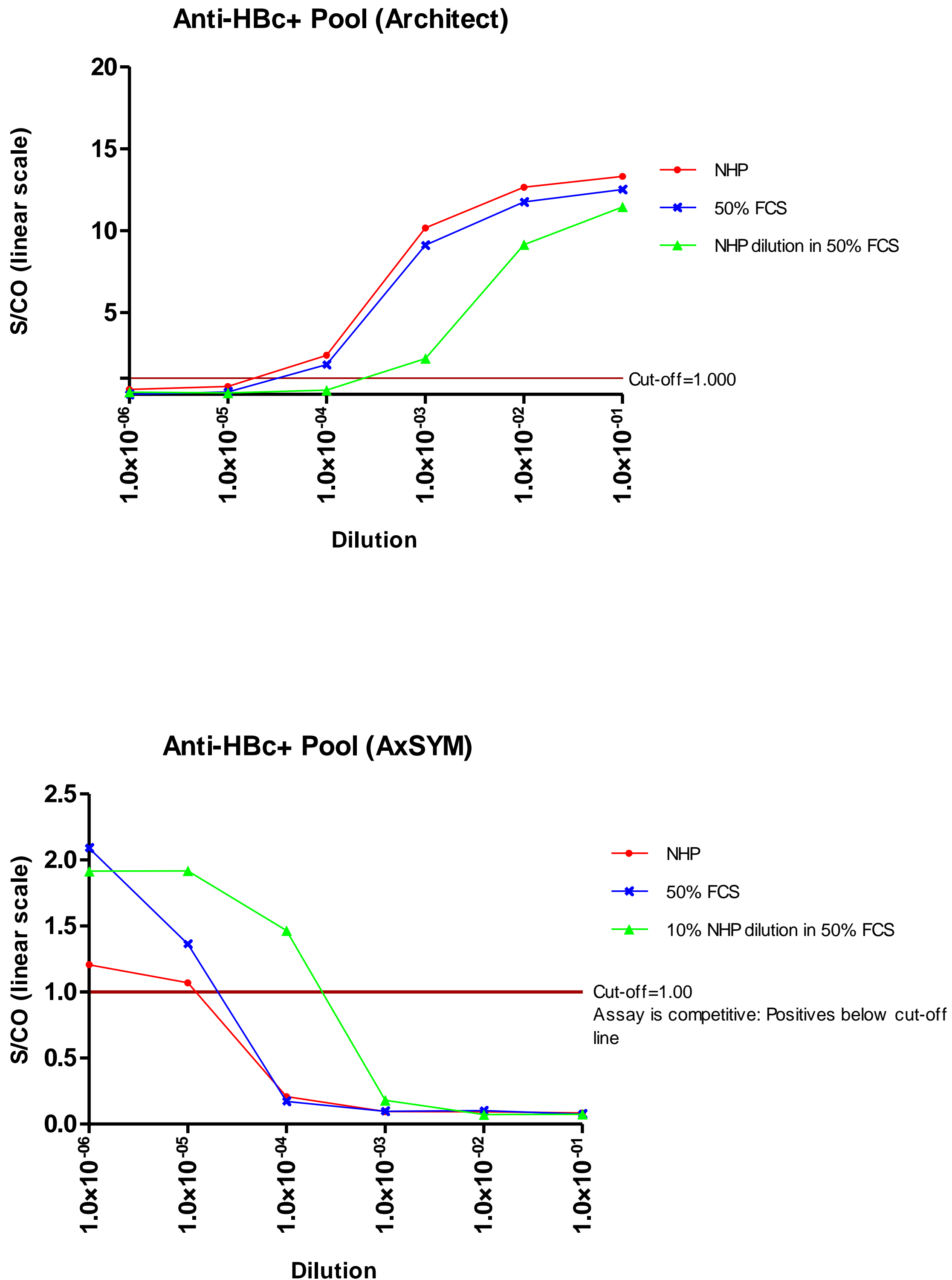

Figure 3. 4: Plot of results of Anti-HBc (total) positive pool testing on the Architect and AxSYM. The three samples used to make the pool had S/CO>10 on ARCHITECT. The X axis represents the S/CO and S/N readings respectively and the Y axis represent the dilutions. On the ARCHITECT curve, all points above the cut-off line are positive samples whereas the points below are negative samples. The opposite is seen on the AxSYM curve because the assay is competitive on the AxSYM.

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3.2.3.3 HBsAg testing

Principle

The HBsAg assay on the ARCHITECT system is a one-step chemiluminescent microparticle immunoassay (CMIA) for the qualitative detection of HBsAg in human serum or plasma. The sample is incubated at first with anti-HBs coated paramagnetic microparticles and anti-HBs acridinium-labeled conjugate, forming a reaction mixture. Incubation allows HBsAg present in the sample to bind to the anti-HBs. Then, the reaction mixture is washed to remove unbound particles. Following these steps, the pre-trigger (Hydrogen peroxide) and trigger (Sodium hydroxide) solutions used here as substrates, are added to the reaction mixture. These two solutions, as the name suggests, trigger a chemiluminescent signal measured as relative light unit (RLUs) and directly proportional to the amount of HBsAg present in the specimen. This reaction is initiated by the addition of the pre-trigger solution which causes the release of the label, being acridinium, from the microparticles into solution. A magnetic separation of the microparticles from the label then occurs. The microparticles are attracted to the inner walls of the reaction vessels and the label remains in solution. Finally, the trigger solution containing base is added to the reaction causing the chemiluminescence **(Wild, 2005)**. The chemiluminescent signal generated in the reaction is compared to a cut-off value determined from an active ARCHITECT HBsAg calibration. If the signal generated by the sample is lower than the cut-off value, then the sample is considered negative for HBsAg and if superior or equal to the cut-off value, the sample is considered positive for HBsAg.

3.2.3.4 Anti-HBs testing

Principle

This is a CMIA for the quantitation of anti-HBs present in serum and plasma of human origin completed in two steps on the ARCHITECT system. Anti-HBs present in the sample binds to recombinant HBsAg (rHBsAg) coated on the paramagnetic microparticles that were incubated with the sample during the first step of the assay. The reaction mixture is washed then incubated with acridinium-labeled rHBsAg conjugate. Following another wash step, the pre-trigger and trigger solutions are added to the reaction mixture for the quantification of anti-HBsAg present in the specimen. The chemiluminescent signal, generated by the pre-trigger and trigger solutions, is directly proportional to the quantity of anti-HBsAg present.

The concentration of anti-HBs in the sample is determined using a previously generated anti-HBs calibration curve generated by the ARCHITECT instrument. According to the criteria of the ARCHITECT anti-HBs assay, a sample is considered positive for anti-HBs if the concentration of anti-HBs calculated is greater or equal to 10,0 mIU/mL. The upper limit of detection of the assay was determined to be 1000 IU/mL **(Kim *et al.,* 2007)**. The lower limit of detection of the assay using dilution was determined during the validation of the assay (See Section 3.2.3.2).

3.2.3.5 Anti-HBc (total) testing

Principle

The anti-HBc (total) assay on the ARCHITECT is also a two-step sandwich CMIA for the qualitative detection of total antibodies against the hepatitis B virus core antigen (anti-HBc (total)) in serum or plasma from human. During the test, the specimen is incubated with recombinant hepatitis B virus core antigen (rHBcAg) coated on paramagnetic microparticles, allowing the binding of anti-HBc (total) present in the sample to the rHBcAg. A washing cycle follows this incubation then the reaction mixture is incubated with anti-human acridinium-labeled rHBcAg conjugate. Once the incubation period is finished, the reaction mixture is again washed and the pre-trigger and trigger solutions are added to it, creating a chemiluminescent signal detected by the optics of the machine. The strength of the signal is directly related to the quantity of anti-HBc (total) detected in the specimen.

The chemiluminescent signal generated in the reaction is compared to a cut-off value determined from an active ARCHITECT anti-HBc (total) calibration curve. If the signal generated by the sample is greater than or equal to the cut-off value, then the sample is considered positive for anti-HBc (total).

Method

Due to the low volume of sample available for testing, samples were at first diluted to 1:10 with 50% inactivated FCS/PBS. The samples were then inserted into the ARCHITECT machine for the testing of all three HBV markers (HBsAg, anti-HBs and anti-HBc (total)) at one time.

3.2.3.6 Quality control of serological assays

To ensure the validity of the serology assays used, each assay had a calibration and quality control procedures. Each test performed on the ARHICTECT i2000SR was calibrated according to the manufacturer's instructions. Calibrators were tested in triplicates for HBsAg and anti-HBc (total) testing and in duplicates for anti-HBs testing, every time a new kit was used. The system of the machine calculates the mean signal-to-cut-off (S/CO) values from the calibrator replicates and uses it to calculate the S/CO values of the samples.

Two controls, negative and positive, are supplied with the kits. Each control was tested once every day during the testing period to evaluate the assay calibration. Each control values were ensured to be within the ranges specified in the package insert of the control before the testing of samples.

During the dilution procedures, clean pipettes and microcentrifuge tubes were used. Samples were centrifuged before being opened to avoid contact with gloves and the formation of droplets in the lid of the tubes, which could result in cross contamination of samples. Tips were changed after each pipetting to avoid cross contamination between samples and contamination of the reagents used for dilution. Before dilution, the NHP was screened for all HBV markers being HBsAg, anti-HBs, anti-HBc (total) and tested negative for all.

3.2.4 Molecular procedures

3.2.4.1 Individual viral HBV DNA extraction

The QIAamp® MinElute® Virus Spin (QIAGEN GmbH, Hilden, Germany) was used for the extraction of viral DNA from HBsAg positive samples. The extraction procedure followed the manufacturer's instructions using 200µl of serum or plasma sample.

A lysis buffer mix was prepared with 200µl of buffer AL (the lysis buffer) containing 5.6µg of carrier RNA (provided with the kit) and 3.5µl of murine cytomegalovirus (mCMV) extract used as internal control (400 copies/µl) prior to extraction. The internal control was received from the Blood Borne Virus Unit (BBVU), PHE (Colindale, UK). A volume of 200µl of each sample was pipetted into 1.5mL microcentrifuge tubes containing 25µl of QIAGEN protease. Then, 200µl of the buffer AL mix was dispensed into the solution. Contents of the microcentrifuge tubes were mixed through pulse-vortexing and incubated at 56ºC for fifteen

minutes on a heating block to ensure efficient lysis of the cells and inactivation of RNases. A brief spin was performed after incubation to remove drops from inside the lid and 250µl of 100% ethanol (Sigma-Aldrich, St Louis, MO) was added. The solution was mixed by pulse-vortexing and incubated at room temperature for five minutes. After brief centrifugation of the microcentrifuge tubes, each sample mix was carefully applied to the QIAamp Mini spin column and centrifuged at 8000 rpm (6000 x g) for a minute. This enabled the binding of the nucleic acid to the silica membrane of the column for further purification. Two washing steps were performed subsequently with 500µl buffer AW1 and 500µl buffer AW2. Each wash was followed by two centrifugations at 8000rpm (6000 x g) for a minute for complete purification of the nucleic acid. The washing done, 500µl of 100% ethanol was added into the QIAamp mini columns for DNA purification. The QIAamp mini columns were centrifuged at 8000 rpm (6000 x g) for three minutes. An additional centrifugation step was performed at full speed 14 000 rpm (20 000 x g) to ensure that there is no more ethanol left in the column. The columns were incubated at 56ºC on a heating block for three minutes with caps opened, for complete dry out of the membrane. Finally 60µl of buffer AVE, the elution buffer, was added to the mini columns. The latter were incubated at room temperature for five minutes then centrifuged at 14 000 rpm (20 000 x g) for a minute for DNA elution.

3.2.4.2 Determination of limit of detection of the in-house real-time PCR assay

The quantitative real-time PCR (qPCR) was established in this laboratory **(Maponga TG, MSc Thesis, Stellenbosch University, 2012)** using for reference the in-house real-time HBV assay used at the BBVU, PHE London
(http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1194947340684**).**

The limit of detection of the assay, D_L IU/ml, was to be determined. A ten-fold dilution of sample, "Chong" standard, with a known viral load of 10^8 IU/mL was made. Additionally, the 10^2 IU/mL dilution was further diluted at 1:2, 1:5 and 1:10 to a concentration of 50 IU/mL, 20 IU/mL and 10 IU/mL respectively (see Table 3.11). A 60µl elute of viral nucleic acid DNA was extracted from the diluted samples using the same QIAamp® MinElute® Virus Spin kit (QIAGEN GmbH, Hilden, Germany). The DNA extracts were run in quadruplicate in one run and in quintuple in a second run during two different days using a real-time PCR protocol defined later. Those results were used to draw a standard curve.

Table 3. 11: Ten-fold dilution of the "Chong" standard

In-house Control	Composition
10 000 000 IU/mL	30µl 100 000 000 IU/mL control + 270µl NHP
1 000 000 IU/mL	30µl 10 000 000 IU/mL control + 270µl NHP
100 000 IU/mL	30µl 1 000 000 IU/mL control + 270µl NHP
10 000 IU/mL	30µl 100 000 IU/mL control + 270µl NHP
1 000 IU/mL	30µl 10 000 IU/mL control + 270µl NHP
100 IU/mL	30µl 1 000 IU/mL control + 270µl NHP
50 IU/mL	250µl 100 IU/mL control + 250µl NHP
20 IU/mL	60µl 100 IU/mL + 140 µl NHP
10 IU/mL	30µl 100 IU/mL control + 270µl NHP
Negative	300µl NHP

NHP: Normal Human Plasma
IU/mL: International units per millilitres

3.2.4.3 Quantification of HBV viral copies using quantitative Real-Time PCR (qPCR)

HBV viral DNA loads of all HBsAg and anti-HBc (total) positive samples were determined using the probe-based real-time PCR assay developed by Garson *et al*. **(Garson *et al.*, 2005)** with a final volume of 25µL per reaction on the Rotor Gene 6000 real-time PCR machine (Corbett Sciences, Australia).

Table 3.12 shows the primers and probes used during the assay. These are specific for highly conserved regions of the HBs gene and for the detection of the internal control, mCMV. The TAMRA fluorochrome was used as a quencher dye and FAM and VIC were used as reporter dyes for HBV and mCMV detection, respectively. These three fluorochromes were used to identify only DNA molecules containing the probe sequence. The green channel of the thermal cycler was used to detect HBV after excitation of FAM and the yellow channel was used to identify the internal control, mCMV, after excitation of VIC. Calculation of the viral loads was done using the standard curve constructed with the real-time PCR standards previously extracted as described in section 3.2.4.2. and quantified in IU/mL.

Table 3. 12: List of primers and probes used for HBV and mCMV detection

Primer / Probe	Sequence
HBV forward primer	5'-GTG TCT GCG GCG TTT TAT CA-3'
HBV reverse primer	5′- GAC AAA CGG GCA ACA TAC CTT-3′
HBV probe	5'FAM-CCT CT(T/G) CAT CCT GCT GCT ATG CCT CAT C-3'-TAMRA
mCMV forward primer	5′-AAC CCG GCA AGA TTT CTA ACG-3′
mCMV reverse primer	5′-ATT CTG TGG GTC TGC GAC TCA -3′
mCMV probe	5'-VIC-CTA GTC ATC GAC GGT GCA CAT CGG C-3'-TAMRA

The real-time PCR master mix was prepared according to the number of samples, controls and standards processed at a time. The standards were run in duplicates. The 2× Quantitect qPCR master mix kit (QIAGEN, GmbH, Hilden, Germany) was used to carry on the assay. Table 3.13 shows details on the reagents used to prepare the real-time PCR master mix. A 10µL volume of each sample, control and standard was added to 15 µL of the master mix. Amplification and detection of the targets were run at the cycling conditions represented on Table 3.14. For purpose of quality control, a negative control (NC), a no template control (NTC) and a working control were added in each run. The working control used was a sample which previously tested positive for HBV viral load hence had a known viral load. This sample was used to ensure the reproducibility and assess the variability of the assay. The NTC was nuclease-free water and the NC was NHP, to assess if no contamination had occurred during the handling and manipulation of the PCR tubes and during viral nucleic acid extraction. An internal control, mCMV, added to the samples during DNA extraction was also used as a quality control. The yellow channel of the machine was used to monitor if the internal control was detected in the assay and was used to validate the results for each sample.

Table 3. 13: Composition of the quantitative real-time PCR master mix

Reagent	Working Concentration	Final concentration	Volume/reaction (µL)
2X Quantitect PCR kit	2X	1X	12.50
HBV forward primer	100µM	400nM	0.10
HBV reverse primer	100 µM	400 nM	0.10
HBV probe	100 µM	200 nM	0.05
mCMV forward primer	100 µM	400 nM	0.10
mCMV reverse primer	100 µM	400 nM	0.10
mCMV probe	100 µM	200 nM	0.05
Water	n/a	n/a	2.00
Total Volume	n/a	n/a	15.00

Table 3. 14: Real-time PCR cycling conditions

Cycling parameters	Cycles	Temperature	Time
Initial denaturation	1	95ºC	15 minutes
Denaturation	40	95ºC	15 seconds
Annealing and extension		60ºC	60 seconds

Acceptance criteria of a valid run

To ensure the validity of a run, a number of criteria had to be considered, including the following:

i. No HBV DNA was to be detected from the negative control (NHP) and the NTC. However, the NC must have a valid internal control cycle threshold (Ct). The cycle threshold is the cycle at which the fluorescence exceeds the background level (transition from negative value to positive value).

ii. The standard curve of the run had to be valid according to the following values:

- The slope of the standard curve (M) should be within -3.0 to -3.6. It determines the efficiency of the qPCR. Ideally, for an efficiency of 100% the slope is -3.32.
- The R^2 value should be between 0.9 and 1.1. R^2 indicates how well data points fit a curve, showing how linear the data are.
- The B value had to be higher than the Ct values of the samples and standards. B represents the highest cycle number at which the viral load obtained from the graph was considered to be reliable.

iii. The Ct values of samples for HBV and mCMV, the internal control, should be no further than 3 standard deviations.

3.2.4.4 Nucleotide sequencing of the Polymerase and Surface *(pol/surface)* HBV ORFs

Positive samples with known HBV DNA viral loads were subjected to Sanger sequencing for the purpose of determining the HBV genotype and detecting the presence of mutations associated with drug resistance or vaccine-escape.

Pre-nested PCR

Remnants of DNA extracted were used to perform pre-nested qualitative polymerase chain reactions (PCRs) on the HBV DNA positive samples for further *pol/surface* sequencing. Each pre-nested PCR reaction required 2.5µl of 10× PCR buffer (Invitrogen, California), 0.5µl of 10 mM deoxynucleotide triphosphate (dNTP) mix (Bioline, London), 0.5µl of the 20 pmol/µl HBV 3 reverse primer, 0.5µl of the 20 pmol/µl HBV Z forward primer (listed in Table 3.15), 0.75µl of 50 mM magnesium chloride ($MgCl_2$) (Invitrogen, California), 0.1µl *Thermus aquaticus* (*Taq*) Polymerase (Invitrogen, California), 5µl of DNA template and 15.15µl of nuclease-free water combined to make a final volume of 25µl. The reaction tubes were

inserted into the GeneAmp PCR system 9700 (Applied Biosystems) and the reaction was run at the cycling conditions shown in Table 3.16.

Table 3. 15: Pre-nested PCR primers of the *pol/surface* region

Primer	Sequence	Binding position from *Eco* R1
HBV Z – Forward	5′- AGC CCT CAG GCT CAG GGC ATA -3′	3134 – 3154
HBV 3 – Reverse	5′- CGT TGC CKD GCA ACS GGG TAA AGG -3′	1170 – 1146

Table 3. 16: pre-nested PCR cycling conditions of the *pol/surface* region

Cycling parameters	Cycles	Temperature	Time
Initial denaturation	1	95ºC	5 minutes
Denaturation	34	94ºC	30 seconds
Annealing		55ºc	30 seconds
Extension		72ºC	1 minute
Final extension	1	72ºC	2 minutes

Nested PCR

A second PCR round was performed to improve the specificity of the products obtained from pre-nested PCR and to obtain sufficient DNA material, of an approximate size of 900 bp, required for cycle sequencing. Pre-nested PCR products were used to carry on the nested PCR. For each reaction, 5µl of 10× PCR buffer (Invitrogen, California), 1µl of 10 mM dNTP mix (Bioline, London), 1µl of HBV P forward primer (20 pmol/µl), 1µl of HBV M reverse primer (20 pmol/µl) (listed in Table 3.17), 1.5µl of 50 mM $MgCl_2$ (Invitrogen, California), 0.2µl *Taq* Polymerase (Invitrogen, California), 1µl of DNA template and 39.3µl of nuclease-free water were mixed to a total volume of 50µl. The reaction was run in the GeneAmp PCR system 9700 (Applied Biosystems) at the cycling conditions shown in Table 3.18.

Table 3. 17: Nested PCR primers of the *pol/surface* region

Primer	Sequence	Binding position from *Eco* R1
HBV P – Forward	5′- TCA TCC TCA GGC CAT GCA GT -3′	3247 – 3266
HBV M – Reverse	5′- GAC ACA CTT TCC AAT CAA TNG G -3′	997– 976

Table 3. 18: Nested PCR cycling conditions of the *pol/surface* region

Cycling parameters	Cycles	Temperature	Time
Initial denaturation	1	95ºC	5 minutes
Denaturation	34	94ºC	30 seconds
Annealing		50ºc	30 seconds
Extension		72ºC	1 minute
Final extension	1	72ºC	7 minutes

Visualization of nested PCR products

Nested PCR products were visualized by gel electrophoresis. A 2% agarose gel was made by heat dissolving 2g of powder SeaKem® LE agarose (LONZA, Rockland, ME, USA) into 100ml of 1× tris-acetate-ethylene diamine tetra acetic acid (TAE) buffer for 2 minutes into a microwave. The 1× TAE buffer was prepared from a 50× TAE stock solution. The latter was made up by dissolving 242g Tris base (Boehringer Mannheim, USA) into 57.1ml glacial acid (Merck Chemicals, Germany), 100mL of 0.5 M ethylenediamine tetra acetic acid (EDTA) of pH= 8.0 and distilled water to make up a 1L volume. The heating gel mix was cooled then poured into the gel mold and left for a few minutes to allow the gel to solidify. Before the loading of samples, the gel was submerged in the 1× TAE buffer. A volume of 5µl of each sample was stained with 1µl Novel Juice (GeneDireX, Taiwan), the loading dye, and loaded into each well of the gel. A 1kb DNA ladder (GeneRuler™ 1 kb DNA Ladder, Fermentas) was loaded at both ends of the gel as a molecular marker for purpose of confirming if the correct DNA size had been amplified during PCR. Following sample loading, electrophoresis was performed at a voltage of 80V for at least 30 minutes. Visualization of the gel was done on the UVItec Prochemi (Cambridge, UK) image acquisition system. The gel was exposed to the transluminator light at a high wavelength. The images resulting from the acquisition system were edited using the UVIband-1D gel analysis software (Cambridge, UK) and stored for interpretation.

Clean-up of nested PCR products

Visualization of the PCR products for the confirmation of the correct size of DNA amplified being done, all positive samples were cleaned up using the QIAGEN® QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's instructions. This kit allows the purification and concentration of DNA and uses the principle: DNA capture, DNA binding, washing and elution.

Five volumes of buffer PB were pipetted into one volume of the nested PCR products. Hence 225µl of buffer PB was added to 45µl of PCR product. The solution was mixed through pulse-vortexing. For DNA binding, the mixture was applied to a silica-membrane column provided in the kit. Centrifugation followed for one minute at 13000 rpm. The flow-through was discarded and the column placed in the same collection tube. A volume of 0.75ml of washing buffer, buffer PE, was then added and the column was centrifuged for one minute at 13000 rpm. The filtrate was discarded and the column placed in the same collection tube. The column was centrifuged for an additional minute at 13000 rpm to remove residual ethanol from the washing buffer from the membrane. Next, the column was placed in a clean 1.5ml microcentrifuge tube and 50µl of elution buffer, buffer EB, was pipetted to the centre of the QIAquick membrane. The purified DNA was eluted by centrifuging the column at 13000 rpm for one minute.

Quantification of the purified HBV DNA concentration was performed spectrophotometrically using the NanoDrop® ND-100 (Thermo Fisher Scientific, USA). The concentration and purity of the nucleic acid was automatically calculated with the "Nucleic Acid" Application module of the NanoDrop Software Version 3.1.0. Samples with a concentration above 20ng/µl were diluted to the recommended range (5-20ng/µl) for the sequencing PCR reaction.

Cycle sequencing PCR reaction

This was performed using the BigDye® Terminator v3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems, California, USA), a pre-mixed format to which the template and specific primers (listed in Table 3.19) were added to perform the reaction. The kit is used to perform fluorescence-based cycle sequencing reactions on single-stranded or double-stranded DNA templates, on PCR fragments, and on large templates. The BigDye® Terminator v3.1 Cycle Sequencing Ready Reaction kit uses the same principle as the Sanger sequencing with the use of fluorescent-labelled dNTPs and nucleotide base analogues called dideoxynucleotide triphosphates (ddNTPs). The latter, unlike dNTPs, lack the 3'-OH group essential in forming the phosphodiester bond between two nucleotides and thus act as chain terminators during the sequencing process. Hence during cycle sequencing, when a dNTP (A, C, G, or T) is added to the 3′ end, chain extension can continue. However, when a ddNTP (ddA, ddC, ddG, or ddT) is added to the 3´ end of the primer, chain extension terminates.

This results in the formation of DNA fragments of various lengths with ddNTPs at the 3′ -OH end.

The reaction was set up on a 96-well reaction plate. A master mix was prepared with 1µl of Terminator Ready Reaction mix, 3µl of ABI sequencing buffer and 4µl of water for each reaction. 1µl of each primer and 1µl of the clean nested PCR product were added into the specific wells. The reaction was executed on the GeneAmp PCR system 9700 (Applied Biosystems) following the cycling parameters indicated in Table 3.20. The latter were used for both sequencing reactions.

Table 3. 19: Oligonucleotide primers used for *pol/surface* sequencing

Primer	Sequence	Binding position from *Eco* R1
HBV P – Forward	5′- TCA TCC TCA GGC CAT GCA GT -3′	3247 – 3266
HBV M – Reverse	5′- GAC ACA CTT TCC AAT CAA TNG G -3′	997 – 976
HBV H – Forward	5′-TAT CAA GGA ATT CTG CCC GTT TGT CCT -3′	628 – 655
HBV N – Reverse	5′-ACT GAG CCA GGA GAA ACG GAC TGA GGC -3′	682 – 656

Table 3. 20: *pol/surface* cycle sequencing cycling conditions

Cycling parameter	Cycles	Temperature	Time
Denaturation	30	96°C	20 seconds
Annealing		55°C	20 seconds
Extension		60°C	4 minutes

Purification of sequencing products

Following cycle sequencing, the sequencing products were purified using the BigDye® Xterminator Purification Kit (Applied Biosystems, Foster City, California, USA) to remove unincorporated BigDye terminators used during the sequencing reaction and salts which might interfere for base calling during capillary electrophoresis. The kit contains two reagents: (1) the XTerminator® solution eliminates unincorporated dye terminators and free salts from the post-cycle sequencing reaction and, (2) the SAM™ solution enhances BigDye XTerminator performance and stabilizes sample after purification.

Purification of each well of the 96-wells reaction plate required a master mix made up of 49.5µL SAM solution and 11µL XTerminator solution per sample. A volume of 55µl of the

master mix was pipetted to the post-cycle sequencing products. The reaction plate was sealed with a MicroAmp Optical sheet (Applied Biosystems, California) and vortexed on a Multi-microplate Genie microplate shaker (Scientific Industries, New York) for thirty minutes at 2000 x g. Following vortexing, the plate was centrifuged for two minutes at 1000 x g.

The 96-well plate was sealed with a septa mat and placed into the ABI Prism 3130*xl* genetic analyzer (Applied Biosystems, California) for capillary electrophoresis. The latter is an instrumental evolution of the polyacrylamide gel separation technique previously used to separate DNA sequencing products.

Sequencing data analysis

The raw data created by the DNA sequencing analysis software from Applied Biosystems, USA, were used for further analysis using the software Sequencher v5 (Gene Codes Corporation, Ann Arbor, Michigan, USA). The quality of sequences was improved through trimming and editing where necessary. Consensus sequences were formed for each sample from the four primer sequences and edited for improvement of the quality using Sequencher v5. These consensus sequences were saved in FASTA format then submitted to the following online genotyping tools and databases: The HBV section of the HIV drug resistance data base from Stanford (http://hivdb.stanford.edu/HBV/HBVseq/development.html), the Max Planck Institute (http://www.geno2pheno.org) and the HepSeq Research genotyping tool (http://www.hepseq.org/Public/Tool/genotype_tool.php) and the NCBI HBV Genotyping tool (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi).

These tools were used for the identification of the genotype of the HBV strains and the detection of mutants related to immune or drug escape.

3.2.4.5 Phylogenetic analysis

Phylogenetics is a tool used to confirm the genotype of the HBV strains and to establish the relationship between sequences. The consensus sequences obtained from the polymerase and surface regions sequencing were aligned with reference sequences of HBV genotype A originating from SA and other countries using the software ClustalW **(Larkin, 2007)**. Reference sequences of HBV from other genotypes were also added to the alignment. These reference sequences were obtained from queries on GenBank, from Maponga TG, MSc Thesis, Stellenbosch University (2012) and Chotun BN, MSc Thesis, Stellenbosch University (2012). The alignments were then submitted to MEGA v6 **(Tamura *et al.,* 2013)** for the

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construction of phylogenetic trees. The latter show the comparison between the sequences forming the alignments. The evolutionary relationship between our sequences was inferred using the Neighbor-Joining method **(Saitou and Nei, 1987)** and the Kimura 2-parameter method **(Kimura, 1980)** was used to calculate the evolutionary distances between them. The Neighbor-Joining method is used when dealing with a large number of data sets and for bootstrapping analysis. The algorithm uses the least distance between pairs of sequences to infer nodes resulting in a tree which represents sequences associated with their most related ancestors. The Kimura 2-parameter generates evolutionary distance between sequences and is able to distinguish between transitions appearing where a purine is replaced by the other one (A<-->G) or a pyrimidine is substituted by another pyrimidine (C<-->T), and transversions where a pyrimidine is substituted by a purine or vice versa (A or G <--> C or T).

3.2.4.6 Quality control of molecular assays

(1) A NC (NHP) tested negative for all HBV markers was added during each extraction procedure to detect contamination. DNA extraction was performed in a dedicated room, under a laminar flow cabinet. The latter is an enclosed bench designed to reduce the risk of contamination during the handling of biological samples. Prior to the processing of samples, they were briefly centrifuge before opening. This precaution was performed in order to avoid contact of any droplets formed in the lid of the tubes with the gloves, which could be a possible source of cross contamination between samples. An internal control, mCMV, was used as a marker successful extraction of nucleic acids.

Setup of the PCR assays was performed in different work areas: reagent preparation, sample loading and amplification had dedicated rooms. A NC and a NTC were added into each HBV PCR procedure. The NTC was used to assess that all reagents used during the assay were not contaminated whereas the NC was used to make sure that all reagents were working as they should be hence ensuring that every positive result was a true positive result. Nuclease free water was used as the NTC and the NC was the extract from NHP performed during DNA extraction. The quantitative HBV PCRs had an additional working control added. The latter was a sample known to have a high viral load and was used to ensure the reproducibility of the assay and assess the variability of the assay hence to ensure the long term validity of the assay. Additionally, standards were run during each quantitative run. These standards were run in duplicate to increase confidence in the interpretation of the results. Successful amplification of the internal control was indicative of a successful run whereas failure in

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amplifying the internal control was indicative of an invalid run. Gel electrophoresis, sequencing and capillary electrophoresis procedures were also executed in dedicated rooms.

(2) Each room had dedicated sets of pipettes and filtered tips to use and those were not moved from one room to the other one. Clean gloves were always worn when executing all procedures and the work benches were disinfected with 10% bleach to denature nucleic acids followed by 70% ethanol to destroy any remaining microorganisms left on the work bench before and after each procedure.

3.3 Results

3.3.1 Sample and data collection

Of the 916 infants from whom blood samples were collected, a total of 851 samples from three different locations of SA: Johannesburg, Durban and Cape Town were received. From Johannesburg 555 samples, 46 samples from Durban and 250 samples from Cape Town. The others 65 samples were found absent in the lots received from those three locations. From the 851 samples received, 836 samples were from "week 48" of the study whereas 3 samples were from "week 12" and 12 samples were from unknown time period.

3.3.2 Serology results

3.3.2.1 Prevalence of HBsAg among study population

Prevalence of HBsAg among screening samples

Out of the 851 samples available for testing, one sample was found insufficient hence e 850 samples were screened for HBsAg using the ARCHITECT i2000R system. A total of three out of the 850 samples, originating from Johannesburg, Durban and Cape Town, were found highly reactive for HBsAg, giving an HBsAg prevalence of 0.4%. Fifteen samples were found HBsAg equivocal i.e. HBsAg S/CO values were in the interval of 1.0 and 4.0.

However due to low sample volumes, HBsAg neutralisation could not be performed to confirm the true HBsAg positivity of these eighteen patients, but HBV DNA testing was performed to confirm the presence or absence of viral replication. Table 3.21 gives a summary of the serology results of all samples truly positive for HBsAg.

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Table 3.21: Serology results of true HBsAg positive samples

Patient ID	Location	HBsAg (S/CO)	Anti-HBs (mIU/mL)	Anti-HBc (total) (S/CO)
350V06029315	Johannesburg	2830.25	2.9	10.83
262V08006189	Durban	6014.78	3.0	8.74
279V07019441	Cape Town	5690.34	0.9	10.75

Prevalence of HBsAg among follow-up samples

The breakdown of data available on the followed up infants are listed below on Table 3.22. However, despite extensive efforts, the infants and mothers from Johannesburg and Durban could not be contacted for follow up.

A serum sample collected at point of entry i.e. “Week 0” but not at “Week 12” of sample 279V07019441, originating from Cape Town, was retrieved. The child and his mother were able to be traced and serum samples were collected from both mother and child. The infant and his mother were both HIV positive. The child was put on antiretroviral treatment (ART), stavudine, 3TC and lopinavir/ritonavir (Kaletra (KLT)), two years prior to this testing. The mother, HIV-infected and referred here as “Cape Town mother”, was also put on ART two years previously. Her regimen consisted of 3TC, TNF and KLT. Both samples tested HBsAg positive. The child sample retrieved from “Week 0” was diluted as described previously (Section 3.2.3.1.) and screened for HBsAg. The specimen, showed strong HBsAg reactivity.

Again, due to low sample volume, HBsAg neutralization was not performed on these samples to confirm the true HBsAg positivity.

Table 3. 22: Demographic data of the followed up infants and their mothers

Patient ID	Location	Sex	Age (years)	HIV status
279V07019441	Cape Town	Male	8	Infected
Cape Town mother	Cape Town	-	34	Infected
262V08006189	Durban	Unknown	Unknown	Unknown
Durban mother	Durban	-	Unknown	Infected
350V6029315	Johannesburg	Unknown	Unknown	Unknown
Johannesburg mother	Johannesburg	-	Unknown	Infected

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3.3.2.2 Prevalence of anti-HBs

A total number of 474 samples (55.8%) were found positive for anti-HBs with protective anti-HBs titres greater than 10 IU/mL. Among those specimens, four had unknown point of entry and one was from “Week 12”. On the other hand, 293 samples displayed anti-HBs titres lower than 10 IU/mL. Three of these samples had unknown point of entry and one was collected from “Week 12”. Moreover, 83 of the 850 samples (9.8%) showed anti-HBs titres lower than 1mIU/mL. Five of these 83 samples had unknown point of entry and one was from “Week 12” leaving 77 samples (9.1%) of “Week 48” with anti-HBs titres < 1mIU/mL.

3.3.2.3 Prevalence of anti-HBc (total)

Among the 850 specimens screened, two samples which tested negative for HBsAg and anti-HBs positive, were anti-HBc (total) positive. The three HBsAg positive samples were also reactive for anti-HBc (total) whereas all fifteen HBsAg equivocal specimens were anti-HBc (total) non-reactive. This shows a 0.6% (5/850) prevalence for anti-HBc (total) in our cohort.

3.3.2.4 Prevalence of HBeAg and anti-HBe among HBsAg positive follow-up samples

Due to the low volume of samples, the “Week 48” specimens positive for HBsAg were not tested for either HBeAg or anti-HBe. However samples collected at “Week 0” or “Week 12” of study and the follow-up samples from both mothers and children were screened for these two markers.

Sample collected at “Week 0” from the child originating from Cape Town was reactive for HBeAg and non-reactive for anti-HBe. However, the follow-up sample was found HBeAg negative and anti-HBe positive, it is likely that the child had seroconverted. The mother was also HBeAg negative and anti-HBe positive. Table 3.23 illustrates the serology status of the follow-up HBsAg positive infant and mother.

Table 3. 21: Serology results of follow-up HBsAg positive infant and mother

Patient ID	Location	HBsAg (S/CO)	Anti-HBs (mIU/mL)	Anti-HBc (total) (S/CO)	HBeAg (S/CO)	Anti-HBe (S/CO)
279V07019441 (12 weeks)	Cape Town	6548.46 (POS)	0.23 (NEG)	10.11 (POS)	190.52 (POS)	29.09 (NEG)
279V07019441 (60 weeks)	Cape Town	5690.34 (POS)	0.9 (NEG)	10.75 (POS)	NT	NT
279V07019441* (8 years old)	Cape Town	4731.37 (POS)	0.02 (NEG)	11.13 (POS)	0.403 (NEG)	0.02 (POS)
Cape Town Mother	Cape Town	4779.94 (POS)	0.45 (NEG)	11.13 (POS)	0.550 (NEG)	0.25 (POS)

* HIV positive and on ARTs being D4T, 3TC, KLT
NT: Not tested

3.3.3 Molecular results

3.3.3.1 Viral HBV DNA extraction

A total number of 815 samples that were found negative for both HBsAg and anti-HBc (total) had no further molecular testing performed. HBV DNA extraction was performed on 36 samples of which the three HBsAg positive samples, the fifteen HBsAg equivocal samples, the two anti-HBc (total) positive samples and an additional number of sixteen samples with HBsAg S/CO values between 0.70 and 1.0. HBV DNA extraction was performed on those sixteen samples to ensure that these samples were truly HBV negative.

HBV DNA was also extracted from the "Week 0" sample of the Cape Town child and his follow-up blood sample. The serum sample from the mother was also processed for HBV DNA extraction.

3.3.3.2 Quantification of HBV viral copies

3.3.3.2.1 Quantification at screening stage

The lower limit of quantitation for the assay on the Rotor Gene 6000 was determined to be 20 IU/ml through repeat testing of standard serial dilutions (**Maponga TG, MSc Thesis, Stellenbosch University, 2012**).

HBV viral load quantitation was achieved on 36/851 HBV DNA extracted samples using our validated in-house real-time PCR assay. The viral load results seen on Table 3.24 showed that 3/36 samples had a high viral load.

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The 15 HBsAg equivocal samples and the 16 samples with low HBsAg S/CO values had no detectable HBV DNA. None of the 2 anti-HBc (total) samples had a detectable HBV viral DNA.

This calculates to an active HBV infection prevalence of 0.4% (3/851) of the whole cohort.

Table 3. 22: HBV viral copies of true HBsAg positive "Week 48" samples

Patient ID	Location	HBV DNA IU/mL	HBV DNA log 10	HBsAg	Anti-HBs	Anti-HBc (total)
350V06029315	Johannesburg	286 976 397 IU/mL	8.46	+	-	+
262V08006189	Durban	147 117 IU/mL	5.17	+	-	+
279V07019441	Cape Town	846 254 976 IU/mL	8.93	+	-	+

3.3.3.2.2 Quantification at follow-up

As shown on Table 3.25, the specimen collected at "Week 0" from the Cape Town infant had a high viral load of 750 746 079 IU/mL but had no detectable viral load at follow up. Serum collected from the mother showed a low detectable viral load of 19 IU/mL.

Table 3. 23: HBV viral copies at follow up

Patient ID	Location	HBV DNA IU/mL	HBsAg	Anti-HBc (total)	HBeAg	Anti-HBe
279V07019441 (Twelve weeks)	Cape Town	750 746 079 IU/mL	+	+	+	-
279V07019441 (8 years old)	Cape Town	Undetectable	+	+	-	+
Cape Town mother	Cape Town	19 IU/mL	+	+	-	+

3.3.3.3 Nucleotide sequencing of the Polymerase and Surface ORFs results

The three infants positive for HBV DNA were sequenced for the *pol/surface* gene to determine the genotype of the HBV strains through pre-nested and nested PCR runs. The "Week 0"sample of the Cape Town infant, with high viral load was also used for sequencing. Due to the low viral load of the Cape Town mother, sample could not be sequenced.Visualization of the PCR products is shown on Figure 3.5. The pre-nested DNA products obtained from the Johannesburg and Cape Town "Week 48"samples were used whereas the nested product from the Durban "Week 48" sample was used for sequencing.

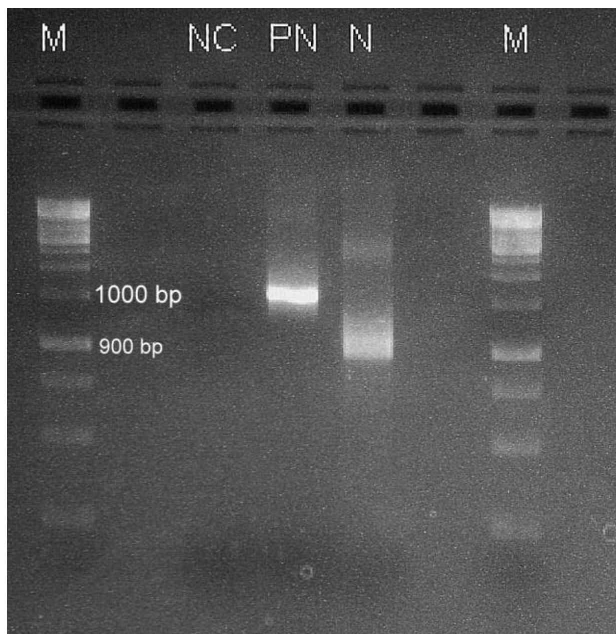

Figure 3. 5: *Pol/surface* clean PCR products obtained from "Week 0" of the Cape Town infant. M: 1kb molecular weight marker; NC: Negative Control; PN: pre-nested; N: Nested.

These products were cleaned up and the DNA products were analyzed via spectrophotometry to determine the purity (A_{260nm} and $A_{260/280nm}$ values) and concentration (ng/µL) of the products, see Table 3.26. The $A_{260/280nm}$ ratio is a measure of the purity of DNA. This ratio should be approximately 1.8 to consider the DNA product "pure".

Table 3. 24: Concentration and purity of *pol/surface* DNA products

Sample ID	ng/µL	A_{260nm}	$A_{260/280nm}$
NC	5.38	0.108	1.84
350V06029315	3.69	0.074	1.33
262V08006189	24.07	0.481	2.11
279V07019441	10.93	0.219	2.20
279V07019441*	42.83	0.857	1.92

* "Week 0" sample
ng/µL: nanogram per microliters
A: Absorbance
NC: Negative Control

The 279V07019441 "Week 0" sample was diluted to the recommended DNA concentration range (5 – 20ng/µl) for the sequencing reaction. The sample was diluted using DNA nuclease-free water. A volume of 1µL of each sample, including the NTC, was used for the

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sequencing reaction. None of the "Week 48"samples were diluted giving the low DNA concentration. The sequences were amplified with four different primers, as previously mentioned.

After the sequencing reaction, the products were cleaned up and analysed on the ABI Prism 3130*xl* genetic analyzer. The sequences obtained were analyzed, trimmed and aligned. A contiguous sequence of approximately 900 bp long was obtained from each infant.

The sequences were then submitted on the HepSeq Research website and the NCBI HBV genotyping tool website and these HBV strains were found to belong to Genotype A, subgenotype A1. The sequences were also submitted to the Max Plank institute (Geno2pheno) website and the HBV section of the HIV drug resistance data base from Stanford to identify the presence or absence of clinically relevant mutations. No drug-resistance or vaccine-escape mutation was identified in *the pol/surface* gene of the infants originating from Johannesburg and Cape Town. The Durban infant showed the M204I mutation on the RT domain of the *polymerase* gene. This mutation is clinically associated with drug resistance to 3TC and telbivudine and convers partial resistance to entecavir. No other clinically significant mutations were identified on any of these three infants.

A phylogenetic tree, seen on Figure 3.6, was constructed using these infant's' HBV sequences. The three sequences clustered with genotype A sequences. A second tree, in which was added the "Week 0" Cape Town infant's sequence, was constructed to compare the sequences from "Week 48" to the sequence from "Week 0"of that infant (Figure 3.7). The two sequences appeared close to each other.

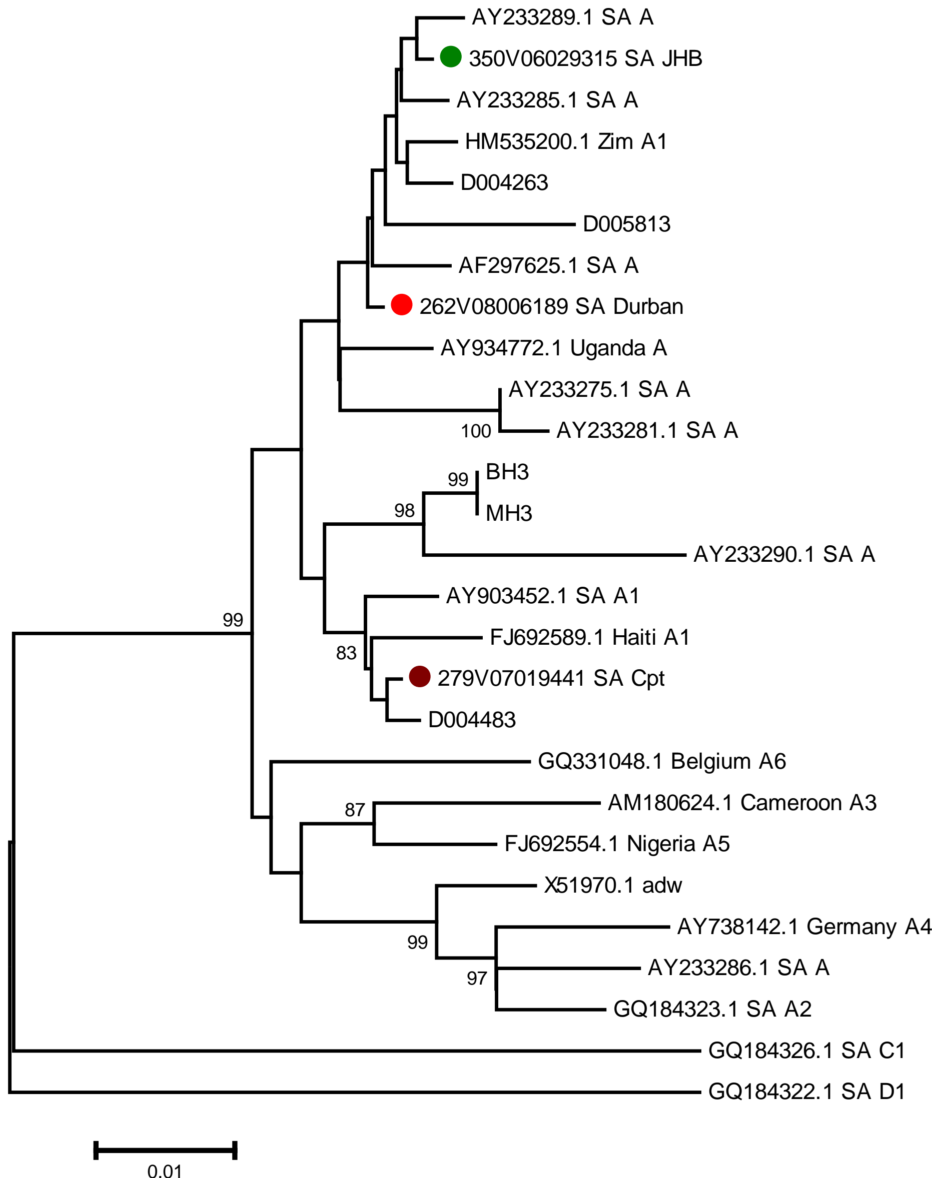

Figure 3. 6: Phylogenetic tree of HBV-infected infants with HBV strains belonging to subgenotype A1 based on *pol/surface* region of the genome.

BH3, MH3, D004263, D005813 and D004483 are sequences from patients from the Western Cape **(Chotun BN and Maponga TG, MSc Thesis, Stellenbosch University, 2012)**. Sequences with accession numbers starting with AY, HM, AF, FJ, GQ, AM and X5 were downloaded from GenBank. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.34175109 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The analysis involved 27 nucleotide sequences. There were a total of 898 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 **(Tamura *et al.,* 2013).**

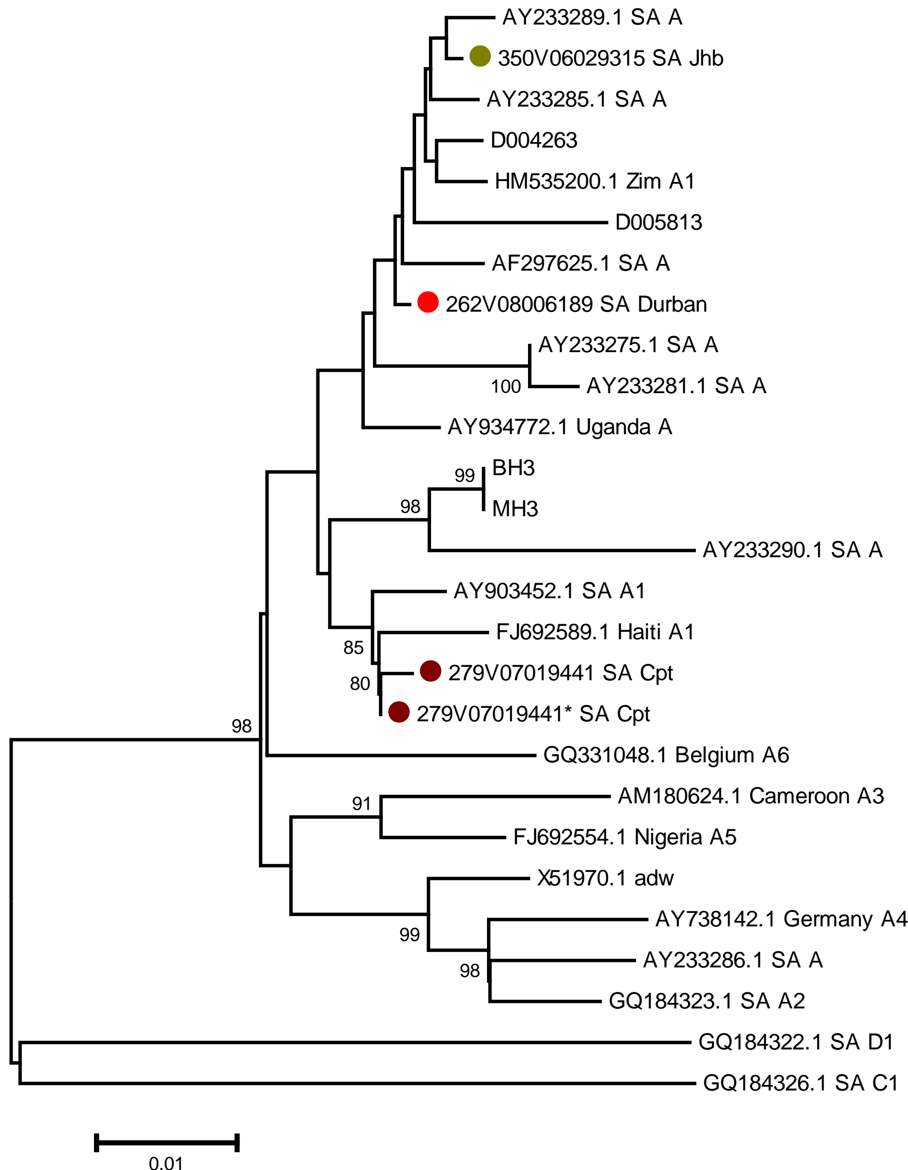

Figure 3. 7: Phylogenetic tree of HBV-infected infants and the "Week 0" sample with HBV strains belonging to subgenotype A1 based on *pol/surface* region of the genome.

279V07019441* represents the "Week 0"sample of the Cape Town infant. BH3, MH3, D004263, D005813 and D004483 are sequences from patients from the Western Cape **(Chotun BN and Maponga TG, MSc Thesis, Stellenbosch University, 2012)**. Sequences with accession numbers starting with AY, HM, AF, FJ, GQ, AM and X5 were downloaded from GenBank.

The evolutionary history was inferred using the Neighbor-Joining method **(Saitou and Nei, 1987)**. The optimal tree with the sum of branch length = 0.33538548 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method **(Kimura, 1980)** and are in the units of the number of base substitutions per site. The analysis involved 27 nucleotide sequences. There were a total of 896 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (**Tamura *et al.,* 2013**).

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3.4 Summary of findings

To summarize, an HBV infection prevalence of 0.6% (5/850) was described in HIV-exposed infants. Three of these five infants were positive for HBsAg whilst two were positive for anti-HBc (total). The three HBsAg positive infants were HBV DNA positive whereas the two anti-HBc (total) positive samples where negative for HBV DNA. No clinically relevant mutation in the analysis of the *pol/surface* of these three HBsAg positive infants was found in two infants. In the third, the 3TC drug-resistant mutation (M204I) in the polymerase gene was found. The significance of these findings will be discussed in chapter 5.

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CHAPTER 4: THE PREVALENCE OF HCC-RELATED HBV MUTATIONS IN HIV/HBV CO-INFECTED AND HBV MONO-INFECTED WOMEN AND IN HIV-EXPOSED CHILDREN

This chapter reports on the prevalence of HBV mutations, which have been associated with HCC, in antenatal samples from the Western Cape Province, SA.

4.1 Introduction

Infants infected through vertical transmission are at a great risk (>90%) of becoming chronic carriers of HBV. Chronic HBV carriers are at a high risk of developing HCC. In chapter 3, three possible cases of perinatal transmission with one confirmed case were described. In a previous antenatal study, a high prevalence of BCP and pre-core mutations was described in HIV-infected pregnant women as compared to HIV-uninfected pregnant women **(Andersson *et al.,* 2013)**. These mutations have also been reported in CHB and HCC patients in SA **(Baptista *et al.,* 1999; Mayaphi *et al.,* 2013)**. Many Asian studies have associated the presence of these specific HBV mutations with a high risk of HCC in CHB patients **(Kao *et al.,* 2003; Tong *et al.,* 2006, 2007; Qu *et al.,* 2011)**. These HBV mutations are currently used as a biomarker in algorithms which are accurately able to predict the risk of HCC development in patients with chronic hepatitis **(Yuen *et al.,* 2009)**. The findings from the transmission study (chapter 3) prompted questions about the prevalence of HCC-related mutations in the context of HIV. This study aimed to investigate the prevalence of HCC-related mutations in HIV-infected and HIV-uninfected antenatal women and HIV-exposed infants.

4.2 Materials and Methods

4.2.1 Ethical aspect

For this study, we made use of samples from three studies conducted in the Division of Medical Virology, Stellenbosch University. All studies had approval from the Health Research Ethics Committee (HREC) of the Faculty of Medicine and Health Sciences,

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Stellenbosch University. The ethics reference numbers are: N11/05/151, N10/04/115 and N09/11/319 for the two paediatric and antenatal studies, respectively.

4.2.2 Sample and data collection

Sixty samples were retrieved from three HBV-related studies conducted in the Division of Medical Virology, Stellenbosch University:

(1) Five samples (three maternal and two paediatric) were collected from the study: The prevalence of hepatitis B virus infection in an HIV-exposed paediatric cohort from the Western Cape, SA, the so-called NHLS Paediatric Study **(Chotun, BN, MSc Thesis, Stellenbosch University, 2012)**

(2) Fifty antenatal samples were collected from the study: An investigation of hepatitis B virus in antenatal women tested for human immunodeficiency virus, in the Western Cape Province of SA **(Andersson *et al.*, 2013)**

(3) Four paediatric samples and one maternal sample from the transmission study (chapter 3) were also included.

Data collection involved retrieving HBV genome sequencing results performed on samples from the NHLS Paediatric Study and the Antenatal Study.

4.2.3 Molecular procedures

4.2.3.1 Nucleotide sequencing of the core ORF

These data were already available for the NHLS Paediatric and the Antenatal studies. Hence, the assay was only performed on the five samples from the transmission study.

Pre-nested PCR

For each pre-nested PCR reaction, 2.5µl of 10× PCR buffer (Invitrogen, California), 0.5µl of 10 mM deoxynucleotide triphosphate (dNTP) mix (Bioline, London), 0.5µl of the 20 pmol/µl H4072, 0.5µl of the 20 pmol/µl Outer core (listed in Table 4.1) , 0.75µl of 50 mM magnesium chloride ($MgCl_2$) (Invitrogen, California), 0.1µl *Taq* Polymerase (Invitrogen, California), 5µl of DNA template and 15.15µl of nuclease-free water were mixed to make a final volume of

25µl. The reaction tubes were inserted into the GeneAmp PCR system 9700 (Applied Biosystems) and the reaction was run at the cycling conditions shown in Table 4.2.

Table 4. 1: Pre-nested PCR primers of the core region

Primer	Sequence	Binding position from *Eco* R1
H4072 – Forward	5'- TCTTGCCCAAGGTCTTACAT 3'	1602 – 1621
Outer core – Reverse	5'- TCCCACCTTATGAGTCCAAG 3'	2509– 2528

Table 4. 2: Pre-nested PCR cycling conditions of the core region

Cycling parameters	Cycles	Temperature	Time
Initial denaturation	1	94°C	2 minutes
Denaturation		94°C	30 seconds
Annealing	35	55°c	30 seconds
Extension		72°C	1 minute
Final extension	1	72°C	2 minutes

Nested PCR

A second PCR round was performed to obtain sufficient DNA material required. The pre-nested PCR products were used to carry on this step. An approximate DNA size of 700 bp was expected. Each reaction required a mix of 5µl of 10× PCR buffer (Invitrogen, California), 1µl of 10 mM dNTP mix (Bioline, London), 1µl of H4072 (20 pmol/µl), 1µl of Inner core (20 pmol/µl) (listed in Table 4.3), 1.5µl of 50 mM $MgCl_2$ (Invitrogen, California), 0.2µl *Taq* Polymerase (Invitrogen, California), 1µl of DNA template and 39.3µl of nuclease-free water to obtain a total volume of 50µl. The reaction was once again run in the GeneAmp PCR system 9700 (Applied Biosystems) at the cycling conditions shown in Table 4.4.

Table 4. 3: Nested PCR primers of the core region

Primer	Sequence	Binding position from *Eco* R1
H4072 – Forward	5'- TCTTGCCCAAGGTCTTACAT 3'	1602 – 1621
Inner core – Reverse	5'- CAGCGAGGCGAGGGAGTTCTTCTT 3'	2422 – 2445

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Table 4. 4: Nested PCR cycling conditions of the core region

Cycling parameters	Cycles	Temperature	Time
Initial denaturation	1	94ºC	2 minutes
Denaturation	35	94ºC	30 seconds
Annealing		55ºc	30 seconds
Extension		72ºC	1 minute
Final extension	1	72ºC	2 minutes

4.2.3.2 Nucleotide sequencing of the pre-Surface (pre-S) ORF

All samples with known HBV DNA viral loads were subjected to pre-S cycle sequencing to determine the nucleotide sequence of the Pre-S region for the mutation analysis. Two PCR reactions were performed using the kit MyFi™ Mix (Bioline, GmbH, Germany). This mix makes use of a high-fidelity DNA enzyme polymerase to which are added specific primers and the DNA template.

Pre-nested PCR

This first round of PCR was used to obtain a fragment size of around ~2.5kb using two specific primers. This pre-nested PCR master mix was made up of 25µL of 2×MyFi™ mix (Bioline, GmbH, Germany), 1µL of 20pmol/µl UBC_7F, 1µL of 20pmol/µl UBC_6R (listed in Table 4.5) and 18µL of water to make up a volume of 45µL per reaction. Five microliters of DNA extract was added to the master mix to make up a final volume of 50µL. The reaction tubes were inserted into the GeneAmp PCR system 9700 (Applied Biosystems) and the reaction was run at the cycling conditions shown in Table 4.6.

Table 4. 5: Pre-nested PCR primers of the pre-S region

Primer	Sequence	Binding position from *Eco* R1
UBC_7 – Forward	5’- CTT TTT CAC TTC TGC CTA ATC ATC -3’	1821 - 1843
UBC_6 – Reverse	5’- AAA AAG TTG CAT GGT GCT GGT G -3’	1825 - 1804

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Table 4. 6: Pre-nested PCR cycling conditions of the pre-S region

Cycling parameter	Cycles	Temperature	Time
Initial denaturation	1 hold	95°C	2 minutes
Denaturation	30 cycles	95°C	15 seconds
Annealing		55°C	30 seconds
Elongation		72°C	2 minutes
Final Elongation	1 hold	72°C	10 minutes

Nested PCR

A second round of PCR was performed to obtain two fragments of around ~1.7kb using two set of primers. Two master mixes were prepared for this reaction. The first master mix was made up with 25µL of 2×MyFi™ mix (Bioline, GmbH, Germany), 1µL of 20pmol/µl P'1, 1µL of 20pmol/µL MD16 (listed in Table 4.7) and 18µL of water to make up a volume of 45µL per reaction. The second master mix was a mixture of 25µL of 2×MyFi™ mix (Bioline, GmbH, Germany), 1µL of 20pmol/µl MD19, 1µL of 20pmol/µl B1as (listed in Table 4.8) and 18µL of water to make up again a volume of 45µL per reaction. For each reaction, 5µL of DNA template was added to 45 µL of master mix to perform the reaction. Both reactions were done at the same cycling conditions, listed in Table 4.9.

Table 4. 7: Nested PCR primers of the pre-S region – first master mix

Primer	Sequence	Binding position from *Eco* R1
P'1 – Forward	5'- TGC CTA ATC ATC TCA TGT TCA TGT CC -3'	1832 – 1857
MD16 - Reverse	5'- GCA GGG GTC CTA GGA ATC CTG ATG -3'	193 – 170

Table 4. 8: Nested PCR primers of the pre-S region – second master mix

Primer	Sequence	Binding position from *Eco* R1
MD19 – Forward	5'- GTG GGT CAC CAT ATT CTT GGG -3'	2818 – 2838
B1as – Reverse	5'- GGC AGC ACA SCC TAG CAG CCA TGG -3'	1395 – 1372

Table 4. 9: Nested PCR cycling conditions for the pre-S region

Cycling parameter	Cycles	Temperature	Time
Initial denaturation	1 hold	95°C	2 minutes
Denaturation	35 cycles	95°C	40 seconds
Annealing		55°C	40 seconds
Elongation		72°C	60 seconds

Nested PCR products were visualized by gel electrophoresis. A 1% agarose gel was made by heat dissolving 1g of powder SeaKem® LE agarose (LONZA, Rockland, ME, USA) into 100ml of 1× TAE buffer for 2 minutes into a microwave. Visualization of these products was done as described previously in chapter 3, section 3.2.4.4. Positive samples were cleaned up following the procedure described previously in chapter 3, section 3.2.4.4.

Cycle sequencing

This reaction was achieved using the BigDye® Terminator v3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems, California, USA), a pre-mixed format to which the template and specific primers (listed in Table 4.10 and Table 4.11) were added to perform the reaction.`

For each sample, 2 pre-S nested PCR products of around ~1.7kb were amplified hence for each sample 4 sequences were to be obtained from the cycle sequencing assay.

The reaction was set up as described previously and executed on the GeneAmp PCR system 9700 (Applied Biosystems) following the cycling parameters indicated in Table 4.12.

Table 4. 10: Oligonucleotide primers used for core sequencing

Primer	Sequence	Binding position from *Eco* R1
H4072 - Forward	5'- TCT TGC CCA AGG TCT TAC AT- 3’	1602 – 1621
Inner Core– Reverse	5'-CAG CGA GGC GAG GGA GTT CTT CTT- 3’	2422 – 2445
CSEQR – Reverse	5’- GGA GGA GTG CGA ATC CAC ACT- 3’	2314 – 2334
RSP – Forward	5’- GTT CAA GCC TCC AAG- 3’	1830 – 1844

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Table 4. 11: Oligonucleotide primers used for pre-S sequencing

Primer	Sequence	Binding position from *Eco* R1
MD19 – Forward	5′- GTG GGT CAC CAT ATT CTT GGG -3′	2818 – 2838
MD16 – Forward	5′- GCA GGG GTC CTA GGA ATC CTG ATG -3′	193 – 170

Table 4. 12: Pre-S and core cycle sequencing cycling conditions

Cycling parameters	Temperature	Time	Cycles
Denaturation	96°C	20 seconds	30
Annealing	55°C	20seconds	30
Extension	60°C	4minutes	30

At the end of cycle sequencing, the sequencing products were purified using the BigDye® Xterminator Purification Kit (Applied Biosystems, Foster City, California, USA) following the procedure described previously (Chapter 3, section 3.2.4.4.).

The raw data created by the DNA sequencing analysis software from Applied Biosystems, USA, were used for further analysis using the software Sequencher v5 (Gene Codes Corporation, Ann Arbor, Michigan, USA). The quality of sequences was improved through trimming and editing where necessary. Consensus sequences were formed for each sample from the four sequences obtained and edited to improve the quality of the consensus, when needed. These consensus sequences were saved in FASTA format. Multiple alignments were formed with all sample sequences, the pre-core gene sequence reference, X gene sequence reference and the pre-S gene sequence reference using the molecular software Geneious v7.1.5 (Biomatters, New Zealand). The specific regions being pre-core, BCP and pre-S, needed for our analysis were extracted using HBV pre-core gene, X gene and Pre-S gene sequences references. These extracts were analysed manually for mutations using BioEdit v7.2.5. Following manual analysis of these sequences, phylogenetic trees were constructed using the MEGA v6 to observe their clustering.

Quality control of molecular assays

This was discussed previously in Chapter 3, section 3.2.3.6.

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4.3 Results

4.3.1 Sample and data collection

Sixty samples in total were identified for this study: fifty samples from the Antenatal Study **(Maponga, TG, MSc Thesis, Stellenbosch University, 2012)**; five samples from the NHLS Paediatric Study **(Chotun, BN, MSc Thesis, Stellenbosch University, 2012)** and five samples from the transmission study. The five samples from the transmission study were from 3 infants each from Johannesburg, Durban and Cape Town ("Week 0"and "Week 48"), and the mother of the Cape Town infant. These samples were all HBV DNA and HBsAg positive.

Thirty five samples from this cohort were HIV positive: thirty mothers from the antenatal study, two (Cape Town infant and mother) from the transmission study and three mothers from the NHLS Paediatric Study.

Concerning data collection, whole genome sequencing data were retrieved for the 5 paediatric specimens whereas core and *pol/surface* sequencing data were retrieved from the 50 antenatal specimens. *Pol/surface* sequencing data were also available (see section 3.3.3.3) for the transmission study samples with the exception of the Cape Town mother for who *pol/surface* sequencing was unsuccessful.

4.3.2 Molecular results

4.3.2.1 Nucleotide sequencing of the core ORF results

All three "Week 48" HBsAg positive samples were used for the pre-core/core sequencing. The samples collected at "Week 0" of Cape Town infant and his mother were also used for the core sequencing. The PCR products obtained are represented on Figure 4.1 and Figure 4.2.

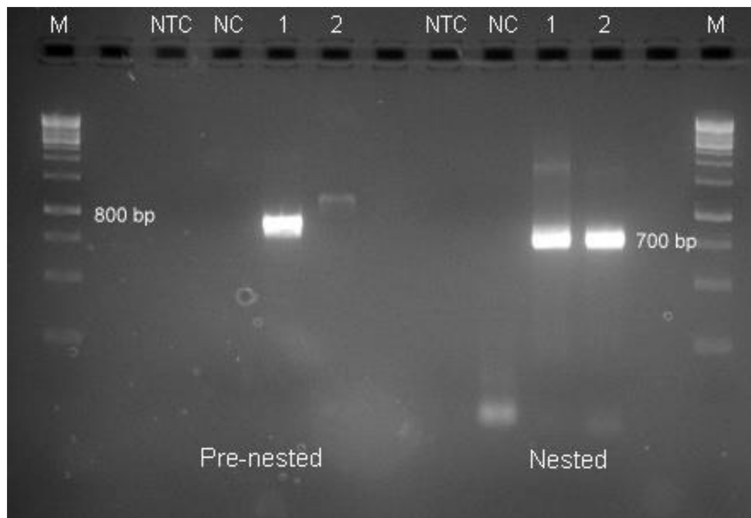

Figure 4. 1: Core clean PCR products of "Week 48"samples collected from the Johannesburg and Durban infants. M: 1kb molecular weight marker; NTC: No Template Control; NC: Negative Control; 1. 350V06029315 (Johannesburg); 2. 262V08006189 (Durban)

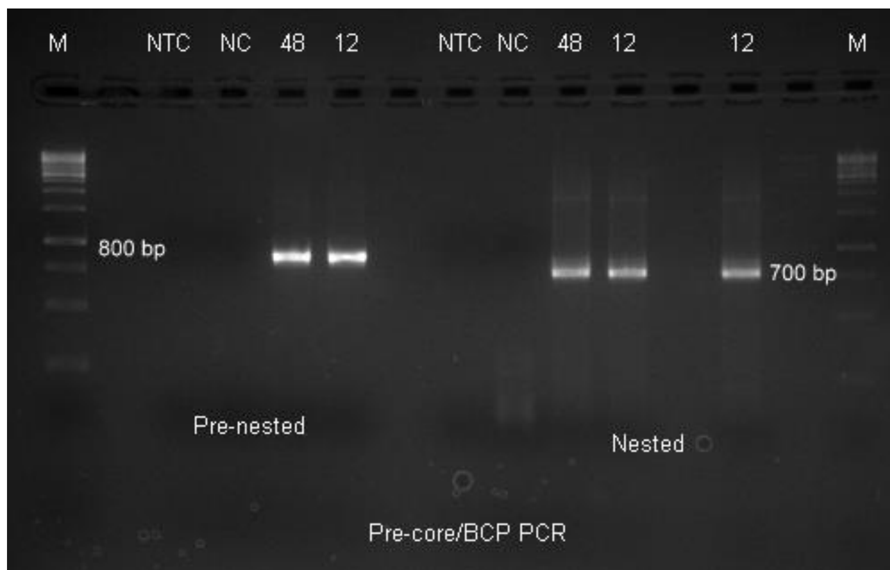

Figure 4. 2: Core clean PCR products of "Week 48"and "Week 0"* samples collected from the Cape Town infant. M: 1kb molecular weight marker; NTC: No Template Control; NC: Negative Control. * represents "Week 0" labeled 12 on the gel.

The DNA products were cleaned up and analyzed on the spectrophotometer to assess the purity and calculate the DNA concentration of the products (See Table 4.13). The DNA concentration of sample 279V07019441 (Week 0) was diluted using DNA nuclease-free water to a concentration of 20ng/µL. A volume of 1µL of each sample, including the NC, was used for the sequencing PCR reaction. Each sequence was amplified with a set of four primers.

Table 4. 13: Concentration and purity of core DNA products

Sample ID	ng/µL	A_{260nm}	$A_{260/280nm}$
NC	19.32	0.386	1.71
350V06029315	29.49	0.59	2.22
262V08006189	23.87	0.477	2.39
279V07019441 (Week 48)	26.64	0.533	1.93
279V07019441 (Week 0)	34.1	0.682	1.9

NC: Negative Control

After the sequencing PCR reaction, the products were cleaned up and analyzed with the ABI Prism 3130*xl* genetic analyzer. The sequences obtained were analyzed, trimmed and aligned. A contiguous sequence of approximately 700 bp long was obtained from each infant. The NC did not amplify, hence no contamination had occurred.

The contiguous sequences obtained were analyzed manually and the K130M/V131I double X mutation was found on both sequences ("Week 0"and "Week 48" sequences) of the Cape Town sample (279V07019441). This mutation is associated with the A1762T/G1764A double mutation on the BCP region of the core gene. The S101P, L116V, L123S, A146S and the P147S mutations were also identified on both sequences, based on analysis of the X gene. No pre-core mutation was found in the infant. Analysis of the BCP sequence from the mother of this infant revealed that she also had the double A1762T/G1764A BCP mutation and no pre-core mutation. No major mutations on the sequences obtained from the Johannesburg (350V06029315) and Durban (262V08006189) samples other than the S101P, L116V, L123S, A146S and the P147S mutations on the core gene.

A phylogenetic tree, showed on Figure 4.3, was constructed using these infant's sequences. The three sequences appeared to be genetically different but all clustered with genotype A sequences. The two sequences 279V07019441 and 279V07019441 mother, representing the Cape Town infant and his mother respectively, appeared to be similar.

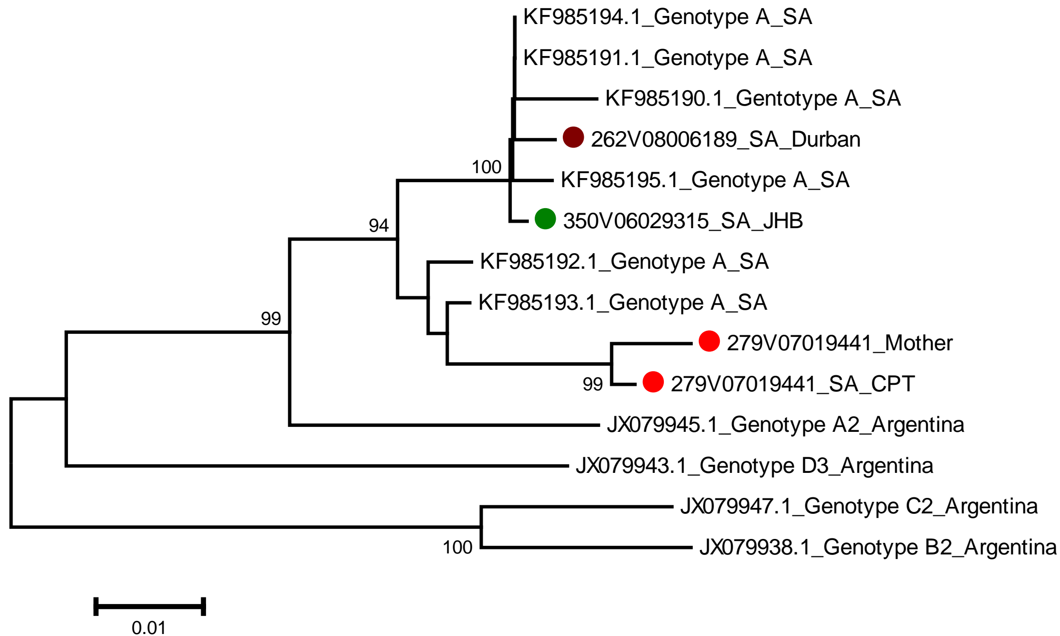

Figure 4. 3: Phylogenetic tree of HBV-infected infants and Cape Town mother with HBV strains belonging to subgenotype A1 based on the core region of the genome.
Sequences with accession numbers starting with JX and KF were downloaded from GenBank.
The evolutionary history was inferred using the Neighbor-Joining method (**Saitou and Nei, 1987**). The optimal tree with the sum of branch length = 0.25525873 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (**Kimura, 1980**) and are in the units of the number of base substitutions per site. The analysis involved 25 nucleotide sequences. There were a total of 883 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (**Tamura *et al.*, 2013**).

4.3.2.2 Nucleotide sequencing of the pre-S ORFs results

On a total of 60 specimens, 42 pre-S sequences were obtained from the pre-S PCR, of which 33 were from the Antenatal Study. Despite numerous efforts, the remaining 18 samples could not be amplified. This was likely to be due to low DNA viral load (HBV DNA viral load < 300 IU/mL). Pre-S sequencing was successful on all 5 samples from the NHLS Paediatric Study. From the transmission study, pre-S sequences were acquired for all samples but the Cape Town mother. Figure 4.4 shows a representation of pre-S positive samples after nested PCR. For each sample, two fragments each of around 1.7kb were amplified during the PCR reaction as seen on Figure 4.4.

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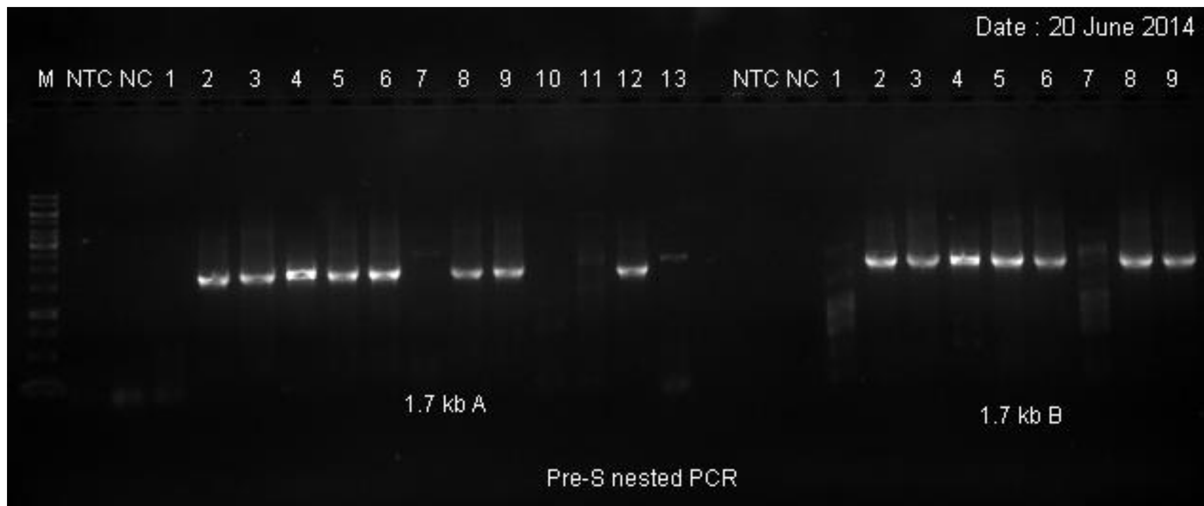

Figure 4. 4: Agarose gel showing successful pre-S amplification. M: 1kb molecular weight marker; NTC: No Template Control; NC: Negative Control; 1.7kb A: segment amplified with P'1 and MD16 primers; 1.7kb B: segment amplified with MD19 and B1as primers.

DNA products were cleaned up then analyzed on the spectrophotometer to assess the purity and calculate the DNA concentration of PCR products. Samples with high DNA concentration were diluted to a final concentration of 20ng/µL using DNA nuclease-free water. A volume of 1µL of each sample, including the NC, was used for the sequencing PCR reaction. For each sample, two fragments were amplified thus for each sample, four sequences were acquired. Following sequencing PCR reaction, the products were cleaned up and analyzed with the ABI Prism 3130*xl* genetic analyzer. The sequences obtained were analyzed, trimmed and aligned. A contiguous sequence of approximately 1700 bp long was obtained from each sample.

All pre-S sequences were aligned with reference sequences received from Guillaume Fallot (personal communication) on Geneious v7.1.5 (Biomatters, New Zealand). Using the alignment, the pre-S1 (119 aa i.e. 357 bp) and pre-S2 (55 aa i.e.165 bp) regions of 522 bp in total were extracted. The extract was analysed manually on BioEdit for the deletions positions.

Analysis of these 42 pre-S sequences revealed 8 sequences with deletions of which 5 were from HIV-infected samples. These 8 sequences, summarized in Table 4.14, originated from women recruited in the Antenatal Study. Four types of pre-S deletions were observed: pre-S1 start codon deletion accompanied with other pre-S1 deletions, pre-S1 deletions alone, pre-S2 deletions alone and pre-S2 start codon deletion accompanied with pre-S1 deletions and other pre-S2 deletions, respectively.

In addition to pre-S deletions, an HIV-uninfected woman (D004659) of 23 years old harbored two pre-S start codon mutations at pre-S1 (ATG/TCA) and pre-S2 (ATG/TCC) leading to a change of the amino acid methionine to serine (M/S) at both positions.

Table 4. 14: Summary of samples with pre-S region deletions

Sample ID	Age (years)	Genotype	HIV status	Pre-S region, aa	Deletion type
300685	36	D	Infected	1-11	Type 1
300843	25	A1	Infected	67-97	Type 2
300030	33	A1	Infected	133-141	Type 3
D004411	28	A1	Infected	135-141	Type 3
303962	27	A1	Infected	119-120, 137-142	Type 4
300214	26	D	Uninfected	1-11	Type 1
D005219	20	A1	Uninfected	2-7	Type 1
300768	29	A1	Uninfected	40, 48-50, 54-97	Type 2

aa: amino acid

All sequencing data on the pre-S, BCP and pre-core regions acquired from all three studies were analyzed together. The following sections will be presenting results from this analysis.

4.3.2.3 Analysis of pre-core and BCP/X gene mutations

Although only 42 pre-S sequences were acquired from pre-S sequencing, pre-core and BCP/X sequences were acquired from all 60 samples included in the study (See Table 4.15).

In the Antenatal Study, six women had the double BCP A1762T/G1764A mutations and no pre-core mutations; six showed the BCP T1753C mutation combined to the double BCP mutants with two also harbouring the A1896T pre-core mutant. These two women were each genotype D and genotype A1. Three patients had the C1766T/T1768A BCP mutations and no pre-core mutation. A 34 year old woman had the combined T1753C, A1762T/G1764A, and C1766T/T1768A BCP mutations accompanied with the A1896T mutation and the stop codon G1899A mutation in the pre-core region. Another 32 years old women had the G1764A and C1766T/T1768A BCP mutations and no pre-core mutant.

Eight HIV-infected women harboured different mutations profiles. These women had no BCP mutations but showed two types of pre-core mutations: six mutations at the initiation codon (1816: M1R or M1L) of the pre-core region and a stop codon at position 1872 (K21*).

The three mothers from the Paediatric Study showed no BCP or pre-core mutations. The core sequences of the Cape Town infant at "Week 0" and "Week 48" from the transmission study revealed the double A1762T/G1764A BCP mutation and no other mutation on the pre-core region. The Cape Town mother had the same double BCP mutation as her child and no pre-core mutation.

The remaining twenty four samples of the cohort were HIV-uninfected. These consisted of twenty women from the Antenatal Study, two babies from the Paediatric Study and two babies from the transmission study. All four babies displayed no BCP or pre-core mutations in their core sequences. However among the twenty women, three had the double A1762T/G1764A BCP mutation, one C1766T/T1768A BCP mutation, two combined G1764A and C1766T/T1768A mutations and one combined T1753C and A1764T/G1764A BCP mutations. Out of the three women harbouring the double A1764T/G1764A BCP mutations, two also had pre-core mutations: the stop codon G1899A and a mutation at the start codon (position 1816). The women harbouring the G1899A stop codon was infected with HBV genotype D strain.

These pre-core and BCP/X gene mutations were analysed with the pre-S deletions observed in the cohort. Samples harbouring these mutations in combination are summarized in the table below, Table 4.16.

Table 4. 15: Samples with BCP/X and pre-core mutations

Sample ID	HIV status	Age	Genotype	BCP	Pre-C
300030	INFECTED	33 years	A1	A1762T/G1764A	NONE
300632	INFECTED	28 years	A1	C1766T/T1768A	NONE
300685	INFECTED	36 years	D	T1753C, A1762T/G1764A	G29D
300843	INFECTED	25 years	A1	T1753C, A1762T/G1764A	NONE
301066	INFECTED	22 years	A1	T1753C, A1762T/G1764A	NONE
301073	INFECTED	31 years	A1	A1762T/G1764A	NONE
301578	INFECTED	28 years	A1	T1753C, A1762T/G1764A	G29D
303374	INFECTED	26 years	A1	NONE	M1L
303484	INFECTED	32 years	A1	NONE	M1L
303557	INFECTED	22 years	A1	C1766T/T1768A	NONE
303962	INFECTED	27 years	A1	A1762T/G1764A	NONE
D000504	INFECTED	32 years	A1	G1764A, C1766T/T1768A	NONE
D000756	INFECTED	36 years	A1	NONE	M1L
D000764	INFECTED	27 years	A1	NONE	M1L
D002436	INFECTED	36 years	A1	NONE	K21*
D002473	INFECTED	37 years	A1	A1762T/G1764A	NONE
D002526	INFECTED	22 years	A1	T1753C, A1762T/G1764A	NONE
D002911	INFECTED	21 years	A1	NONE	M1R
D003934	INFECTED	34 years	A1	T1753C, A1762T/G1764A, C1766T/T1768A	G29D, W28*
D004066	INFECTED	30 years	A1	T1753C, A1762T/G1764A	NONE
D004411	INFECTED	28 years	A1	NONE	M1L
D004528	INFECTED	19 years	A1	A1762T/G1764A	NONE
D005512	INFECTED	22 years	A1	C1766T/T1768A	NONE
279V07019441	INFECTED	12, 60 weeks	A1	A1762T/G1764A	NONE
Cape Town mother	INFECTED	34 years	A1	A1762T/G1764A	NONE
300032	UNINFECTED	27 years	A1	NONE	M1L
300706	UNINFECTED	27 years	A1	A1762T/G1764A	NONE
301433	UNINFECTED	28 years	A1	NA	V17L
302490	UNINFECTED	26 years	A1	NONE	M1L
302798	UNINFECTED	25 years	A1	NONE	M1L
302977	UNINFECTED	36 years	D	A1762T/G1764A	W28*
303153	UNINFECTED	28 years	A1	C1766T/T1768A	NONE
303521	UNINFECTED	38 years	A1	A1762T/G1764A	M1L
D004015	UNINFECTED	31 years	A1	NONE	M1L
D004483	UNINFECTED	27 years	A1	T1753C, A1764T/G1764A	NONE
D004659	UNINFECTED	23 years	A1	G1764A, C1766T/T1768A	NONE

NA: Not Available

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Table 4. 16: Summary of sample with combined BCP, pre-core mutations and pre-S deletions

Sample ID	HIV status	Age (years)	Genotype	BCP mutations	Pre-C mutations	Pre-S deletions
300030	INFECTED	33	A1	A1762T/G1764A	NONE	Type 3
300685	INFECTED	36	D	T1753C, A1762T/G1764A	G29D	Type 1
300843	INFECTED	25	A1	T1753C, A1762T/G1764A	NONE	Type 2
301578	INFECTED	28	A1	T1753C, A1762T/G1764A	G29D	None
303962	INFECTED	27	A1	A1762T/G1764A	NONE	Type 4
D003934	INFECTED	34	A1	T1753C, A1762T/G1764A, C1766T/T1768A	G29D, W28*	NA
302977	UNINFECTED	36	D	A1762T/G1764A	W28*	NA

NA: Not available
BCP: Basal Core Promoter
Pre-C: Pre-core

4.4 Summary of findings

In summary, none of the paediatric samples were harbouring pre-S mutations or a combination of all pre-S, BCP and pre-core HBV mutations. However, eight maternal samples of which five were HIV-infected and three were HIV-uninfected harboured pre-S deletions. Moreover, a higher rate of combination of these mutations was found in HIV-infected (6/7) as compared to HIV-uninfected mothers (1/7). In the following chapter, the interpretation of these results and the implications of these findings will be discussed.

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CHAPTER 5: DISCUSSION

This study has found an HBV infection prevalence of 0.6% (5/850) in a cohort of 850 HIV-exposed infants. The HBsAg prevalence was 0.4% (3/850) whilst two of the five infants had evidence of past exposure to HBV. Of the three HBsAg positive infants, one harboured the mutation M204I associated with lamivudine-drug resistance. Unfortunately, this child was lost to follow up. A second infant who was followed up was HIV-infected, HBsAg positive and HBeAg negative. This child had been on ART treatment for a period of approximately 2 years. The mother of this child was also HBsAg positive and HBeAg negative. The mother-infant pair had similar core gene sequences, harbouring the double A1762T/G1764A BCP mutation. The latter has been well described in CHB and HCC patients and has been associated with the evolution of primary liver malignancy. These observations provided evidence of transmission of mutant viruses from mother to child and led to the investigation of HCC-related mutations in antenatal women with a risk of perinatal transmission. The screening of antenatal samples in this study revealed 15% (8/53) of women harbouring pre-S, BCP and pre-core mutations which have previously been described as high risk factors of development of liver tumours in Asia. Five of these women were HIV-infected, suggesting a possible impact of HIV on HBV. This is the first study reporting the transmission of mutant virus from mother to child and describing the presence of HCC-related mutations in antenatal women.

HBsAg prevalence

A total HBsAg prevalence of 0.4% (3/850) was detected in this cohort of HIV-exposed infants. These results are similar to results obtained in a previous paediatric study conducted in the Division of Medical Virology, Stellenbosch University. One thousand HIV-exposed babies below 18 months of age were screened for HBV infection, of whom 0.3% (3/1000) were positive for HBsAg. Using antenatal HBsAg prevalence in pregnant women **(Andersson *et al.,* 2013)** and the HBsAg prevalence in infants in that study, the rate of MTCT was about 12% **(Chotun, BN, MSc Thesis, Stellenbosch University, 2012)**. However, the observed prevalence in infants was higher than that reported in a recent study conducted by Hoffmann *et al.* 2014. The latter was a prospective study which followed up 189 HIV/HBV co-infected pregnant women and their babies in Soweto, Gauteng, SA. One

hundred and eighty four women were enrolled in the PMTCT programme to prevent perinatal child transmission of HIV prior to presenting in labour. Of these women, 23% (44/184) received zidovudine monotherapy, 10% (20/184) received stavudine or zidovudine and 63% (120/184) were on TNF. Fourteen (7.4%) of these mothers were found positive for HBsAg and these 14 mother-child pairs were followed up. An HBsAg seropositivity of 7.14% (1/14) was described among the babies born of those 14 HIV/HBV co-infected mothers. The mother of this HBsAg positive infant, although on an ART regimen which included stavudine, 3TC and efivarenz, was HBeAg positive and had an HBV DNA viral load of $\log_{10}$ 8.3 IU/mL prior to delivery. This infant did not receive any immunization against HBV at birth and was only vaccinated at six weeks of age **(Hoffmann *et al.,* 2014)**. The low prevalence of HBsAg described in these HIV-exposed infants could be due to the fact that 63% were on TNF and a further 10% were likely to be on 3TC, both of which have anti-HBV activity.

Earlier studies have described a higher HBsAg prevalence in children older than five years when compared to younger infants. The high HBV prevalence in children and adolescents was associated with horizontal transmission **(Vos *et al.,* 1980; Prozesky *et al.,* 1983; Abdool Karim *et al.,* 1988)**. This was used as a background for the administration of the first dose of the HB vaccine at the age of six weeks within the EPI in SA. However, Vardas and colleagues described an HBsAg prevalence which had not been previously observed in infants in SA, suggesting that perinatal transmission might be more important than previously thought. They conducted a large seroepidemiological study in unvaccinated rural and urban children from 0 to 6 years of age with the aim of determining the age of acquisition of HBV infection in SA. The highest HBsAg seroprevalences, of 8.1% and 8.9%, were reported in infants in the age groups of 0 – 6 months and 7 – 12 months, respectively **(Vardas *et al.,* 1999)**. The high prevalence of HBsAg in infants aged below one year of age suggested that perinatal transmission should not be an underestimated mode of HBV transmission.

Furthermore, the HBsAg prevalence observed in the current study is different to what was observed in the earliest years of the HB vaccine era. Westwood tested 326 children below 14 years of age, all of whom tested negative for HBsAg **(Westwood, 2001)**. The same HBsAg prevalence was reported by Tsebe *et al.* who tested 578 babies aged between 8 to 72 months **(Tsebe *et al.,* 2001)**. Results from these two studies have shown the positive impact of the HB vaccine on the incidence of HBV in children in SA. The studies by Westwood and Tsebe *et al.* investigated the general paediatric population without specifying HIV status, while this

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study adds to the current literature as it has investigated the impact of HIV exposure on HBV prevalence.

Mother to child transmission

Pre-HB vaccine studies reported a higher prevalence of HBsAg among children older than 5 years old of age as compared to infants below one year old (**Prozesky *et al.,* 1983; Botha *et al.,* 1984**). MTCT was then considered as a neglected risk of transmission in the South African population. Vardas *et al.* reported a high HBs antigenemia in unvaccinated infants and suggested that MTCT might be underestimated as a contributing factor to the endemicity of HBV (**Vardas *et al.,* 1999**).

Thirty years ago, in Namibia, Botha *et al.* **(Botha *et al.,* 1984)** described a greater risk of HB perinatal transmission in mothers positive for both HBsAg and HBeAg as compared to HBsAg positive but HBeAg negative mothers. They found that 63% (12/19) of mothers positive for both HBsAg and HBeAg had HBsAg positive children compared to 17% (16/92) of mothers who were positive for HBsAg and anti-HBe. The authors took into consideration that mothers with older children might have been HBeAg positive at the time of pregnancy and could have seroconverted, and only determined the prevalence of HBeAg among mothers who were not expected to have seroconverted. Thus, among mothers with children aged less than 2 years old, 16% (16/97) were HBeAg positive. Cord blood testing revealed an 80% (12/15) HBsAg prevalence in unvaccinated babies born to 15 HBsAg positive women. (**Botha *et al.,* 1984**). This report triggered a series of investigations which led to the implementation of the HB vaccine in SA based on the importance of horizontal transmission only. Since then, many studies have reported the efficacy of the HB vaccine but none have specifically looked at perinatal transmission of HBV until recently by Hofmann *et al.,* 2014. The authors tested 189 HIV-infected women for HBV markers and 14 were found HBsAg positive, of whom 6 were also HBeAg positive. They tested the infants born from those 14 women and reported four vertical transmissions. Among these four infants, one was both HBsAg and HBV DNA positive and the three others were HBV DNA positive but HBsAg negative. The authors also mentioned that due to the unavailability of routine HBV immunization at birth, no intervention was performed on these infants (**Hoffman *et al.,* 2014)**; however 97.4% mothers were on anti-HBV therapy as part of PMTCT.

In this study, one case of MTCT was confirmed through analysis of the core sequences from both mother and child originating from Cape Town. Analysis of the *pol/surface* region would have been the best comparison between the mother-pair sequences. However, due to unsuccessful *pol/surface* sequencing for the mother, the core sequences were used. Both sequences had similar core protein sequences and were shown to be very close to each other on the phylogenetic tree (Figure 4.3). This case is very likely to represent MTCT. Unfortunately, information neither on the mothers HBsAg status, nor on the PMTCT regimes are unknown.

This study highlighted that vertical transmission may be an important route of transmission particularly in HIV/HBV co-infected women. Immuno-suppression is one mechanism by which HIV hastens HBV pathogenesis by delaying HBsAg and HBeAg seroconversion and increasing HBV replication **(Thio, 2009)**. A recent antenatal study observed a loss of immune control and high prevalence of both HBeAg (18.9%) and HBsAg (3.4%) in 1543 HIV-infected pregnant women compared to HIV-uninfected women, in the Western Cape **(Andersson *et al.,* 2013)**. As mentioned earlier (Section 2.1), HBeAg is a marker of active viral replication and is often used as a marker of infectivity. This antigen has been also proven to cross the placenta to infect the foetus **(Wang & Zhu, 2000)**. HBeAg positivity and high HBV DNA viral loads have been associated with higher chance of perinatal transmission by Dwevedi *et al.* They studied 4000 women with the aim of determining the risk factors for vertical transmission of HBV. Of these women, 0.9% (37/4000) tested seropositive for HBsAg and 56.8% (21/37) of the HBsAg positives were HBeAg positive. Babies born from these HBsAg positive mothers were also tested for HBV infection. MTCT occurred in 65% (13/20) and 9.1% 91/11) of babies born from HBeAg and HBV DNA positive mothers and HBeAg and HBV DNA negative mothers, respectively **(Dwevedi *et al.,* 2011)**. Furthermore, a study from Kwazulu-Natal investigated the burden of HBV in HIV-infected and HIV-uninfected women. The study included 570 pregnant women, of whom 215 were HIV-infected. Sixteen (7.4%) of these HIV-infected women were HBsAg positive and among them six (37.5%) were HBeAg positive. In the HIV-uninfected group, fourteen (4.8%) were carrying HBsAg but none were HBeAg positive **(Thumbiran *et al.,* 2014)**. The higher presence of HBeAg in the HIV/HBV co-infected group would suggest high viral loads and hence constitute a significant reservoir as compared to HBV mono-infected women, putting the HIV/HBV co-infected women at greater risk of transmitting HBV to their infants.

The introduction of the HB vaccine has brought a major change in the epidemiology of hepatitis B in many regions of the world including SA. However, the benefits of the vaccine could be improved if the first dose was delivered within 24-hours of birth as recommended by the WHO instead of six weeks of age as is currently the case. Thumbiran and colleagues advocated for the implementation of antenatal screening for the identification of all HBV-exposed infants hence allowing active immunization at birth for those babies (**Thumbiran *et al.,* 2014**). Administration of the HB vaccine immediately after birth has been proven necessary to avoid vertical transmission in infants at risk in Asia (**Wong *et al.,* 1984; Lee *et al.,* 2006b**).

Anti-HBs prevalence

Anti-HBs testing of all infants revealed that 474 out of 850 (55.8%) of the babies had protective anti-HBs levels (>10mIU/mL). Tsebe *et al.* tested the efficacy of the HB vaccine after its implementation and focused on vaccinated children aged between 8 months and 6 years old. Approximately 86.8% of these children presented with protective anti-HBs titres. In comparison to the current study, the anti-HBs positivity described in that study is higher but may be due to that the study was conducted in HIV-unexposed infants as compared to the current study (**Tsebe *et al.,* 2001**). This observation was confirmed thirteen years later by a pre- versus post-HBV immunization analysis of 1206 children aged one to twenty five years. These patients were stratified by age into pre- and post-vaccine introduction and the two groups were compared for evidence of immunity and chronic carriage. The analysis revealed (1) an overall increased immunity to HBV infection from 13% to 57% which decreased with increasing age due to the waning levels of anti-HBs and, (2) a decreased HBV chronic carriage from 4.2% to 1.4% (**Amponsah-Dacosta *et al.,* 2014**).

However, 376 infants, 44.2% (376/850) had a low anti-HBs seroprotective status (anti-HBs< 10 IU/mL). Simani *et al.* investigated the prevalence and exposure to hepatitis B in vaccinated babies aged between 5 and 24 months. Three hundred and three children, of whom 243 were from the EPI clinic and 60 from a paediatric outpatient clinic (OPD), were included. Twenty four per cent of these babies were HIV-infected and had a lower rate of seroprotection as compared to the HIV-uninfected group (78.1% vs. 85.7%; p=0.125). The low rate of seroprotection in the HIV-infected group could have been a result to immune

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suppression rather than a failure to the HB vaccine (**Simani *et al.,* 2009**). HIV status of the babies tested in this study was unknown, so it is not clear whether the low levels of seroprotection of the 376 babies was due to the immune suppression or poor immune response as a result of HIV infection or was just an indicator of the waning levels of anti-HBs with time after vaccination.

Furthermore, among infants with non-protective anti-HBs levels, 22.1% (83/376) of the babies in this study had a very poor response to the HB vaccine characterized by anti-HBs titres less than 1mIU/ml. A nonresponse to HB vaccine has been previously observed in HIV-exposed infants. Abramczuk and colleagues investigated the difference in the humoral response to HB vaccination between 45 HIV-exposed, uninfected (HEU) and 112 HIV-unexposed infants. They observed that 6.7% (3/45) of the HEU infants did not respond to the HB vaccine (anti-HBs titre, <10 mIU/ml) as compared to only 3.6% (4/112) nonresponders in the HIV-unexposed group (**Abramczuk *et al.,* 2011**). Such observations have been explained by the effect of HIV on thymic maturation in HEU infants which lead to an immature immune system. These infants show decreased CD4 T cell numbers but increased CD8 T cells due to the interaction between HIV soluble particles through placental transfer from the mother and the infant's immune response *in-utero* (**Clerici *et al.,* 2000**). That interaction triggers (1) a minimal T cells immune response in the infant preventing intrauterine infection and, (2) other defects such as abnormalities in antigen presenting cells (**Velilla *et al.,* 2008**) and low naïve CD4 T cell counts (**Nielsen *et al.,* 2001**).

The observations regarding the immaturity of the neonatal immune system of HEU infants described above could thus explain the rate of nonresponse to HB vaccine in the 83 infants, that is, 9.8% of the cohort in this study.

HBV genotyping and phylogenetic analysis significance

Genotyping and phylogenetic analysis were performed on samples positive for HBV DNA to assess the molecular diversity of the HBV strains present in this cohort. Three samples positive for HBV DNA were sequenced to determine the genotype of the HBV strains present in this cohort. Sequencing of the *pol/surface* region of the HBV genome revealed that all the HBV strains belonged to genotype A, subgenotype A1. This subgenotype is considered to be the most common HBV strain in circulation in the country (**Bowyer *et al.,* 1997; Kimbi *et***

***al.,* 2004; Kramvis & Kew, 2007)**. However, very few sequences have been described in patients from the Western Cape Province, in particular HIV-exposed infants.

Analysis of these *pol/surface* sequences also revealed a drug-resistance mutation in one of the three HBsAg positive infants. This infant harbored a mutation in the RT domain of the *polymerase* gene at position 204 (rtM204I). This mutation is found on the catalytic or C domain also called the YMDD locus of the *polymerase* gene and has been associated to resistance to the drug 3TC **(Stuyver *et al.,* 2000)**. Mutations in the YMDD locus related to 3TC resistance have been previously reported in HBV mono-infected treatment-naïve and those treated with 3TC in SA **(Selabe *et al.,* 2007)**. To date, no data is available regarding transmission of HBV drug-resistant mutant viruses from mother to child. This is the first study, reporting possible vertical transmission of a drug-resistant mutation.

Analysis of the phylogenetic tree constructed with the *pol/surface* sequences showed that they clustered with other South African subgenotype A1 sequences. At follow up the child was found to be HBsAg positive. He is therefore a chronic carrier. Chronic HBV carriers are at risk of developing HCC. Lin and Kao reported that HBV genotype A-infected patients have a higher tendency of developing HBV chronicity and hence have a higher rate of HCC development **(Lin & Kao, 2011)**. Studies in SA, where genotype A predominates, have described a high rate of HCC in patients with HBV genotype A infections as compared to patients with non-A HBV infections **(Kew *et al.,* 1979; Kimbi *et al.,* 2004; Kew *et al.,* 2005)**. In Southern Africa, the highest HCC rates have also been shown in patients under the age of 30 as compared to those over 60 years of age (73.5% vs. 28.6%) **(Kew *et al.,* 1979)** and males are at a greater risk of developing the cancer as compared to females **(Kew *et al.,* 1983)**.

This study has described one case of perinatal transmission where the mother had transmitted a mutant virus. The transmitted virus had the double T1762/A1764 BCP mutation which has been described in chronic and HCC patients. The latter mutation has been associated with a high risk of developing HCC. A high prevalence of BCP and pre-core mutations, also associated with HCC, has been reported in HIV-infected compared to HIV-uninfected pregnant women in Cape Town, Western Cape **(Maponga, TG, MSc Thesis, Stellenbosch University, 2012)**. In addition to BCP and pre-core HBV mutations, pre-S

deletions/mutations have also been associated to HCC development in Asia where genotypes B and C predominate. These mutations were thus investigated in a cohort of HIV-infected and HIV-uninfected women and in HIV-exposed infants.

Pre-S mutations/deletions prevalence and significance

There has been an on-going accumulation of pre-S variants in the sera and malignant tissues in HCC patients observed in Asia, a highly HBV endemic region. The changes brought about by these deletions have been shown to trigger a cascade of events leading to genomic instability and abnormal proliferation of liver cells **(Wang *et al.,* 2006)**. Although these mutants are well recognized in Asia, where HBV genotypes B and C predominate, as marker of HCC development, the prevalence and association of these mutants with HCC remain unknown with regards to other HBV genotypes. Makondo and colleagues have previously described the presence of pre-S mutants in a cohort of HIV/HBV co-infected individuals. However, they did not discuss the meaning of the mutations **(Makondo *et al.,* 2012)**. Based on the importance of these mutations in Asia and background data on the presence of these variants in HIV/HBV South African individuals, this study investigated their epidemiology in an antenatal setting.

The prevalence of pre-S mutants was assessed on 60 samples recruited from three studies: an antenatal study conducted during the period 2010-2012 **(Maponga, TG, MSc Thesis, Stellenbosch University, 2012)**, a paediatric study conducted during 2011-2012 **(Chotun, BN, MSc Thesis, Stellenbosch University, 2012)** and the current study.

None of the samples from the current study or any of the paediatric samples harboured pre-S mutants but 8(8/59 (13.6%)) samples, from the Antenatal Study, were harbouring pre-S mutations/deletions at the 5′end of the pre-S1 region and 3′end of the pre-S2 region. Asian studies have investigated mutations in individuals with CHB and severe liver diseases such as cirrhosis and HCC. An accumulation of pre-S mutations during the course of CHB was observed **(Fan *et al.,* 2001)** and the association between pre-S deletions/mutations with the development of HCC has been established **(Chen *et al.,* 2006; Yeung *et al.,* 2011)**. Among the pre-S mutations found in CHB or HCC patients, a high proportion of pre-S2 mutations compared to pre-S1 mutations were found. The latter were confirmed to be found in type II

ground glass hepatocytes (GGHs) whereas pre-S1 mutants are found in type I GGHs, a histological hallmark of CHB **(Wang *et al.,* 2003)**.

Unlike the high prevalence of pre-S2 mutations observed in the Asian population, a high proportion of the pre-S mutations observed in this cohort were found in the pre-S1 region. Three samples of which two were HIV-uninfected showed pre-S1 start codon deletion and one HIV-uninfected sample had a pre-S1 start codon point mutation (ATG/TCA). This codon is responsible for encoding the LHBs protein hence its deletion or a point mutation at that position could abrogate synthesis of the L protein. The SHBs and LHBs surface proteins together are necessary for virion production **(Bruss & Ganem, 1991)** hence the lack of LHBs proteins in these samples would decrease the formation of virions. However, wild type viruses were found to co-exist with pre-S1 deletions strains to compensate for the defectiveness of those mutants to produce LHBs proteins hence sustaining the life cycle of the virus **(Melegari, Bruno & Wands, 1994)**. Other than being important for the synthesis of LHBs proteins, the pre-S1 region contains B and T cell epitopes and other functional sites such as the hepatocyte binding site (aa 21-47); the transactivator domain (aa 21-90), the transcriptional binding sites and the nucleocapsid binding site **(Chen *et al.,* 2006)**. Pre-S1 deletions at aa 54-77 and 67-97 were found in samples 300768 and 300843 respectively, partially overlapped with the region involved in virion secretion (aa 3-77) during the infection step of the life cycle of the virus. Hence, hepatocytes bearing these deletions could have a mild defect in HBsAg secretion as proven by Le Seyec *et al.* **(Le Seyec *et al.,* 1999)**. One HIV/HBV co-infected sample had a deletion of aa 119 which is found in the nucleocapsid binding site. This domain is responsible for the virus morphogenesis thus deletion in this region could have an impact on virion formation **(Bruss, 1997)**.

Although the number of pre-S1 mutants is higher, three samples (300030, 303962 and D00441) showed pre-S2 deletions. These samples presented deletions from aa 133 to aa 142 approximately. In addition to this deletion, sample 303962 also had a pre-S2 start codon deletion. The latter could lead to a decrease in the formation of MHBs proteins whose deficiency would result in an accumulation of LHBs and formation of type II GGHs **(Wang *et al.,* 2003)**. The latter could also happen in case of point mutations at the pre-S2 start codon like the one found in an HIV-uninfected sample from this study (ATG/TCC). Furthermore, the deleted pre-S2 region in these sample's' sequences covers a big part of the B epitope situated in the pre-S2 region (aa 120-145). These deletions were thought to be a mechanism

of escape of the virus from the immune B cells causing an accumulation of LHBs in the ER of type II GGHs (**Wang *et al.*, 2003**). Persistence of these oxidative products may deactivate certain signals involved in the suppressions of tumours or oncogenes, resulting to cellular transformation and HCC. Hsieh *et al.* presented a model explaining hepatocarcinogenesis whereby accumulation of unfolded LHBs causes oxidative stress and oxidative DNA lesions which in turn activates the DNA repair mechanism. These DNA lesions prompt mutagenesis which possibly leads to genomic instability (**Hsieh *et al.*, 2004**). Five of eight of the pre-S mutations/deletions positive samples of the studied cohort were from HIV/HBV co-infected women. The high rate of pre-S mutations among HIV/HBV co-infected individuals observed in this cohort is in accordance with Makondo *et al.* who characterized HBV strains in 71 HIV/HBV co-infected individuals from Southern Africa. Five of the seventy one (7.04%) patients had pre-S mutants (**Makondo *et al.*, 2012**). Furthermore, we observed that pre-S2 deletions were all found in HIV-infected samples with the exception of the sample harbouring the pre-S2 start codon point mutation. Andersson *et al.* reporting on these women, showed a loss of immune control over HBV in HIV-infected women. The formation of pre-S2 mutants could be a mechanism to escape immune surveillance in these HIV/HBV co-infected women. Moreover, it was observed that the HIV-infected women with pre-S2 deletions had low HBV DNA titres < 1500 IU/mL). This is in accordance with Fan *et al.* who observed that pre-S2 mutants prevailed over pre-S1 mutants during the low or non-replicative phases of the chronic infection (HBV viral load $<10^6$ genomes/mL) (**Fan *et al.*, 2001**).

Pre-S deletions have always been thought to be more predominant in genotype B and C due to the high number of reports of these mutations in Asian and Korean studies where HBV genotype B and C strains predominate. In this study, pre-S deletions/mutations were found in 2 samples from genotype D and 6 genotype A1 samples.

Combination of BCP, pre-core and pre-S mutations/deletions prevalence and significance

A pattern of combination of BCP, pre-core and pre-S mutations was observed among seven (12%) samples in our cohort. Each of these mutations has been previously found in CHB patients as well as HCC patients and is well recognized as risk factor for primary liver cancer.

Kramvis *et al.* investigated the pre-core gene in 71 black African sera and 16 additional black African tumorous and non-tumorous liver tissues from HCC patients for the identification of mutations that might play a role in hepatocarcinogenesis. Five sera harboured the stop codon at nucleotide 1896 (W28*), preventing the synthesis of HBeAg and 10 had a mutation at nucleotide 1899 (G to A), of which 3 also had the 1896 stop codon. The authors concluded that further investigations of the X and *pol* genes were necessary to have a full understanding of the lack of HBV DNA replication in HCC patients **(Kramvis, Kew & Bukofzer, 1998)**.

In another South African study, a year later, the BCP and enhancer II region in HCC patients and HBV asymptomatic carriers (ASCs) were investigated for the presence of mutations which might cause the interruption of HBeAg production and contribute to hepatocarcinogenesis. The authors recruited 52 ASCs and 59 HCC patients. The authors described the double A1762T/G1764A BCP mutation in 33 (66%) patients with HCC compared to only 5 (11%) of HBV asymptomatic carriers. The sera harbouring this mutation appeared to have a significant decrease in HBeAg titres **(Baptista, Kramvis & Kew, 1999)**. Although these types of mutations have been described in South African HCC patients, the effect of the combination of mutations has not been investigated. In Asia, the presence of mutations in the BCP and pre-core regions serve as a predictor of HCC development **(Tong *et al.*, 2006, 2007)**. The risk for hepatocarcinogenesis is shown to highly increase with the combination of pre-S deletions, T1762/A1764, and A1899 rather than the presence of a single mutation, although each is an independent risk factor **(Chen *et al.*, 2008)**.

Extrapolating from observations from Asian studies, it could be hypothesized that the seven samples with these combined mutations at the BCP, pre-core and pre-S regions of the HBV strains are at an increased risk of developing HCC as compared to the ones harbouring single mutations. However, a longitudinal study would be required to test this hypothesis.

The chronic HBV HIV-exposed infant from Cape Town presented in chapter 3, was found to have the double T1762/A1764 BCP mutation at 12 weeks of age. Based on the hypothesis that different HBV mutations appear with time at different replicative phases of CHB, the presence of the double BCP mutation at such an early age in this infant could be used as a marker of prediction of hepatocarcinogenesis at a later stage of the disease.

The mutations associated with a high risk of liver cancer found in this cohort (antenatal and paediatric) could be used as markers of risk of HCC development, as has previously been

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done in Asia, thus allowing a better screening of CHB patients for those at high risk of HCC. Further work is needed to explore this hypothesis.

Strengths and limitations of study

The strengths of this study were the large sample size available for screening and study design. Moreover, because perinatal transmission has been underestimated in SA, this study is the first to report on MTCT of mutant viruses and to report on the prevalence of HCC-related mutation in an antenatal setting.

However, the study had a few limitations which could affect the interpretation of these results. The absence of maternal ART information would have been useful to determine if the reduced maternal HBV viral load had an impact on the HBsAg prevalence described in these HIV-exposed infants. Furthermore, maternal HBV status was unknown and could have helped to determine the risk of perinatal transmission. Lastly, the samples were collected more than two years ago from three different locations and patients were lost to follow up, making a prediction of chronic infection impossible. We were only able to trace and follow up one HBV-infected infant.

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CHAPTER 6: CONCLUSION

This study has found an HBV infection prevalence of 0.6% (5/850) in a cohort of HIV-exposed infants. Only one infant was able to be contacted for follow up and showed persistence of HBsAg. He was likely to have been infected vertically. Phylogenetic analysis revealed similarities between the mother and infant pair sequences on the core region of the HBV genome with a mutation in the BCP domain. No mutation related to drug resistance or immune escape was found in the infant at 14 months of age. As he was HIV positive, he had been on an ART regimen including 3TC for approximately two years with no detectable viral load at follow up. However, the similarity between the mother and baby pair sequences with the same BCP mutation was evidence of the transmission of a mutant virus from the mother to her child.

A number of factors such as HBeAg positivity, high HBV DNA viral load **(Dwevedi *et al.,* 2011)** and HIV status **(Thumbiran *et al.,* 2014)** have been associated with an increased risk of vertical transmission in HBV-infected pregnant women. However, these variables were unknown at the screening , but the fact that vertical transmission occurred suggests that the mother could have been positive for HBeAg with a high viral load at the time of birth. Although this study only reports few infant HBV infections, five other studies conducted in SA have reported the high risk of vertical transmission in HIV-infected pregnant women with high HBV viral load **(Burnett *et al.,* 2007; Andersson *et al.,* 2012, 2013; Hoffman *et al.,* 2014; Thumbiran *et al.,* 2014)**. This calls for the importance of screening pregnant women for HBV markers and possibly HBV viral load during their pregnancy with the aim of suppressing viral load and reducing the risk of transmitting HBV infection to their infants. Moreover, HB vaccination around the time of birth, as recommended by the WHO, reduces the risk of acquiring infection. HB vaccination at birth has been shown to reduce HBV transmission from in SSA as compared to a 6-week dose of the vaccine **(Ekra *et al.,* 2008)**. Longitudinal studies, assessing the impact of screening and treating pregnant women and administering birth dose vaccine on HBV perinatal transmission, need to be performed to show the reduction of mother to child transmission in Africa.

Furthermore, the BCP mutation found in the followed up infant, transmitted from the mother, led to the questions of whether these mutations could be transmitted from mothers and whether HIV could impact on the evolution of these mutants so they could be at greater

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risk of HCC. The transmitted mutation, A1762T/G1764A BCP mutation has been shown to be a risk factor for liver tumour development in CHB patients in Asia. Other HCC-related mutations in the pre-S and pre-core regions were described in antenatal women at high risk of perinatal transmission. The combination of these mutations has been established as a higher risk of liver malignancy formation as compared to single mutations **(Chen *et al.,* 2008)**. Based on the evidence that mutant virus could be transmitted vertically, this leaves the infants born from these mothers at high risk of acquiring these mutations hence at a greater risk of developing liver tumours. This study provides preliminary results which need to be confirmed with further longitudinal studies to determine whether there is an increased risk for transmission of these mutant viruses to infants and whether they might increase the risk of HCC.

Perinatal HBV transmission of mutant virus, a problem that has not been well-described in HBV infected children in SA, is described in this study. Furthermore, the higher presence of HCC-related mutations in HIV-infected pregnant women as compared to HIV-uninfected represents an important public health problem which deserves further study. More work is needed to (1) determine why a higher prevalence of HCC-related mutations was shown in HIV-infected women as compared to HIV-uninfected and, (2) to determine the risk of HBV transmission in infants born from HBV mono-infected as compared to HIV/HBV co-infected women. It is also crucial to assess how HBV antenatal screening and a birth dose of the HB vaccine could be introduced in SA for a better control and possible eradication of mother to child transmission in SSA.

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REFERENCES

Abdool Karim, S.S., Coovadia, H.M., Windsor, I.M., Thejpal, R., van den Ende, J. & Fouche, A. 1988. The Prevalence and Transmission of Hepatitis B Virus Infection in Urban, Rural and Institutionalized Black Children of Natal/KwaZulu, South Africa. *International Journal of Epidemiology,* 17(1):168-173.

Abramczuk, B.M., Mazzola, T.N., Moreno, Y.M., Zorzeto, T.Q., Quintilio, W., Wolf, P.S., Blotta, M.H., Morcillo, A.M., da Silva, M.T. & Dos Santos Vilela, M.M. 2011, "Impaired humoral response to vaccines among HIV-exposed uninfected infants", *Clinical and vaccine immunology : CVI,* 18(9):1406-1409.

Adewole, O.O., Anteyi, E., Ajuwon, Z., Wada, I., Elegba, F., Ahmed, P., Betiku, Y., Okpe, A., Eze, S. & Ogbeche, T. 2009, "Hepatitis B and C virus co-infection in Nigerian patients with HIV infection", *The Journal of Infection in Developing Countries,* 3(5):369-375.

Alter, M.J. 2006, "Epidemiology of viral hepatitis and HIV co-infection", *Journal of hepatology,* 44(1):6-9.

Amponsah-Dacosta, E., Lebelo, R.L., Rakgole, J.N., Burnett, R.J., Selabe, S.G. & Mphahlele, M.J. 2014, "Evidence for a change in the epidemiology of hepatitis B virus infection after nearly two decades of universal hepatitis B vaccination in South Africa", *Journal of medical virology,* 86(6):918-924.

Andersson, M.I., Maponga, T.G., Ijaz, S., Theron, G., Preiser, W. & Tedder, R.S. 2012, "High HBV viral loads in HIV-infected pregnant women at a tertiary hospital, South Africa", *Journal of acquired immune deficiency syndromes (1999),* 60(4):111-112.

Andersson, M., Maponga, T., Ijaz, S., Barnes, J., Theron, G., Meredith, S., Preiser, W. & Tedder, R. 2013, "The epidemiology of hepatitis B virus infection in HIV-infected and HIV-uninfected pregnant women in the Western Cape, South Africa", *Vaccine,* 31(47):5579-5584.

Arrazola, M., De Juanes, J., Aragón, A., de Codes, A.G. & Ramos, J. 1995, "Hepatitis B vaccination in infants of mothers infected with human immunodeficiency virus", *Journal of medical virology,* 45(3):339-341.

Baptista, M., Kramvis, A. & Kew, M.C. 1999. High Prevalence of 1762T 1764A Mutations in the Basic Core Promoter of Hepatitis B Virus Isolated from Black Africans with Hepatocellular Carcinoma Compared with Asymptomatic Carriers. *Hepatology,* 29(3):946-953.

Beasley, R.P., Trepo, C., Stevens, C.E. & Szuness, W. 1977, "The e antigen and vertical transmission of hepatitis B surface antigen", *American Journal of Epidemiology,* 105(2):94-98.

Beasley, R.P., Lin, C., Hwang, L. & Chien, C. 1981. Hepatocellular Carcinoma and Hepatitis B Virus: A Prospective Study of 22 707 Men in Taiwan. *The Lancet,* 318(8256):1129-1133.

Beasley, R.P. & Hwang, L.Y. 1984. Hepatocellular Carcinoma and Hepatitis B Virus. *Seminars in Liver Disease,* 4(2):113-121.

Bertoletti, A. & Gehring, A.J. 2013. Immune Therapeutic Strategies in Chronic Hepatitis B Virus Infection: Virus Or Inflammation Control? *PLoS Pathogens,* 9(12):1003784.

Blumberg, B.S. & Alter, H.J. 1965, "A" new" antigen in leukemia sera", *JAMA: the journal of the American Medical Association,* 191(7):541-546.

Blumberg, B.S., Larouze, B., London, W.T., Werner, B., Hesser, J.E., Millman, I., Saimot, G. & Payet, M. 1975, "The relation of infection with the hepatitis B agent to primary hepatic carcinoma", *The American journal of pathology,* vol. 81, no. 3, pp. 669-682.

Botha, J., Dusheiko, G., Ritchie, M., Mouton, H. & Kew, M. 1984, "Hepatitis B virus carrier state in black children in Ovamboland: role of perinatal and horizontal infection", *The Lancet,* 323(8388):1210-1212.

Bouchard, M.J. & Schneider, R.J. 2004, "The enigmatic X gene of hepatitis B virus", *Journal of virology,* 78(23):12725-12734.

Bowyer, S.M., van Staden, L., Kew, M.C. & Sim, J. 1997, "A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa.", *Journal of General Virology,* 78(7):1719-1729.

Bowyer, S. & Sim, J. 2000, "Relationships within and between genotypes of hepatitis B virus at points across the genome: footprints of recombination in certain isolates", *Journal of General Virology,* 81(2):379-392.

Brandt, L., Teferi, T., Angala, M.P., Kalibbala, M., Mendai, R. & Egodhi, M.M. 2012, "Prevalence of HBV infection in HIV-infected children in northern Namibia; baseline ALT as an indicator of immune-tolerant HBV disease and selection of a lamivudine-sparing HAART regimen", 19th International AIDS Conference 2012, abstract WEPE048.

Bressac, B., Kew, M., Wands, J. & Ozturk, M. 1991a. Selective G to T Mutations of p53 Gene in Hepatocellular Carcinoma from Southern Africa. *Nature,* 350(6317):429-431.

Bressac, B., Puisieux, A., Kew, M., Volkmann, M., Bozcall, S., Bella Mura, *et al*. 1991b. P53 Mutation in Hepatocellular Carcinoma After Aflatoxin Exposure. *The Lancet,* 338(8779):1356-1359.

Broderick, A. & Jonas, M.M. 2004, "Management of hepatitis B in children.", *Clinics in liver disease,* 8(2):387-401.

Bruss, V. & Ganem, D. 1991. The Role of Envelope Proteins in Hepatitis B Virus Assembly. *Proceedings of the National Academy of Sciences of the United States of America,* 88(3):1059-1063.

Bruss, V. 1997. A Short Linear Sequence in the Pre-S Domain of the Large Hepatitis B Virus Envelope Protein Required for Virion Formation. *Journal of Virology,* 71(12):9350-9357.

Burnett, R., François, G., Kew, M., Leroux-Roels, G., Meheus, A., Hoosen, A. & Mphahlele, M. 2005, "Hepatitis B virus and human immunodeficiency virus co-infection in sub-Saharan Africa: a call for further investigation", *Liver international,* 25(2):201-213.

Burnett, R., Ngobeni, J., François, G., Hoosen, A., Leroux-Roels, G., Meheus, A. & Mphahlele, M. 2007, "Increased exposure to hepatitis B virus infection in HIV-positive South African antenatal women", *International Journal of STD & AIDS,* 18(3):152-156.

Cacciola, I., Pollicino, T., Squadrito, G., Cerenzia, G., Orlando, M.E. & Raimondo, G. 1999, "Occult hepatitis B virus infection in patients with chronic hepatitis C liver disease", *New England Journal of Medicine,* 341(1):22-26.

Cao, G. 2009, "Clinical relevance and public health significance of hepatitis B virus genomic variations", *World journal of gastroenterology,* 15(46):5761-5769.

Cento, V., Mirabelli, C., Dimonte, S., Salpini, R., Han, Y., Trimoulet, P., Bertoli, A., Micheli, V., Gubertini, G., Cappiello, G., Spano, A., Longo, R., Bernassola, M., Mazzotta, F.,

Stellenbosch University http://scholar.sun.ac.za

De Sanctis, G.M., Zhang, X.X., Verheyen, J., D'Arminio Monforte, A., Ceccherini-Silberstein, F., Perno, C.F. & Svicher, V. 2013, "Overlapping structure of hepatitis B virus (HBV) genome and immune selection pressure are critical forces modulating HBV evolution", *The Journal of general virology,* 94(1):143-149.

Chadwick, D., Stanley, A., Sarfo, S., Appiah, L., Ankcorn, M., Foster, G., Schwab, U., Phillips, R. & Geretti, A.M. 2013, "Response to antiretroviral therapy in occult hepatitis B and HIV co-infection in West Africa", *AIDS,* 27(1):139-141.

Chan, H.L., Hui, A., Wong, M., Tse, A.M., Hung, L.C., Wong, V.W. & Sung, J.J. 2004, "Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma", *Gut,* 53(10):1494-1498.

Chang, M. 2007, "Hepatitis B virus infection", *Seminars in Fetal and Neonatal Medicine*, 12(3):160-167.

Chazouilleres, O., Mamish, D., Kim, M., Carey, K., Wright, T., Ferrell, L., Roberts, J. & Ascher, N. 1994, "" Occult" hepatitis B virus as source of infection in liver transplant recipients", *The Lancet,* 343(8890):142-146.

Chen, C., Eng, H., Lee, C., Fang, Y., Kuo, F. & Lu, S. 2004, "Correlations between hepatitis B virus genotype and cirrhotic or non-cirrhotic hepatoma", *Hepatogastroenterology,* 51(56):552-555.

Chen, B., Liu, C., Jow, G., Chen, P., Kao, J. & Chen, D. 2006, "High prevalence and mapping of pre-S deletion in hepatitis B virus carriers with progressive liver diseases", *Gastroenterology,* 130(4):1153-1168.

Chen, C.H., Changchien, C.S., Lee, C.M., Hung, C.H., Hu, T.H., Wang, J.H., Wang, J.C. & Lu, S.N. 2008, "Combined mutations in pre-s/surface and core promoter/precore regions of hepatitis B virus increase the risk of hepatocellular carcinoma: a case-control study", *The Journal of infectious diseases,* 198(11):1634-1642.

Chen, D. 2009, "Hepatitis B vaccination: The key towards elimination and eradication of hepatitis B", *Journal of hepatology,* 50(4):805-816.

Chen, D. & Chen, P. 2011, "CHAPTER 66 - Hepatitis B and Deltavirus Infections" in *Tropical Infectious Diseases: Principles, Pathogens and Practice (Third Edition)*, Third edition edn, W.B. Saunders, Edinburgh, pp. 433-440.

Chen, X., Chen, J., Wen, J., Xu, C., Zhang, S., Zhou, Y. & Hu, Y. 2013, "Breastfeeding Is Not a Risk Factor for Mother-to-Child Transmission of Hepatitis B Virus", *PloS one,* 8(1):55303.

Chotun BN, The prevalence of hepatitis B virus infection in an HIV-exposed paediatric cohort from the Western Cape, South Africa. Stellenbosch University, *MSc Thesis*, 2012. Available online at: http://scholar.sun.ac.za/handle/10019.1/71771

Chu, C., Hussain, M. & Lok, A.S. 2002, "Hepatitis B virus genotype B is associated with earlier HBeAg seroconversion compared with hepatitis B virus genotype C",*Gastroenterology,* 122(7):1756-1762.

Clements, C.J., Coghlan, B., Creati, M., Locarnini, S., Tedder, R.S. & Torresi, J. 2010, "Global control of hepatitis B virus: does treatment-induced antigenic change affect immunization?", *Bulletin of the World Health Organization,* 88(1):66-73.

Clerici, M., Saresella, M., Colombo, F., Fossati, S., Sala, N., Bricalli, D., Villa, M.L., Ferrante, P., Dally, L. & Vigano', A. 2000, "T-lymphocyte maturation abnormalities in uninfected newborns and children with vertical exposure to HIV", *Blood,* 96(12):3866-3871.

Collier, L. & Oxford, J. 2006, "The blood-borne hepatitis viruses B and Delta" in *Human Virology*, Third edn, Oxford University Press Inc., New York, United States, pp. 161-164.

Conjeevaram, H.S. & Lok, A.S. 2001, "Occult hepatitis B virus infection: a hidden menace?", *Hepatology,* 34(1):204-206.

Cooreman, M.P., Leroux-Roels, G. & Paulij, W.P. 2001, "Vaccine- and hepatitis B immune globulin-induced escape mutations of hepatitis B virus surface antigen", *Journal of Biomedical Science,* 8(3):237-247.

Diop-Ndiaye, H., Touré-Kane, C., Etard, J., Lo, G., Diaw, P., Ngom-Gueye, N., Gueye, P., Ba-Fall, K., Ndiaye, I. & Sow, P. 2008, "Hepatitis B, C seroprevalence and delta viruses in HIV-1 Senegalese patients at HAART initiation (retrospective study)", *Journal of medical virology,* 80(8):1332-1336.

Stellenbosch University http://scholar.sun.ac.za

Dwivedi, M., Misra, S.P., Misra, V., Pandey, A., Pant, S., Singh, R. & Verma, M. 2011, "Seroprevalence of hepatitis B infection during pregnancy and risk of perinatal transmission", *Indian Journal of Gastroenterology,* 30(2):66-71.

Ekra, D., Herbinger, K., Konate, S., Leblond, A., Fretz, C., Cilote, V., Douai, C., Da Silva, A., Gessner, B.D. & Chauvin, P. 2008, "A non-randomized vaccine effectiveness trial of accelerated infant hepatitis B immunization schedules with a first dose at birth or age 6 weeks in Côte d'Ivoire", *Vaccine,* 26(22):2753-2761.

Fan, Y. 2001. Prevalence and Significance of Hepatitis B Virus (HBV) pre-S Mutants in Serum and Liver at Different Replicative Stages of Chronic HBV Infection. *Hepatology,* 33(1):277-286.

Fang, Z., Hué, S., Sabin, C.A., Li, G., Yang, J., Chen, Q., Fang, K., Huang, J., Wang, X. & Harrison, T.J. 2011, "A complex hepatitis B virus (X/C) recombinant is common in Long An county, Guangxi and may have originated in southern China", *Journal of General Virology,* 92(2):402-411.

Ferrari, C., Cavalli, A., Penna, A., Valli, A., Bertoletti, A., Pedretti, G., Pilli, M., Vitali, P., Neri, T.M. & Giuberti, T. 1992, "Fine specificity of the human T-cell response to the hepatitis B virus preS1 antigen",*Gastroenterology,* 103(1):255-263.

Firnhaber, C., Reyneke, A., Schulz, D., Malope, B., Macphail, P., Sanne, E. & Biseglie, A. 2008, "The Prevalence of Hepatitis B Co infection in a South African (SA) Urban government HIV Clinic", *South African Medical Journal,* 98(7):541-544.

Firnhaber, C., Viana, R., Reyneke, A., Schultze, D., Malope, B., Maskew, M., Di Bisceglie, A., MacPhail, P., Sanne, I. & Kew, M. 2009, "Occult hepatitis B virus infection in patients with isolated core antibody and HIV co-infection in an urban clinic in Johannesburg, South Africa", *International Journal of Infectious Diseases,* 13(4):488-492.

Ganem, D. & Prince, A.M. 2004, "Hepatitis B virus infection—natural history and clinical consequences", *New England Journal of Medicine,* 350(11):1118-1129.

Garson, J., Grant, P., Ayliffe, U., Ferns, R. & Tedder, R. 2005. Real-Time PCR Quantitation of Hepatitis B Virus DNA using Automated Sample Preparation and Murine Cytomegalovirus Internal Control. *Journal of Virological Methods,* 126(1):207-213.

Geretti, A.M., Patel, M., Sarfo, F.S., Chadwick, D., Verheyen, J., Fraune, M., Garcia, A. & Phillips, R.O. 2010, "Detection of highly prevalent hepatitis B virus coinfection among HIV-seropositive persons in Ghana", *Journal of clinical microbiology,* 48(9):3223-3230.

Guidozzi, F., Schoub, B.D., Johnson, S. & Song, E. 1993. Should Pregnant Urban South African Women be Screened for Hepatitis B? *South African Medical Journal*, 83(2):103-105.

Harrison, T. 2008, "Hepatitis B Virus: Molecular Biology" in *Encyclopedia of Virology*, ed. Mahy, B.W.J. & Van Regenmortel, M.H.V., Third edn, Academic Press, Oxford, UK, pp. 360-367.

Harrison, T.J., Dusheiko, G.M. & Zuckerman, A.J. 2009, "Hepatitis viruses", *Principles and Practice of Clinical Virology, Sixth Edition,* , pp. 273-320.

Healy, S.A., Gupta, S. & Melvin, A.J. 2013, "HIV/HBV coinfection in children and antiviral therapy", *Expert Review of Anti-infective Therapy,* 11(3):251-263.

Hino, K., Katoh, Y., Vardas, E., Sim, J., Okita, K. & Carman, W.F. 2001, "The effect of introduction of universal childhood hepatitis B immunization in South Africa on the prevalence of serologically negative hepatitis B virus infection and the selection of immune escape variants", *Vaccine,* 19(28):3912-3918.

Hoffmann, C.J., Charalambous, S., Martin, D.J., Innes, C., Churchyard, G.J., Chaisson, R.E., Grant, A.D., Fielding, K.L. & Thio, C.L. 2008, "Hepatitis B virus infection and response to antiretroviral therapy (ART) in a South African ART program", *Clinical Infectious Diseases,* 47(11):1479-1485.

Hoffmann, C.J., Mashabela, F., Cohn, S., Hoffmann, J.D., Lala, S., Martinson, N.A. & Chaisson, R.E. 2014, "Maternal hepatitis B and infant infection among pregnant women living with HIV in South Africa", *Journal of the International AIDS Society,* 17(1):18871.

Hogan, D.R., Salomon, J.A., Canning, D., Hammitt, J.K., Zaslavsky, A.M. & Bärnighausen, T. 2012, "National HIV prevalence estimates for sub-Saharan Africa: controlling selection bias with Heckman-type selection models", *Sexually transmitted infections,* 88(2):17-23.

Hsieh, Y.H., Su, I.J., Wang, H.C., Chang, W.W., Lei, H.Y., Lai, M.D., Chang, W.T. & Huang, W. 2004, "Pre-S mutant surface antigens in chronic hepatitis B virus infection induce oxidative stress and DNA damage",*Carcinogenesis,* 25(10):2023-2032.

Hsu, H., Chen, D., Chuang, C., Lu, J.C., Jwo, D., Lee, C., Lu, H., Cheng, S., Wang, Y. & Wang, C.C. 1988, "Efficacy of a mass hepatitis B vaccination program in Taiwan", *JAMA: the journal of the American Medical Association,* 260(15):2231-2235.

Hu, K. 2002, "Occult hepatitis B virus infection and its clinical implications", *Journal of viral hepatitis,* 9(4):243-257.

Hübschen, J., Mbah, P., Forbi, J., Otegbayo, J., Olinger, C., Charpentier, E. & Muller, C. 2011, "Detection of a new subgenotype of hepatitis B virus genotype A in Cameroon but not in neighbouring Nigeria", *Clinical Microbiology and Infection,* 17(1):88-94.

Hui, A.Y., Hung, L.C., Tse, P.C., Leung, W., Chan, P.K. & Chan, H.L. 2005. Transmission of Hepatitis B by Human bite—Confirmation by Detection of Virus in Saliva and Full Genome Sequencing. *Journal of Clinical Virology,* 33(3):254-256.

Hunt, C.M., McGill, J.M., Allen, M.I. & Condreay, L.D. 2000, "Clinical relevance of hepatitis B viral mutations", *Hepatology,* 31(5):1037-1044.

Huo, T., Wu, J., Lee, P., Chau, G., Lui, W., Tsay, S., Ting, L., Chang, F. & Lee, S. 1998, "Sero-clearance of hepatitis B surface antigen in chronic carriers does not necessarily imply a good prognosis", *Hepatology,* 28(1):231-236.

Huy, T.T.T., Ngoc, T.T. & Abe, K. 2008, "New complex recombinant genotype of hepatitis B virus identified in Vietnam", *Journal of virology,* 82(11):5657-5663.

Jammeh, S., Tavner, F., Watson, R., Thomas, H.C. & Karayiannis, P. 2008, "Effect of basal core promoter and pre-core mutations on hepatitis B virus replication", *The Journal of general virology,* 89(4):901-909.

Jonas, M.M. 2013, "Hepatitis B virus infection in children", *Clinical Liver Disease,* 2(1):41-44.

Jonas, M.M., Kelley, D.A., Mizerski, J., Badia, I.B., Areias, J.A., Schwarz, K.B., Little, N.R., Greensmith, M.J., Gardner, S.D. & Bell, M.S. 2002, "Clinical trial of lamivudine in children with chronic hepatitis B", *New England Journal of Medicine,* 346(22)1706-1713.

Jonas, M.M., Kelly, D., Pollack, H., Mizerski, J., Sorbel, J., Frederick, D., Mondou, E., Rousseau, F. & Sokal, E. 2008, "Safety, efficacy, and pharmacokinetics of adefovir dipivoxil

in children and adolescents (age 2 to< 18 years) with chronic hepatitis B", *Hepatology,* 47(6):1863-1871.

Kao, J., Chen, P., Lai, M. & Chen, D. 2003, "Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers",*Gastroenterology,* 124(2):327-334.

Kapembwa, K.C., Goldman, J.D., Lakhi, S., Banda, Y., Bowa, K., Vermund, S.H., Mulenga, J., Chama, D. & Chi, B.H. 2011, "HIV, hepatitis B, and hepatitis C in Zambia", *Journal of global infectious diseases,* 3(3):269.

Karayiannis, P. & Thomas, H.C 2008, "Hepatitis B Virus: General Features" in *Encyclopedia of Virology*, ed. Mahy, B.W.J. & Van Regenmortel, M.H.V., Third edn, Academic Press, Oxford, UK, pp. 350-360.

Kew, M., Reis, P. & Macnab, G. 1973, "The witch-doctor and tribal scarification of the skin and the hepatitis B antigen", *J.Urol,* 43(1):439.

Kew, M., Miller, G., Stevenson, C., Macnab, G. & Bersohn, T. 1974. The Prevalence of Virus-B Hepatitis South African Blacks. *South African Medical Journal,* 48(43):1837-1838.

Kew, M.C., Desmyter, J., Bradburne, A.F. & Macnab, G.M. 1979, "Hepatitis B virus infection in southern African blacks with hepatocellular cancer", *Journal of the National Cancer Institute,* 62(3):517-520.

Kew, M.C., Rossouw, E., Paterson, A., Hodkinson, J., Whitcutt, M. & Dusheiko, G. 1983. Hepatitis B Virus Status of Black Women with Hepatocellular Carcinoma. *Gastroenterology,* 84(4):693-696.

Kew, M.C., Kramvis, A., Yu, M.C., Arakawa, K. & Hodkinson, J. 2005. Increased Hepatocarcinogenic Potential of Hepatitis B Virus Genotype A in Bantu-speaking sub-saharan Africans. *Journal of Medical Virology,*75(4):513-521.

Kew, M. 2010. Epidemiology of Chronic Hepatitis B Virus Infection, Hepatocellular Carcinoma, and Hepatitis B Virus-Induced Hepatocellular Carcinoma. *Pathologie Biologie,* 58(4):273-277.

Kew, M.C. 2012, "Hepatitis B virus/human immunodeficiency virus co-infection and its hepatocarcinogenic potential in sub-saharan black africans", *Hepatitis monthly,* 12(10 HCC).

Kiire, C. 1996, "The epidemiology and prophylaxis of hepatitis B in sub-Saharan Africa: a view from tropical and subtropical Africa.", *Gut,* 38(2):5-12.

Kim, S., Lee, J. & Ryu, W. 2009, "Four conserved cysteine residues of the hepatitis B virus polymerase are critical for RNA pregenome encapsidation", *Journal of virology,* 83(16):8032-8040.

Kim, H., Oh, E.J., Kang, M.S., Kim, S.H. & Park, Y.J. 2007. Comparison of the Abbott Architect i2000 Assay, the Roche Modular Analytics E170 Assay, and an Immunoradiometric Assay for Serum Hepatitis B Virus Markers. *Annals of Clinical and Laboratory Science,* 37(3):256-259.

Kimbi, G.C., Kramvis, A. & Kew, M.C. 2004, "Distinctive sequence characteristics of subgenotype A1 isolates of hepatitis B virus from South Africa", *Journal of general virology,* 85(5):1211-1220.

Kimura, M. 1980. A Simple Method for Estimating Evolutionary Rates of Base Substitutions through Comparative Studies of Nucleotide Sequences. *Journal of Molecular Evolution,*16(2):111-120.

Kourtis, A.P., Bulterys, M., Hu, D.J. & Jamieson, D.J. 2012, "HIV–HBV Coinfection—A Global Challenge", *New England Journal of Medicine,* 366(19):1749-1752.

Kramvis, A., Kew, M.C. & Bukofzer, S. 1998. Hepatitis B Virus Precore Mutants in Serum and Liver of Southern African Blacks with Hepatocellular Carcinoma. *Journal of Hepatology,* 28(1):132-141.

Kramvis, A., Kew, M. & François, G. 2005, "Hepatitis B virus genotypes", *Vaccine*, 23(19):2409-2423

Kramvis, A. & Kew, M. 2005, "Relationship of genotypes of hepatitis B virus to mutations, disease progression and response to antiviral therapy", *Journal of viral hepatitis,* 12(5):456-464.

Kramvis, A. & Kew, M.C. 2007, "Epidemiology of hepatitis B virus in Africa, its genotypes and clinical associations of genotypes", *Hepatology Research,* 37(1):9-19.

Kurbegov, A.C. & Sokol, R.J. 2009, "Hepatitis B therapy in children", *Expert review of gastroenterology & hepatology,* 3(1):39-49.

Lai, C., Shouval, D., Lok, A.S., Chang, T., Cheinquer, H., Goodman, Z., DeHertogh, D., Wilber, R., Zink, R.C. & Cross, A. 2006, "Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B", *New England Journal of Medicine,* 354(10):1011-1020.

Larkin, M.A. 2007. Clustal W and Clustal X Version 2.0. *Bioinformatics (Oxford, England),* 23(21):2947-2948.

Le Seyec, J., Chouteau, P., Cannie, I., Guguen-Guillouzo, C. & Gripon, P. 1999. Infection Process of the Hepatitis B Virus Depends on the Presence of a Defined Sequence in the Pre-S1 Domain. *Journal of Virology,*73(3):2052-2057.

Lee, W.M. 1997, "Hepatitis B virus infection", *New England journal of medicine,* 337(24):1733-1745.

Lee, C., Gong, Y., Brok, J., Boxall, E. & Gluud, C. 2006a, "Hepatitis B immunisation for newborn infants of hepatitis B surface antigen-positive mothers", *Cochrane Database Syst Rev,*2.

Lee, C., Gong, Y., Brok, J., Boxall, E.H. & Gluud, C. 2006b, "Effect of hepatitis B immunisation in newborn infants of mothers positive for hepatitis B surface antigen: systematic review and meta-analysis", *BMJ (Clinical research ed.),* 332(7537):328-336.

Lee, J.H., Han, K.H., Lee, J.M., Park, J.H. & Kim, H.S. 2011, "Impact of hepatitis B virus (HBV) x gene mutations on hepatocellular carcinoma development in chronic HBV infection", *Clinical and vaccine immunology*,18(6):914-921.

Leung, N., Peng, C., Hann, H., Sollano, J., Lao-Tan, J., Hsu, C., Lesmana, L., Yuen, M., Jeffers, L. & Sherman, M. 2009, "Early hepatitis B virus DNA reduction in hepatitis B e antigen–positive patients with chronic hepatitis B: A randomized international study of entecavir versus adefovir", *Hepatology,* 49(1):72-79.

Li, X., Shi, M., Yang, Y., Shi, Z., Hou, H., Shen, H. & Teng, B. 2004, "Effect of hepatitis B immunoglobulin on interruption of HBV intrauterine infection.", *Age (yr),* 26(1.8):27.8-22.8.

Liang, T., Chen, E. & Tang, H. 2013, "Hepatitis B Virus Gene Mutations and Hepatocarcinogenesis", *Asian Pacific Journal of Cancer Prevention,* 14(8):4509-4513.

Liaw, Y., Chien, R., Yeh, C., Tsai, S. & Chu, C. 1999, "Acute exacerbation and hepatitis B virus clearance after emergence of YMDD motif mutation during lamivudine therapy",*Hepatology,* 30(2):567-572.

Liaw, Y. & Chu, C. 2009, "Hepatitis B virus infection", *The lancet,* 373(9663):582-592.

Lin, C. & Kao, J. 2011, "The clinical implications of hepatitis B virus genotype: recent advances", *Journal of gastroenterology and hepatology,* 26(1):123-130.

Livingston, S.E., Simonetti, J.P., Bulkow, L.R., Homan, C.E., Snowball, M.M., Cagle, H.H., Negus, S.E. & McMahon, B.J. 2007, "Clearance of hepatitis B e antigen in patients with chronic hepatitis B and genotypes A, B, C, D, and F", *Gastroenterology,* 133(5):1452-1457.

Locarnini, S.A. 2003, "Hepatitis B virus surface antigen and polymerase gene variants: potential virological and clinical significance", *Hepatology,* 27(1):294-297.

Locarnini, S. 2004, "Molecular virology of hepatitis B virus", *Seminars in liver disease,* 24(1):3-10.

Locarnini, S. & Zoulim, F. 2010. Molecular Genetics of HBV Infection. *Antivir Ther,* 15(3):3-14.

Loeb, D.D., Hirsch, R.C. & Ganem, D. 1991, "Sequence-independent RNA cleavages generate the primers for plus strand DNA synthesis in hepatitis B viruses: implications for other reverse transcribing elements.", *The EMBO journal,* 10(11):3533-3540.

Lok, A.S., Hussain, M., Cursano, C., Margotti, M., Gramenzi, A., Luca Grazi, G., Jovine, E., Benardi, M. & Andreone, P. 2000, "Evolution of hepatitis B virus polymerase gene mutations in hepatitis B e Antigen–negative patients receiving lamivudine therapy", *Hepatology,* 32(5):1145-1153.

Lok, A.S. & McMahon, B.J. 2007, "Chronic hepatitis B", *Hepatology,* 45(2):507-539.

Luedde, T. & Schwabe, R.F. 2011, "NF-κB in the liver—linking injury, fibrosis and hepatocellular carcinoma", *Nature Reviews Gastroenterology and Hepatology,* 8(2):108-118.

Lukhwareni, A., Burnett, R.J., Selabe, S.G., Mzileni, M.O. & Mphahlele, M.J. 2009. Increased Detection of HBV DNA in HBsAg-positive and HBsAg-negative South African

Stellenbosch University http://scholar.sun.ac.za

HIV/AIDS Patients Enrolling for Highly Active Antiretroviral Therapy at a Tertiary Hospital. *Journal of Medical Virology,* 81(3):406-412.

Luo, Z., Li, L. & Ruan, B. 2012, "Impact of the implementation of a vaccination strategy on hepatitis B virus infections in China over a 20-year period", *International Journal of Infectious Diseases,* 16(2):82-88.

Makondo, E., Bell, T.G. & Kramvis, A. 2012. Genotyping and Molecular Characterization of Hepatitis B Virus from Human Immunodeficiency Virus-Infected Individuals in Southern Africa. *PloS One,* 7(9):46345.

Maman, Y., Blancher, A., Benichou, J., Yablonka, A., Efroni, S. & Louzoun, Y. 2011. Immune-Induced Evolutionary Selection Focused on a Single Reading Frame in Overlapping Hepatitis B Virus Proteins. *Journal of Virology,* 85(9):4558-4566.

Maponga TG, "An investigation of hepatitis B virus in antenatal women tested for human immunodeficiency virus, in the Western Cape Province of South Africa" Stellenbosch University, *MSc Thesis*, 2012.

Available online at: https://scholar.sun.ac.za/handle/10019.1/38955

Mayaphi, S.H., Martin, D.J., Mphahlele, M.J., Blackard, J.T. & Bowyer, S.M. 2013. Variability of the preC/C Region of Hepatitis B Virus Genotype A from a South African Cohort Predominantly Infected with HIV. *Journal of Medical Virology,* 85(11):1883-1892.

McMahon, B.J., Alward, W.L., Hall, D.B., Heyward, W.L., Bender, T.R., Francis, D.P. & Maynard, J.E. 1985, "Acute hepatitis B virus infection: relation of age to the clinical expression of disease and subsequent development of the carrier state", *Journal of infectious diseases,* 151(4):599-603.

McMahon, B.J. 2009a, "The influence of hepatitis B virus genotype and subgenotype on the natural history of chronic hepatitis B", *Hepatology international,* 3(2):334-342.

McMahon, B.J. 2009b, "The natural history of chronic hepatitis B virus infection", *Hepatology,* 49(5)45-55.

Melegari, M., Bruno, S. & Wands, J.R. 1994. Properties of Hepatitis B Virus Pre-S1 Deletion Mutants. *Virology,* 199(2):292-300.

Modi, A.A. & Feld, J.J. 2007, "Viral hepatitis and HIV in Africa", *Aids Rev,* 9(1):25-39.

Moore, S., Hesseling, P., Wessels, G. & Schneider, J. 1997, "Hepatocellular carcinoma in children", *Pediatric surgery international,* 12(4):266-270.

Moore, S.W., Millar, A.J., Hadley, G., Ionescu, G., Kruger, M., Poole, J., Stones, D., Wainwright, L., Chitnis, M. & Wessels, G. 2004, "Hepatocellular carcinoma and liver tumors in South African children", *Cancer,* 101(3):642-649.

Moore, S., Davidson, A., Hadley, G., Kruger, M., Poole, J., Stones, D., Wainwright, L. & Wessels, G. 2008, "Malignant liver tumors in South African children: a national audit", *World journal of surgery,* 32(7):1389-1395.

Mphahlele, M., Francois, G., Kew, M., Van Damme, P., Hoosen, A. & Meheus, A. 2002. Epidemiology and Control of Hepatitis B: Implications for Eastern and Southern Africa. *South Afr J Epidemiol Infect,* 17(1):12-17.

Mphahlele, M.J., Lukhwareni, A., Burnett, R.J., Moropeng, L.M. & Ngobeni, J.M. 2006. High Risk of Occult Hepatitis B Virus Infection in HIV-Positive Patients from South Africa. *Journal of Clinical Virology,* 35(1):14-20.

Muro, F.J., Fiorillo, S.P., Sakasaka, P., Odhiambo, C., Reddy, E.A., Cunningham, C.K. & Buchanan, A.M. 2013, "Seroprevalence of Hepatitis B and C Viruses Among Children in Kilimanjaro Region, Tanzania", *Journal of the Pediatric Infectious Diseases Society,* 2(4):320-326.

Mutwa, P.R., Boer, K.R., Rusine, J.B., Muganga, N., Tuyishimire, D., Reiss, P., Lange, J.M. & Geelen, S.P. 2013, "Hepatitis B virus prevalence and vaccine response in HIV-infected children and adolescents on combination antiretroviral therapy in Kigali, Rwanda", *The Pediatric infectious disease journal,* 32(3):246-251.

Nassal, M. & Schaller, H. 1993, "Hepatitis B virus replication", *Trends in microbiology,* 1(6):221-228.

National Department of Health, South Africa 2008. “POLICY and GUIDELINES FOR THE IMPLEMENTATION OF THE PMTCT PROGRAMME” Available online at: http://southafrica.usembassy.gov/root/pdfs/2008-pmtct.pdf [Accessed on 06 August 2014]

National Department of Health, South Africa 2009. “2008 National Antenatal Sentinel HIV and Syphilis Prevalence Survey” Available online at: http://indicators.hst.org.za/indicators/HIV_AIDS/antenatal_2008.pdf [Accessed on 28 July 2014]

Ndirangu, J., Bärnighausen, T., Tanser, F., Tint, K. & Newell, M. 2009, "Levels of childhood vaccination coverage and the impact of maternal HIV status on child vaccination status in rural KwaZulu-Natal, South Africa*", *Tropical Medicine & International Health,* 14(11):1383-1393.

Nebbia, G., Peppa, D. & Maini, M. 2012, "Hepatitis B infection: current concepts and future challenges", *Qjm,* 105(2):109-113.

Neurath, A., Kent, S., Strick, N. & Parker, K. 1986, "Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus", *Cell,* 46(3):429-436.

Ni, Y., Chang, M., Wu, J., Hsu, H., Chen, H. & Chen, D. 2012, "Minimization of hepatitis B infection by a 25-year universal vaccination program", *Journal of hepatology,* 57(4):730-735.

Nielsen, S.D., Jeppesen, D.L., Kolte, L., Clark, D.R., Sorensen, T.U., Dreves, A.M., Ersboll, A.K., Ryder, L.P., Valerius, N.H. & Nielsen, J.O. 2001, "Impaired progenitor cell function in HIV-negative infants of HIV-positive mothers results in decreased thymic output and low CD4 counts", *Blood,* 98(2):398-404.

Ohto, H., Tohyama, H., Lin, H., Kawana, T. & Etoh, T. 1987, "Intrauterine transmission of hepatitis B virus is closely related to placental leakage", *Journal of medical virology,* 21(1):1-6.

Olinger, C.M., Venard, V., Njayou, M., Oyefolu, A.O.B., Maïga, I., Kemp, A.J., Omilabu, S.A., le Faou, A. & Muller, C.P. 2006, "Phylogenetic analysis of the precore/core gene of hepatitis B virus genotypes E and A in West Africa: new subtypes, mixed infections and recombinations", *Journal of general virology,* 87(5):1163-1173.

Ott, J., Stevens, G. & Wiersma, S. 2012, "The risk of perinatal hepatitis B virus transmission: hepatitis B e antigen (HBeAg) prevalence estimates for all world regions", *BMC Infectious Diseases,* 12(1):131.

Owiredu, W.K., Kramvis, A. & Kew, M.C. 2001, "Hepatitis B virus DNA in serum of healthy black African adults positive for hepatitis B surface antibody alone: possible association with recombination between genotypes A and D", *Journal of medical virology,* 64(4):441-454.

Paganelli, M., Stephenne, X. & Sokal, E.M. 2012. Chronic Hepatitis B in Children and Adolescents. *Journal of Hepatology,* 57(4):885-896.

Paar, D. 2001, "Hepatitis B Virus: Transmission, Prevention, Treatment and HIV Co-Infection", [Online] . Available online at: http://www.thebody.com/content/art12963.html.

Paganelli, M., Stephenne, X. & Sokal, E.M. 2012, "Chronic hepatitis B in children and adolescents", *Journal of hepatology,* 57(4):885-896.

Pande, C., Kumar, A., Patra, S., Trivedi, S.S., Dutta, A.K. & Sarin, S.K. 2008, "High Maternal Hepatitis B Virus DNA Levels But Not HBeAg Positivity Predicts Perinatal Transmission of Hepatitis B to the Newborn", *Gastroenterology,* 134(4):760.

Parekh, S., Zoulim, F., Ahn, S.H., Tsai, A., Li, J., Kawai, S., Khan, N., Trepo, C., Wands, J. & Tong, S. 2003, "Genome replication, virion secretion, and e antigen expression of naturally occurring hepatitis B virus core promoter mutants", *Journal of virology,* 77(12):6601-6612.

Pol, S., Corouge, M. & Fontaine, H. 2011, "Hepatitis B virus infection and pregnancy", *Clinics and research in hepatology and gastroenterology,* 35(10):618-622.

Pollicino, T., Saitta, C. & Raimondo, G. 2011, "Hepatocellular carcinoma: the point of view of the hepatitis B virus", *Carcinogenesis,* 32(8):1122-1132.

Pourkarim, M.R., Lemey, P., Amini-Bavil-Olyaee, S., Maes, P. & Van Ranst, M. 2010, "Novel hepatitis B virus subgenotype A6 in African-Belgian patients", *Journal of Clinical Virology,* 47(1):93-96.

Prozesky, O.W., Szmuness, W., Stevens, C.E., Kew, M.C., Harley, E.J., Hoyland, J.A., Scholtz, J.E., Mitchell, A.D., Shabangu, A. & Kunene, E. 1983, "Baseline epidemiological studies for a hepatitis B vaccine trial in Kangwane", *South African medical journal,* 64(23):891-893.

Puoti, M., Airoldi, M., Bruno, R., Zanini, B., Spinetti, A., Pezzoli, C., Patroni, A., Castelli, F., Sacchi, P. & Filice, G. 2002, "Hepatitis B virus co-infection in human immunodeficiency virus-infected subjects", *AIDS rev,* 4(1):27-35.

Puoti, M., Torti, C., Bruno, R., Filice, G. & Carosi, G. 2006, "Natural history of chronic hepatitis B in co-infected patients", *Journal of hepatology,* 44(1):65-70.

Qu, L., Liu, T., Jin, F., Guo, Y., Chen, T., Ni, Z. & Shen, X. 2011, "Combined pre-S deletion and core promoter mutations related to hepatocellular carcinoma: A nested case-control study in China", *Hepatology Research,* 41(1):54-63.

Qu, L., Kuai, X., Liu, T., Chen, T., Ni, Z. & Shen, X. 2013. Pre-S Deletion and Complex Mutations of Hepatitis B Virus Related to Young Age Hepatocellular Carcinoma in Qidong, China. *PloS One,* 8(3):59583.

Quarleri, J. 2014, "Core promoter: A critical region where the hepatitis B virus makes decisions", *World J Gastroenterol,* 20(2):425-435.

Raimondo, G., Caccamo, G., Filomia, R. & Pollicino, T. 2013, "Occult HBV infection", *Seminars in Immunopathology* Springer, 35(1):39-52

Ramezani, A., Banifazl, M., Mohraz, M., Rasoolinejad, M. & Aghakhani, A. 2011, "Occult hepatitis B virus infection: a major concern in HIV-infected patients: occult HBV in HIV",*Hepatitis monthly,* 11(1):7-10.

Saitou, N. & Nei, M. 1987. The Neighbor-Joining Method: A New Method for Reconstructing Phylogenetic Trees. *Molecular Biology and Evolution,* 4(4):406-425.

Selabe, S.G., Lukhwareni, A., Song, E., Leeuw, Y.G., Burnett, R.J. & Mphahlele, M.J. 2007. Mutations Associated with lamivudine-resistance in therapy-naïve Hepatitis B Virus (HBV) Infected Patients with and without HIV co-infection: Implications for Antiretroviral Therapy in HBV and HIV co-infected South African Patients. *Journal of Medical Virology,* 79(11):1650-1654.

Sheldon, J. & Soriano, V. 2008, "Hepatitis B virus escape mutants induced by antiviral therapy", *The Journal of antimicrobial chemotherapy,* 61(4):766-768.

Shen, T. & Yan, X. 2014, "Hepatitis B virus genetic mutations and evolution in liver diseases", *World journal of gastroenterology*, 20(18):5435-5441.

Shepard, C.W., Simard, E.P., Finelli, L., Fiore, A.E. & Bell, B.P. 2006, "Hepatitis B virus infection: epidemiology and vaccination", *Epidemiologic reviews,* 28(1):112-125.

Shouval, D. & Samuel, D. 2000, "Hepatitis B immune globulin to prevent hepatitis B virus graft reinfection following liver transplantation: a concise review", *Hepatology,* 32(6):1189-1195.

Simani, O.E., Leroux-Roels, G., François, G., Burnett, R.J., Meheus, A. & Mphahlele, M.J. 2009, "Reduced detection and levels of protective antibodies to hepatitis B vaccine in under 2-year-old HIV positive South African children at a paediatric outpatient clinic", *Vaccine,* 27(1):146-151.

Sokal, E.M., Kelly, D.A., Mizerski, J., Badia, I.B., Areias, J.A., Schwarz, K.B., Vegnente, A., Little, N.R., Gardener, S.D. & Jonas, M.M. 2006, "Long-term lamivudine therapy for children with HBeAg-positive chronic hepatitis B", *Hepatology,* 43(2):225-232.

Stevens, C.E., Beasley, R.P., Tsui, J. & Lee, W. 1975, "Vertical transmission of hepatitis B antigen in Taiwan.", *The New England journal of medicine,* 292(15):771-774.

Stuyver, L., De Gendt, S., Van Geyt, C., Zoulim, F., Fried, M., Schinazi, R.F. & Rossau, R. 2000, "A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness", *Journal of General Virology,* 81(1):67-74.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution,* 30(12):2725-2729.

Tang, B., Kruger, W.D., Chen, G., Shen, F., Lin, W.Y., Mboup, S., London, W.T. & Evans, A.A. 2004, "Hepatitis B viremia is associated with increased risk of hepatocellular carcinoma in chronic carriers", *Journal of medical virology,* 72(1):35-40.

Tatematsu, K., Tanaka, Y., Kurbanov, F., Sugauchi, F., Mano, S., Maeshiro, T., Nakayoshi, T., Wakuta, M., Miyakawa, Y. & Mizokami, M. 2009, "A genetic variant of hepatitis B virus divergent from known human and ape genotypes isolated from a Japanese patient and provisionally assigned to new genotype J", *Journal of virology,* 83(20):10538-10547.

Thio, C.L. 2009, “Hepatitis B and human immunodeficiency virus coinfection”, *Hepatology,* 49(5):138-145.

Thumbiran, N.V., Moodley, D., Parboosing, R. & Moodley, P. 2014. Hepatitis B and HIV Co-Infection in Pregnant Women: Indication for Routine Antenatal Hepatitis B Virus Screening in a High HIV Prevalence Setting. *SAMJ: South African Medical Journal,* 104(4):307-309.

Tong, M.J., Blatt, L.M., Kao, J., Cheng, J.T. & Corey, W.G. 2006. Precore/basal Core Promoter Mutants and Hepatitis B Viral DNA Levels as Predictors for Liver Deaths and Hepatocellular Carcinoma. *World Journal of Gastroenterology,* 12(41):6620-6626.

Tong, M.J., Blatt, L.M., Kao, J., Cheng, J.T. & Corey, W.G. 2007. Basal Core Promoter T1762/A1764 and Precore A1896 Gene Mutations in Hepatitis B Surface antigen-positive Hepatocellular Carcinoma: A Comparison with Chronic Carriers. *Liver International,* 27(10):1356-1363.

Tong, S., Li, J., Wands, J.R. & Wen, Y. 2013, "Hepatitis B virus genetic variants: biological properties and clinical implications", *Emerging Microbes & Infections,* 2(3):10.

Torresi, J. 2002, "The virological and clinical significance of mutations in the overlapping envelope and polymerase genes of hepatitis B virus", *Journal of clinical virology,* 25(2):97-106.

Tsebe, K.V., Burnett, R.J., Hlungwani, N.P., Sibara, M.M., Venter, P.A. & Mphahlele, M.J. 2001, "The first five years of universal hepatitis B vaccination in South Africa: evidence for elimination of HBsAg carriage in under 5-year-olds", *Vaccine,* 19(28):3919-3926.

Umar, M., Umar, S. & Khan, H.A. 2013, "HBV Perinatal Transmission", *International journal of hepatology,* 2013:875791-875797.

Van Bömmel, F., Zöllner, B., Sarrazin, C., Spengler, U., Hüppe, D., Möller, B., Feucht, H., Wiedenmann, B. & Berg, T. 2006, "Tenofovir for patients with lamivudine-resistant hepatitis B virus (HBV) infection and high HBV DNA level during adefovir therapy", *Hepatology,* 44(2):318-325.

Vardas, E., Mathai, M., Blaauw, D., McAnerney, J., Coppin, A. & Sim, J. 1999. Preimmunization Epidemiology of Hepatitis B Virus Infection in South African Children. *Journal of Medical Virology*, 58(2):111-115.

Velilla, P.A., Montoya, C.J., Hoyos, A., Moreno, M.E., Chougnet, C. & Rugeles, M.T. 2008, "Effect of intrauterine HIV-1 exposure on the frequency and function of uninfected newborns' dendritic cells", *Clinical Immunology,* 126(3): 243-250.

Vos, G.H., Rose, E.F. & Marimuthu, T. 1980. Hepatitis B Antigen and Antibodies in Rural and Urban Southern African Blacks. *South African Medical Journal,* 57(21):868-870.

Wang, J. & Zhu, Q. 2000, "Infection of the fetus with hepatitis B e antigen via the placenta", *The Lancet,* 355(9208):989.

Wang, H., Wu, H., Chen, C., Fausto, N., Lei, H. & Su, I. 2003, "Different types of ground glass hepatocytes in chronic hepatitis B virus infection contain specific pre-S mutants that may induce endoplasmic reticulum stress", *The American journal of pathology,* 163(6):2441-2449

Wang, H., Huang, W., Lai, M. & Su, I. 2006, "Hepatitis B virus pre-S mutants, endoplasmic reticulum stress and hepatocarcinogenesis", *Cancer science,* 97(8):683-688.

Westwood, A.T. 2001, "Childhood acute viral hepatitis in Cape Town", *South African medical journal,* (2):135-136.

Wild, D. 2005, *The immunoassay handbook,* Third edn, Elsevier Ltd, Oxford, UK.

Wiseman, E., Fraser, M.A., Holden, S., Glass, A., Kidson, B.L., Heron, L.G., Maley, M.W., Ayres, A., Locarnini, S.A. & Levy, M.T. 2009, "Perinatal transmission of hepatitis B virus: an Australian experience", *Medical Journal of Australia,* 190(9):489-492.

Wong, V.C., Lee, A. & Ip, H.M. 1980, "Transmission of hepatitis B antigens from symptom free carrier mothers to the fetus and the infant", *An International Journal of Obstetrics & Gynaecology,* 87(11):958-965.

Wong, V.W., Reesink, H., Ip, H.H., Nco Lelie, P., Reerink-Brongers, E., Yeung, C. & Ma, H. 1984, "Prevention of the HBsAg carrier state in newborn infants of mothers who are chronic carriers of HBsAg and HBeAg by administration of hepatitis-B vaccine and hepatitis-B

immunoglobulin: double-blind randomised placebo-controlled study", *The Lancet,* 323(8383):921-926.

World Health Organization 2010, "Hepatitis B vaccines: WHO position paper-recommendations", *Vaccine,* 28(3):589-590.

Wright, T.L. 2006, "Introduction to chronic hepatitis B infection", *The American Journal of Gastroenterology,* 101(1):1-6.

Xu, D., Yan, Y., Choi, B.C., Xu, J., Men, K., Zhang, J., Liu, Z. & Wang, F. 2002, "Risk factors and mechanism of transplacental transmission of hepatitis B virus: A case-control study",*Journal of medical virology,* 67(1): 20-26.

Xu, D., Yan, Y., Zou, S., Choi, B.C., Wang, S., Liu, P., Bai, G., Wang, X., Shi, M. & Wang, X. 2001, "Role of placental tissues in the intrauterine transmission of hepatitis B virus",*American Journal of Obstetrics and Gynecology,* 185(4):981-987.

Yang, H., Lu, S., Liaw, Y., You, S., Sun, C., Wang, L., Hsiao, C.K., Chen, P., Chen, D. & Chen, C. 2002, "Hepatitis B e antigen and the risk of hepatocellular carcinoma", *New England Journal of Medicine,* 347(3):168-174.

Yeung, P., Wong, D.K., Lai, C.L., Fung, J., Seto, W.K. & Yuen, M.F. 2011. Association of Hepatitis B Virus Pre-S Deletions with the Development of Hepatocellular Carcinoma in Chronic Hepatitis B. *The Journal of Infectious Diseases,* 203(5):646-654.

Yu, M.W., Yeh, S.H., Chen, P.J., Liaw, Y.F., Lin, C.L., Liu, C.J., Shih, W.L., Kao, J.H., Chen, D.S. & Chen, C.J. 2005, "Hepatitis B virus genotype and DNA level and hepatocellular carcinoma: a prospective study in men", *Journal of the National Cancer Institute,* 97(4):265-272.

Yuen, M., Fung, J., Wong, D.K. & Lai, C. 2009. Prevention and Management of Drug Resistance for Antihepatitis B Treatment. *The Lancet Infectious Diseases,* 9(4):256-264.

Zhang, S., Han, X. & Yue, Y. 1998, "Relationship between HBV viremia level of pregnant women and intrauterine infection: neated PCR for detection of HBV DNA", *World J Gastroenterol,* 4(1):61-63.

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Zuccotti, G., Riva, E., Flumine, P., Locatelli, V., Fiocchi, A., Tordato, G. & Giovannini, M. 1994, "Hepatitis B vaccination in infants of mothers infected with human immunodeficiency virus", *The Journal of pediatrics,* 125(1):70-72.

Zuckerman, J. 2007, "Review: hepatitis B immune globulin for prevention of hepatitis B infection", *Journal of medical virology,* 79(7):919-921.