THE PREVALENCE OF HEPATITIS B VIRUS INFECTION IN AN HIV-EXPOSED PAEDIATRIC COHORT FROM THE WESTERN CAPE, SOUTH AFRICA

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DECLARATION

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ABSTRACT

Despite the availability of Hepatitis B virus (HBV) vaccination for over three decades, this infection remains a major public health problem. Whilst the WHO recommends giving a birth dose of the vaccine, in South Africa, routine infant HBV vaccination commences at six weeks of age. This schedule is based on data from the pre-HIV era which showed transmission occurred via the horizontal, rather than the vertical route. In the era of HIV however, maternal HIV co-infection may release HBV from immune control, resulting in higher HBV loads and increasing the risk of vertical transmission. The aim of this study was to determine the prevalence and character of HBV infection in HIV-exposed infected and uninfected infants.

Residual plasma samples from routine HIV nucleic acid testing of 1000 HIV-exposed infants aged between 0 and 18 months from the Western Cape were tested. Samples were tested for HBsAg by ELISA (Murex HBsAg Version 3) and confirmed by neutralisation. HBV DNA was quantified using an in-house real-time PCR assay. Infants with HBsAg positive samples were followed up and a blood sample was collected from mother and child. Those HBsAg positive samples were tested for HBeAg/antiHBe (Diasorin) and HBsAg negative samples were tested for antiHBs. HBV DNA was quantified. The *surface* gene was sequenced and the HBV genotype determined by phylogenetic analysis using HepSEQ (www.hepseq.org.uk). Whole genome sequencing was also performed.

Of 1000 samples tested, four samples were positive for HBsAg and/or HBV DNA, indicating a prevalence of HBV transmission of 0.4%. At follow-up, two of three infected infants were positive for HBsAg, with HBV viral loads of greater than 10^8 IU/ml. The third infant was found to have cleared his infection and the fourth child was lost to follow up. These infected infants had all received HBV vaccination. All four mothers were HBeAg positive. Sequencing analysis showed the HBV strains from the two infants and four mothers belonged to subgenotype A1. The two mother-child paired sequences were identical.

The data from this study shows that vertical transmission of HBV infection in HIV-exposed infants from the Western Cape is occurring, despite vaccination. Data from the Western Cape, showing an HBV prevalence of 3.4% in HIV-infected pregnant women, and those presented here suggest a vertical transmission rate of HBV of 12%. This is despite the widespread use of tenofovir and lamivudine in HIV-infected women with low CD4 counts. This study provides data supporting calls to bring HBV vaccination closer to the time of birth. Further work is urgently needed to confirm these findings and to determine the rates of transmission in HIV-unexposed infants.

OPSOMMING

Ten spyte van die beskikbaarheid van die Hepatitis B virus (HBV) inenting vir meer as drie dekades, hierdie infeksie bly 'n groot openbare gesondheid probleem. Terwyl die WGO aan beveel dat'n geboorte dosis van die entstof, in Suid-Afrika, roetine baba HBV inenting op die ouderdom van ses weke gegee word. Hierdie skedule is gebaseer op data van die pre-MIV era wat getoon het dat die oordrag plaasgevind het via die horisontale, eerder as die vertikale roete. In die era van MIV egter, moeder MIV ko-infeksie kan HBV vrylaat van immuun beheer, wat lei in hoër HBV vlakke en die verhoging van die risiko van vertikale oordrag. Die doel van hierdie studie was om die voorkoms en karakter van die HBV infeksie in MIV-besmette en onbesmette babas te bepaal.

Residuele plasma monsters van roetine-MIV-nukleïensuur toetse van 'n 1000 MIV-blootgestelde babas tussen die ouderdomme van 0 en 18 maande van die Wes-Kaap was getoets. Monsters was getoets vir HBsAg deur ELISA (Murex HBsAg Version 3) en bevestig deur neutralisering. HBV DNA is gekwantifiseer deur gebruik te maak van 'n in-huis real-time PCR assay. Babas met HBsAg positiewe monsters was opgevolg en 'n bloedmonster is versamel van moeder en kind. Die HBsAg positiewe monsters was getoets vir HBeAg/antiHBe (Diasorin) en HBsAg negatiewe monsters was getoets vir antiHBs. HBV DNA was gekwantifiseer. Die oppervlak gene volgorde en genotipes was bepaal deur filogenetiese analise met behulp van HepSEQ (www.hepseq.org.uk). Die hele genoom-volgordebepaling was ook uitgevoer.

Van die 1000 monsters wat getoets was, was vier monsters positief vir HBsAg en of HBV DNA, dit dui op 'n voorkoms van HBV oordrag van 0.4%. By op volg, twee van die drie besmette babas was positief vir HBsAg, met HBV virale vlakke van groter as 10^8 IE/ml. Die derde baba was gevind dat sy infeksie opgeklaar het en die vierde kind was verlore as gevolg van op volg. Hierdie besmette babas het almal HBV inenting ontvang. Al vier moeders was HBeAg positief. Volgordebepaling analise het getoon die HBV stamme van die twee babas en vier moeders behoort aan subgenotype A1. Die twee moeder-kind gepaarde rye was homoloë.

Die data van hierdie studie toon dat die vertikale oordrag van HBV infeksie in MIV-blootgestelde babas van die Wes-Kaap vind plaas, ten spyte van inenting. Data van die Wes-Kaap, wat 'n HBV voorkoms van 3.4% in MIV-besmette swanger vroue, en dié wat hier aangebied is dui op 'n vertikale oordrag koers van 12% van die HBV. Dit is ten spyte van die wydverspreide gebruik van tenofovir en lamivudine in MIV-geïnfekteerde vroue met 'n lae CD4-telling. Hierdie studie bied data wat ondersteunende oproepe van HBV inenting nader aan die tyd van die geboorte bring. Verdere werk is dringend nodig om die bevindinge te bevestig en die pryse van die oordrag in MIV-blootgestelde babas te bepaal.

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To Chacha Rafick – « Le vrai tombeau des morts c'est le coeur des vivants ».

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LIST OF ABBREVIATIONS AND SYMBOLS

ABI − Applied Biosystems Incorporated

ALT – Alanine amino transferase

AntiHBc − Antibody to hepatitis B core antigen

AntiHBe − Antibody to hepatitis B e antigen

AntiHBs − Antibody to hepatitis B surface antigen

ART – Antiretroviral therapy

BBVU – Blood Borne Viruses Unit

bp − base pair

ccc – covalently closed circular

C_t – Cycle threshold

dNTPs – deoxynucleoside triphosphate

Eco R1 − *Escherichia coli* restriction enzyme 1

ELISA − Enzyme Linked Immuno Sorbent Assay

EPI – Expanded Programme on Immunization

HBcAg – Hepatitis B core antigen

HBeAg − Hepatitis B e antigen

HBIg – Hepatitis B immunoglobulin

HBsAg − Hepatitis B surface antigen

HBV − Hepatitis B Virus

HBx – Hepatitis B X protein

HCC – Hepatocellular carcinoma

HEU − HIV-exposed uninfected

HIV − Human Immunodeficiency Virus

HPA − Health Protection Agency

HRP − Horse Radish Peroxidase

IU − International Unit

kb − Kilo base

LHBs − Large hepatitis B surface protein

MHBs – Middle hepatitis B surface protein

mCMV − Murine Cytomegalovirus

MTCT – Mother-to-child transmission

mRNA− Messenger RNA

MTCT − Mother-to-child transmission

n/a − not applicable

NHLS − National Health Laboratory Service

NHP − Normal Human Plasma

NTC − No-Template Control

OBI – Occult hepatitis B infection

OD − Optical Density

ORF − Open Reading Frame

Pol – Polymerase

PRF – Poliomyelitis Research Foundation

RCF – Relative centrifugal force

RNAse – Ribonuclease

SHBs – Small hepatitis B surface protein

Taq − *Thermus aquaticus*

TMB − 3,3', 5,5'-tetramethylbenzidine

WHO – World Health Organization

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CHAPTER ONE: INTRODUCTION

Throughout the world, more than 350 million people are chronically infected with hepatitis B virus (HBV) (Lavanchy 2004). This is despite the availability of a safe and effective vaccine for more than three decades. Africa and Asia carry the burden of this infection with up to 58 million and 130 million chronic carriers respectively (Custer et al. 2004). HBV is endemic to subSaharan Africa (Custer et al. 2004), but the rate of HBV infection varies between 5% and 19% between different African countries (Custer et al. 2004) and even within African countries (Kew 1996).

South Africa introduced HBV vaccination in 1995 to the local Expanded Programme on Immunization (EPI). The vaccine is first administered to infants at the age of six weeks, although the World Health Organization (WHO) recommends giving the first dose of the vaccine within 24 hours of birth. The South African schedule was based on epidemiological data available at the time showing horizontal transmission was the most important route of transmission. Guidozzi et al. (1993) showed that only 1.21% of pregnant women were positive for hepatitis B surface antigen (HBsAg) and only 4.6% of these women were positive for hepatitis B e antigen (HBeAg). Thus very few women were at risk of transmitting vertically. Vos et al. (1980) found the prevalence of HBsAg in children between the ages of two and four to be 10%. A study in 1983 by Prozesky et al. showed that the prevalence of HBsAg was only 1% in 103 unvaccinated infants aged less than six months compared to 6% in 256 older children between 0.5 and 5 years. The same prevalence was seen in Namibian infants younger than six months while 12.7% of children between the ages of one and six years were found to be HBsAg positive (Botha et al. 1984). Abdool Karim et al. (1988) found none of 51 infants less than one year old to be positive for HBsAg, but showed that 1.7% of 343 children between the ages of one and four were HBsAg positive. In a study from the Gambia, no infant below the age of six months was found to be infected with HBV, but infants between the ages of two and four from one village had a prevalence of HBsAg of 17.6% (Whittle et al. 1983). These studies showed that African mothers had a low risk of transmitting the virus to their children perinatally and that children were being infected after the age of one year, indicating that the major mode of HBV transmission was horizontal.

However, these studies were carried out when the human immunodeficiency virus (HIV) epidemic was not yet established in South Africa. HIV is known to impact the outcome of infection with HBV. In immunosuppressed patients, viral replication is poorly controlled by the host immune system resulting in high viral loads (Colin et al. 1999) and lower rates of HBeAg clearance (Thio 2009), which increase the risks of mother-to-child (MTCT) transmission of HBV (Burk et al. 1994). Recent research has shown a varying prevalence of HBsAg from 0.4% to 23% (Barth et al. 2010; Barth et al. 2011; Boyles and Cohen, 2011; Lukhwareni et al. 2009) and a high prevalence of so-called occult hepatitis B infections (OBI), which are characterised by the presence of HBV DNA in the absence of HBsAg, in HIV-infected adults in South Africa (Barth et al. 2011; Firnhaber et al. 2009; Lukhwareni et al. 2009). In contrast, fewer studies have been carried out in the South African paediatric population in the HIV-era (Hino et al. 2001; Tsebe et al. 2001; Simani et al. 2009) and none have looked at vertical transmission of HBV.

Currently, HIV-infected patients are not routinely screened for HBV infection, unless they fail first line antiretroviral therapy (ART) (National Department of Health, South Africa and South African National AIDS Council 2010a). HIV-infected adults are only started on ART, which may include two antiretroviral agents, tenofovir and lamivudine, that also have anti-HBV activity, if their CD4 counts are below 350 cells/mm^3 (National Department of Health, South Africa and South African National AIDS Council, 2010b). However, renal impairment is a contraindication to tenofovir therapy and HBV-HIV co-infected patients may be on lamivudine monotherapy (National Department of Health, South Africa and South African National AIDS Council 2010b). With the roll-out of perinatal ART in South Africa, the majority of infants born to HIV-infected mothers are HIV-exposed but uninfected (Jones et al. 2011). These infants are born with immune deficiencies (Filteau 2009) and have less transfer of maternal antibodies (Filteau 2009; Jones et al. 2011) potentially making them more susceptible to infections with more severe outcomes than HIV-unexposed infants (Filteau 2009). HIV-infected infants are not routinely tested for HBsAg and are started on ART, including lamivudine as the only antiHBV agent, as soon as they are diagnosed with HIV (National Department of Health, South Africa and South African National AIDS Council 2010b).

The use of lamivudine in monotherapy leads to the emergence of drug-resistant mutants within the first year of use through mutations in the *polymerase (pol)* gene of the HBV genome (Honkoop 1997). These may alter the overlapping *surface* gene (Torresi 2002) resulting in the expression of a mutated immunogenic epitope on the surface protein (HBsAg) (Clements et al. 2010). The wild-type HBsAg is usually used in HB vaccines and the mutated epitope may lead to the emergence of potential vaccine-escape mutants not neutralized by immunization-induced antibody (Clements et al. 2010). There is little data on the viral strains circulating in South Africa and whether any immune escape variants associated with drug resistance are being transmitted in the general population.

To date, no post-vaccine studies have looked at vertical transmission of HBV or characterised the HBV strains in the HIV-exposed paediatric population. Furthermore, there is limited data on the epidemiology of HBV in the Western Cape. Extrapolation of the data from other South African provinces to the Western Cape is difficult since there are regional differences in the prevalence of HIV (Sherman and Lilian 2011) and HBV infections (Kew 1996).

This study primarily sought to look at the prevalence of HBV infection in HIV-exposed infants and to investigate whether vertical transmission of HBV is occurring in South Africa in the context of the HIV epidemic.

The aims of the study were:

1. To determine the prevalence of active HBV infection in a sample population of HIV-exposed infants
2. To follow-up infected infants to determine the prevalence of persistent HBV infection
3. To trace mothers of infected infants to investigate whether the transmission was vertical
4. To characterise HBV and HBV infection in infected patients (infants and mothers) by determining viral load, HBeAg status, the HBV genotype and any drug-resistant/vaccine-escape mutations in the *pol/surface* gene.

CHAPTER TWO: LITERATURE REVIEW

2.1 STRUCTURE OF HBV

Hepatitis B is the smallest known DNA virus with a genome of only 3.2 kilobases. The mature virion is known as a Dane particle and is 42nm in diameter (Dane et al. 1970). Its genome is enclosed in a nucleocapsid made of core protein dimers, which is itself surrounded by an envelope composed of surface proteins (Harrison, Dusheiko and Zuckerman 2009).

The virus is unusual in several ways. Firstly, it is the only known partially-double stranded virus, with a complete minus and an incomplete plus strand (Delius et al. 1983). Secondly, unlike other DNA viruses, it uses the enzyme reverse transcriptase for its replication (Seeger et al. 1986). Thirdly, since its genome is so small, it has four open reading frames (ORFs) which all overlap each other in varying degrees (Nassal and Schaller 1993) as illustrated in Figure 2.1. These four ORFs code for structural proteins, secreted antigens and other proteins necessary for the virus's replication in the host cell (Nassal and Schaller 1993; Seeger and Mason 2000).

Polymerase ORF

The polymerase ORF which is the longest ORF of the virus has four domains (Locarnini et al. 2003):

1. a primase domain which codes for a primase which primes the genome for replication and is covalently attached to the 5' end of the minus strand in the mature virion,
2. a spacer which does not seem to have any function,
3. a polymerase domain, further divided into seven conserved subdomains named A to G (Bartholomeusz et al. 2004), which codes for a reverse transcriptase which is the largest and most important protein as it is responsible for replication of the virus and reverse transcription of the pregenomic ribonucleic acid (RNA) into DNA in the mature virion and
4. a ribonuclease-H (RNase-H) domain which codes for RNAse which removes the RNA strand from the DNA-RNA hybrid formed by reverse transcription of the pregenomic RNA. It is also thought to play a role in viral RNA packaging, in optimizing priming of

the minus-strand DNA synthesis and in elongation of the minus-strand viral DNA (Chen, Robinson and Marion 1994; Locarnini et al. 2003).

Within the polymerase subdomain C, is a tyrosine-methionine-aspartate-aspartate amino acid motif (the so-called YMDD motif) which is essential for reverse transcriptase activity and in which drug-resistance mutations to lamivudine may arise (Honkoop et al. 1997).

Surface ORF

The surface ORF is divided into three domains:

1. the S domain codes for 226 amino acids which make up the small hepatitis B surface protein (SHBs) which is also known as the surface antigen. The smallest antigen is also the most prolifically secreted antigen produced by the virus, but is non-infectious in nature. The marketed recombinant vaccines against HBV commonly mimic the 'a' determinant, an immunogenic determinant which is present within the SHBs of all genotypes (Clements et al. 2010). Drug-resistance mutations in the polymerase domain affect this region as a result of overlap between the polymerase and surface ORFs and may result in vaccine-escape mutants (Carman et al. 1990; Clements et al. 2010) and escape detection by some surface antigen assays (Weber 2006).
2. the PreS2 domain codes for 55 amino acids. The PreS2 and the S domain together code for the middle surface antigen (MHBs). Although it is known that MHBs can bind polymerized human serum albumin (Pontisso et al. 1989), its role still needs to be elucidated.
3. the PreS1 region codes for 108 or 119 amino acids depending on the genotype. The PreS1, PreS2 and the S domains collectively code for the large surface antigen protein (LHBs) (Locarnini et al. 2003). This protein has been implicated in the attachment of the virus to the host receptor although the receptor itself has not yet been identified (Gripon et al. 1995; Pontisso et al. 1989).

The three proteins produced by the surface ORF differ in size and are all found on the envelope of the virus in varying amounts; the most common is SHBs followed by MHBs and LHBs is the least present on the envelope of the virus (Locarnini et al. 2003).

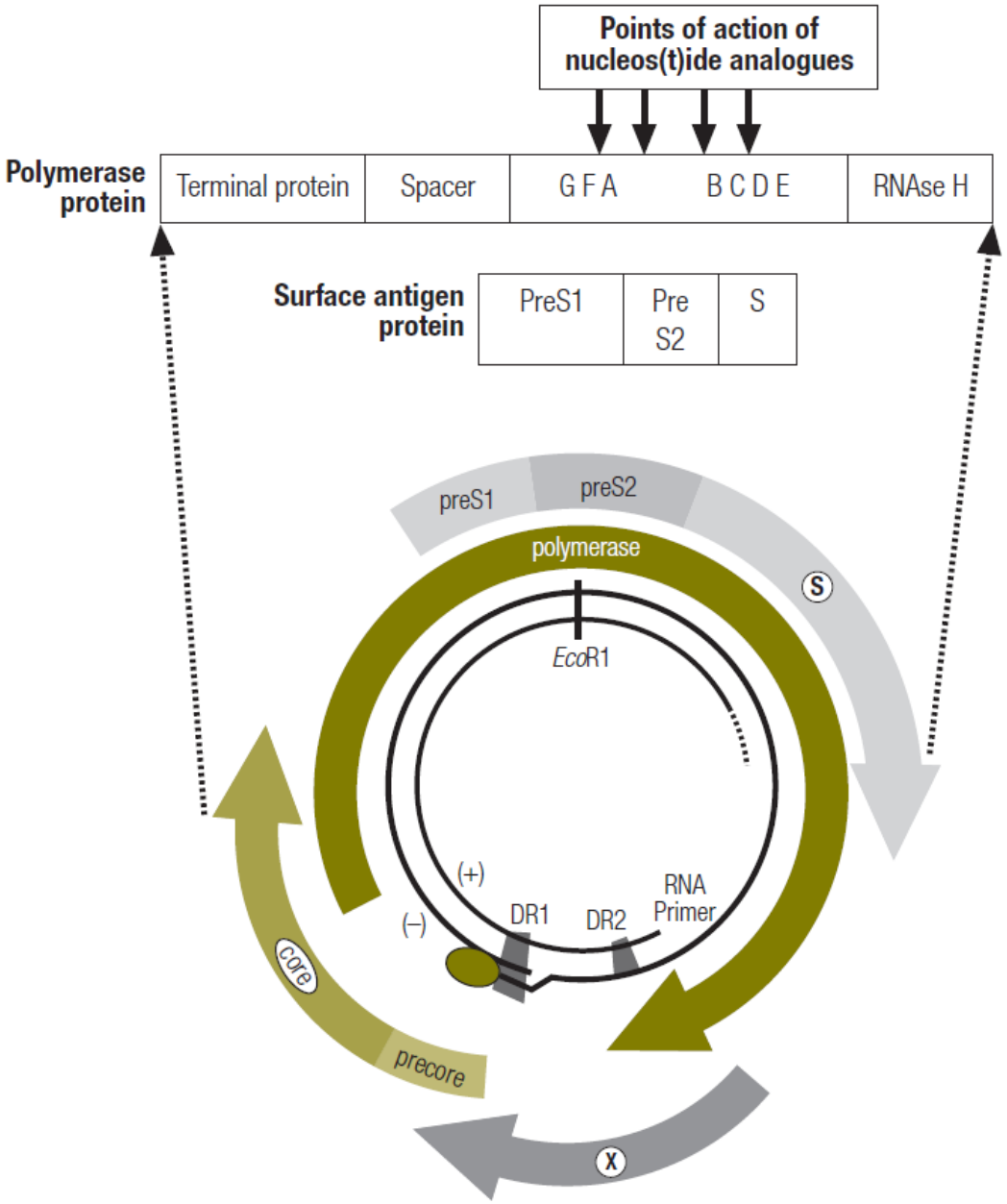

Figure 2. 1 HBV Genome showing overlapping ORFs and the potential for drug-resistance mutations in the *pol* region to influence the surface antigen protein (Source: Clements et al. (2010) Reproduced with permission)

GFABCDE:conserved regions in polymerase domain; DR1, direct repeat sequence 1; DR2, direct repeat sequence 2; EcoR1, the cut site of the restriction endonuclease EcoR1 derived from E. coli; X, X gene encoding the HBV X protein; PreS1 and PreS2, large envelope proteins; S, the small envelope protein.

Core ORF

The core ORF codes for two proteins, the core protein and the ‘e’ protein. The core protein is a structural protein making up the nucleocapsid of the virus (Harrison, Dusheiko and Zuckerman 2009) and is also involved in viral replication whereas the ‘e’ protein is a secreted antigen with immunoregulatory properties (Chen et al. 2005) which has been implicated as a potential tolerogen (Milich et al. 1990). The two proteins have distinct functions and are recognized as being different entities by antibodies but cross-react at the T-cell level (Chen et al. 2005; Milich et al. 1990).

The hepatitis B e antigen (HBeAg) is associated with high levels of replication of the virus and by extension, is a marker of infectiousness. However, ‘pre-core’ mutant strains of HBV have been identified in which a stop codon mutation in the pre-core region of the genome prevents the expression of the HBeAg (Hadziyannis and Vassilopoulos 2001). The viral loads in patients infected with these pre-core mutants can nonetheless be very high and they are infectious despite the absence of HBeAg (Fattovich 2003). Patients infected with precore mutants have been found to be at higher risk of fulminant infection and of developing more severe disease during chronic infections (Omata et al. 1991).

X ORF

The X ORF codes for the smallest product encoded by the virus, the hepatitis B x (HBx) protein which is only composed of 154 amino acids. HBx protein is known to be a transactivator of viral replication and has been shown to be essential for continued replication of the virus in vitro (Lucifora et al. 2011). HBx has been shown to be oncogenic in vitro although the exact mechanisms involving HBx and HBV-induced hepatocellular carcinoma (HCC) development are unclear (Kew 2011). Among those proposed are, the ability of HBx to inactivate the tumor-suppressor p53, its anti- and pro-apoptotic effects and its ability to induce hypo-and hyper-methylation of DNA (Kew 2011).

2.2 LIFE CYCLE AND REPLICATION

Hepatitis B virus infects and replicates in human hepatocytes (Seeger and Mason 2000) although the viral DNA has been isolated in other cells of the body (Bouffard et al. 1990) including the human ovary (Yu et al. 2012). The entry mechanism and the receptor it binds to are currently unknown although the LHBs protein is thought to be the most likely candidate for binding to the host cell surface (Nassal and Schaller 1993; Locarnini et al. 2003). The steps following binding of the virus including penetration and uncoating of the virus are also not well-described (Seeger and Mason 2000), but lead to release of the nucleocapsid into the cell cytoplasm (Locarnini et al. 2003).

The core enclosing the viral DNA migrates to the nucleus where the genome becomes completely double-stranded and the ends are ligated to form a covalently closed circular (ccc) genome (Locarnini et al. 2003). The cccDNA is associated with histone and non-histone proteins to form the minichromosome (Bock et al. 2001) which is then transcribed to produce genomic and subgenomic messenger RNAs (mRNAs) (Seeger and Mason 2000). The subgenomic transcript of 2.4 kilobases (kb) is translated in the rough endoplasmic reticulum to the LHBs protein, the 2.1kb subgenomic transcript is translated to the MHBs and SHBs proteins and the 0.7kb subgenomic mRNA is translated to the HBx protein (Locarnini et al. 2003).

The genomic transcripts are 3.5kb in size and include the precore mRNA which is translated to the HBeAg and a smaller bicistronic pregenomic mRNA which codes for the core and polymerase proteins and is reverse transcribed by the polymerase within the core to form the incomplete genome (Locarnini et al. 2003).

Nucleocapsids containing reverse transcribed RNA are selectively enveloped with the surface proteins in the Golgi apparatus and the viral particles are secreted from the cell through the secretory pathway (Seeger and Mason 2000). The virus is not cytopathic, that is, replication, production and release of mature virions do not result in lysis of the host cell (Harrison, Dusheiko and Zuckerman 2009).

One distinctive characteristic of HBV infection is chronicity. This persistence is maintained by the recycling of some core particles via the intracellular conversion pathway (Locarnini et al. 2003). Some nucleocapsids are not secreted and are instead used to increase the numbers of cccDNA in the nucleus of the hepatocyte thus maintaining a constant pool of cccDNA in the cell without the need for multiple rounds of re-infection (Locarnini et al. 2003). The life cycle of HBV is summarised in Figure 2.2.

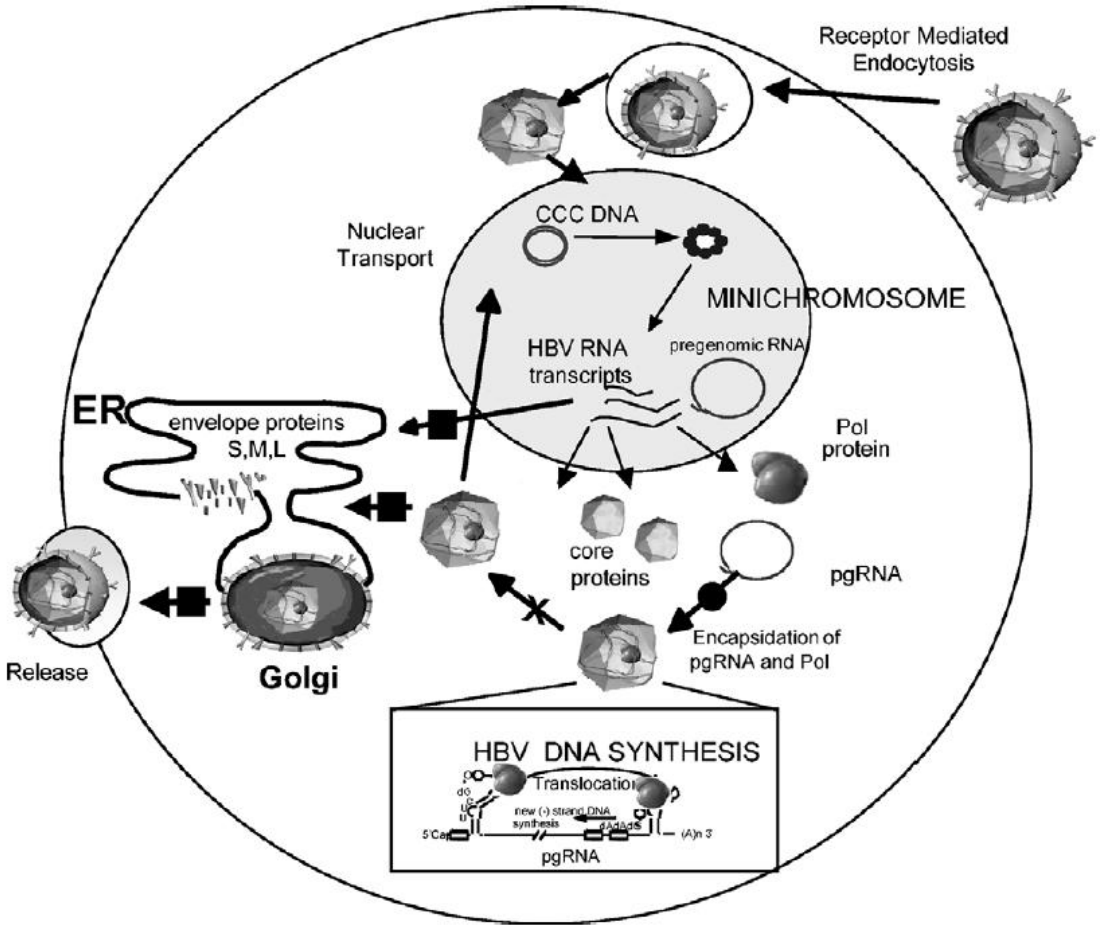

Figure 2. 2 HBV life cycle HBV infection, replication, encapsidation and release from hepatocytes (Source: Feld and Locarnini (2002) Reproduced with permission.)
ER: endoplasmic reticulum; ccc: covalently closed circular; pg: pregenomic; S: small; M: medium; L: large

2.3 EPIDEMIOLOGY OF HBV WITH EMPHASIS ON SOUTH AFRICA

An estimated two billion people have been infected with the hepatitis B virus worldwide and approximately 378 million have chronic liver infections (Franco et al. 2012). Between 500 000 and 1.2 million people die every year due to the acute or chronic consequences of hepatitis B (Lavanchy 2004).

Africa and Asia have the highest prevalence of HBV. Africa itself carries 18% of the burden of disease associated with HBV infections (Kramvis and Kew 2007). In South Africa alone, it is estimated that three to four million black people are chronically infected (Kew 2008). The prevalence of HBsAg across the African continent is illustrated in Figure 2.3.

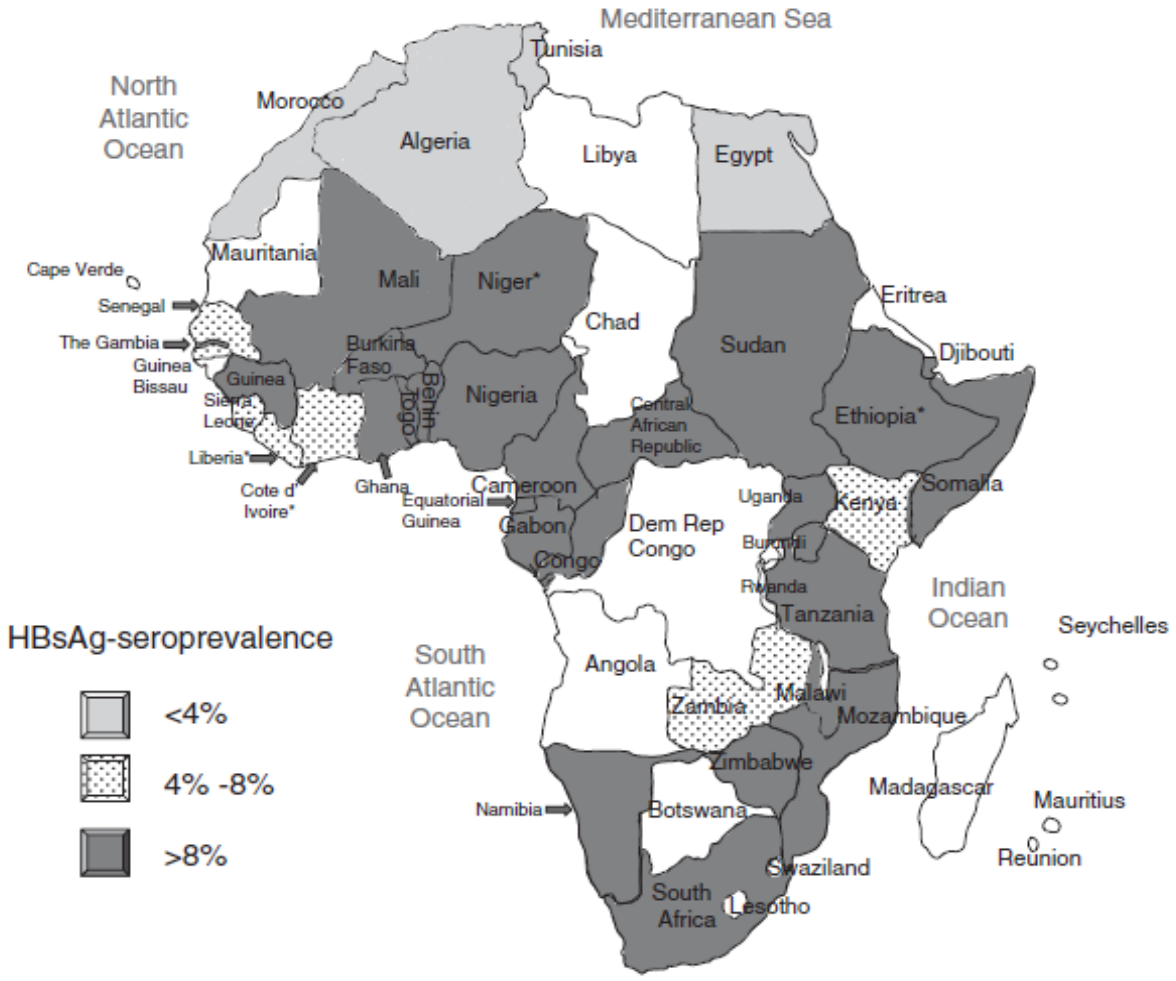

Figure 2. 3 Prevalence of HBsAg across the African continent The prevalence in the non-shaded countries is not known. (Source: Kramvis and Kew (2007) Reproduced with permission).

In South Africa, epidemiological studies have shown the prevalence of HBV infection to vary according to the region and the population under study. Song et al. (1988) reported a prevalence of HBsAg of 6.1% in South African Chinese women of child-bearing age. In contrast, a study by Kew et al. (1975) showed the prevalence of HBsAg in white mothers to be 0.16% whereas a later study by Kew et al. (1987) showed a prevalence of HBsAg of 1.31% in black women.

The Kew study from 1987 also revealed a higher prevalence of HBV infection in rural-born women (4.0%) compared to the urban-born women (1.3%). A similar trend was observed in children in a study by Abdool Karim et al. (1988) where 6.3% of children from an urban area were found to be HBsAg positive compared to 18.5% of children from a rural area. The same study also found the prevalence of HBsAg to be much higher in institutionalized children (35.4%).

Studies which were conducted in South Africa before the introduction of universal HBV vaccination provide a baseline against which the current prevalence of HBV can be compared. A study in 1983 by Prozesky et al. showed that the prevalence of HBsAg was only 1.0% in 103 infants aged less than six months. Abdool Karim et al. (1988) found the prevalence of HBsAg to be 1.5% in unvaccinated urban infants aged less than two years but none of the infected infants were less than one year old. A larger study conducted at the time of HB vaccine introduction to South Africa between 1995 and 1996 by Vardas et al. (1999) found a much higher prevalence of HBsAg of 9% in unvaccinated infants aged less than two years. The large discrepancy observed between these studies can be attributed to the small sample size investigated for that age group in the Prozesky and Abdool Karim studies and possible regional differences in the prevalence of HBV as has been been described previously (Kew et al. 1996). Studies after the introduction of the HB vaccine have shown a decrease in the prevalence of HBsAg in the vaccinated population. Hino et al. (2001) compared the rate of HBV infection between vaccinated children and unvaccinated children and found a decrease in the prevalence of HBV DNA from 6.5% in the unvaccinated cohort to 0.3% in the vaccinated group. Simani et al. (2009) reported 3/303 of their cohort of vaccinated children to be positive for HBsAg. A study by Tsebe et al. (2001) found none of the tested children to be positive for HBsAg.

However, these studies were carried out before the HIV epidemic was established in South Africa when horizontal transmission of HBV was thought to predominate. In the HIV-era, the viral loads in co-infected mothers may be higher as a consequence of loss of immune control (Thio 2009) potentially making vertical transmission a bigger problem than previously thought.

2.4 DISTRIBUTION OF GENOTYPES AND THEIR CLINICAL SIGNIFICANCE

Currently, there are eight major genotypes of HBV, named A to H, which are in circulation worldwide. Genomic differences of at least 8% are required for sequences to qualify as a new genotype (Kramvis et al. 2005). Following this criteria, two additional genotypes, I and J, have recently been described but they are not as well characterised (Huy et al. 2008; Tatematsu et al. 2009) and the classification of genotype I is controversial (Kurbanov et al. 2008). Within some of the genotypes, HBV strains which differ by at least 4% but less than 8% are classified into subgenotypes. These genotypes and their subgenotypes are distributed in specific regions of the world (Figure 2.4) (Kramvis et al. 2005).

Within Africa, HBV strains belonging to genotypes A, D and E predominate. Genotype A is most prevalent in Southern and East Africa and subgenotype A1 has been commonly isolated in African patients including South Africa (Kramvis and Kew 2007). Other less common subgenotypes, A3 to A7, have been described in West and Central African countries (Kramvis and Kew 2007; Hübschen et al. 2010; Pourkarim et al. 2010a) although it is suggested that subgenotypes A3, A 4 and A5 might have been misclassified (Pourkarim et al. 2010b). Subgenotype A2 on the other hand is most commonly seen in Europe and Japan (Kramvis et al. 2005; Tamada et al. 2012). Genotype A does not produce pre-core mutants as typically seen with genotype D with a stop codon at position 1896 of the precore region (Kramvis 2008). This is because of the instability this mutation would cause in the folding of the pregenomic mRNA during encapsidation (Kramvis 2008). However, mutants with a stop codon at position 1862 of the precore region have been identified and have been hypothesised to reduce the expression of HBeAg (Kramvis 2008).

Genotype D is mostly concentrated in the Mediterranean region (Kramvis et al. 2005) and is therefore the most common genotype in North Africa. Isolates belonging to genotype D have been described in South Africa. However, the strains found in these two different geographical areas belong to different subgenotypes (Kramvis and Kew 2007).

Genotype E which is an African genotype is most commonly seen in Central and West Africa (Kramvis and Kew 2007).

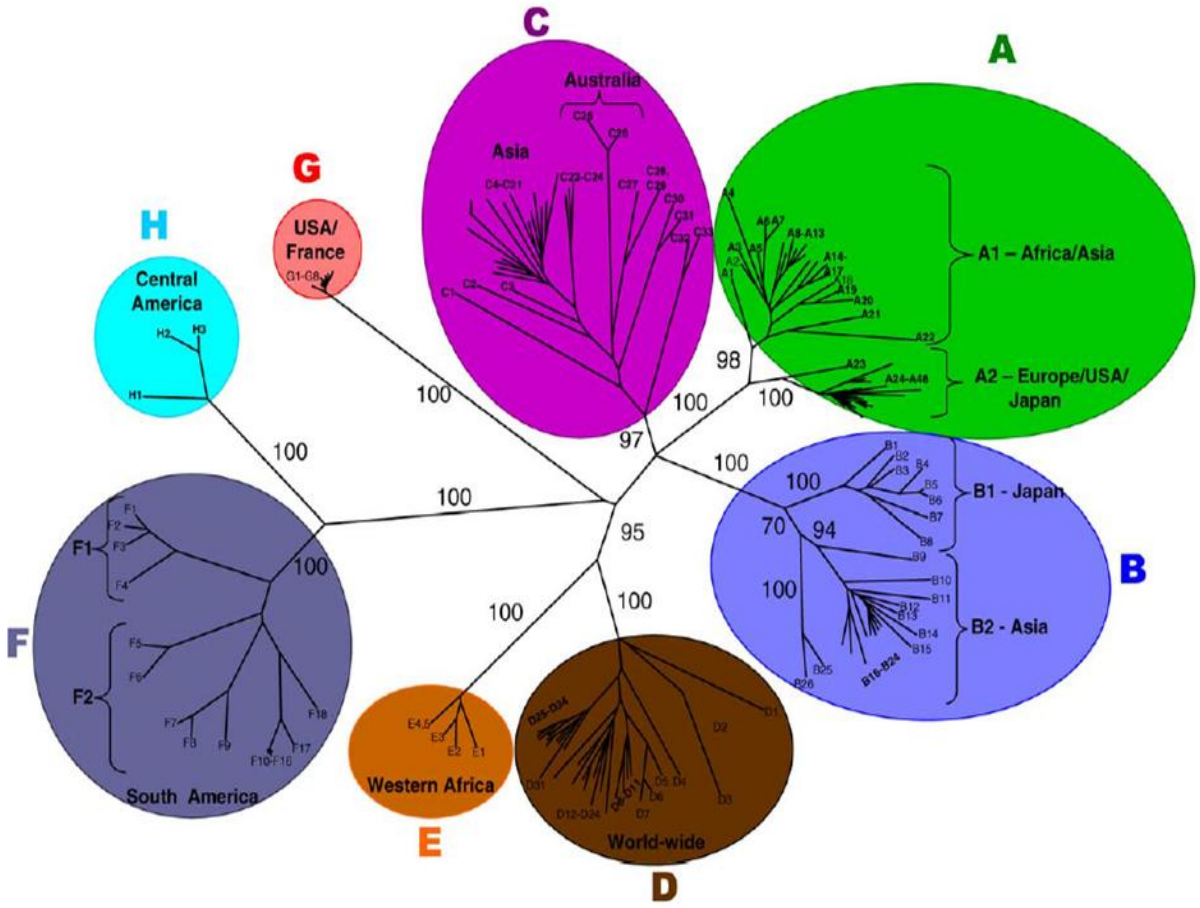

Figure 2. 4 Phylogenetic tree representing the worldwide distribution of HBV genotypes
(Source: Kramvis et al. (2005) Reproduced with permission.)

These different genotypes have an impact on treatment as they show different disease progression have different susceptibilities to therapy. These are summarised in Table 2.1.

Table 2. 1 Comparison of clinical and virological differences among hepatitis B virus genotypes. (Adapted from Lin and Kao (2011) Reproduced with permission.)

GENOTYPE	**A**	**D**	**E**
Clinical characteristics			
Modes of transmission	Horizontal	horizontal	horizontal
Tendency of chronicity	Higher	Lower	ND
Positivity of HBeAg	Higher	Lower	ND
HBeAg seroconversion	Earlier	Later	ND
HBsAg seroclearance	More	Less	ND
Histologic activity	Lower	Higher	ND
Clinical outcome (cirrhosis and HCC)	Worse	Worse	ND
Response to interferon alpha	Higher	Lower	ND
Response to nucleos(t)ide analogues		ND	ND
Virologic characteristics			
Serum HBV DNA level	ND	ND	ND
Frequency of precore A 1896 mutation	Lower	Higher	ND
Frequency of basal core promoter T1762/A1764 mutation	Higher	Lower	ND
Frequency of pre-S deletion mutation	ND	ND	ND

ND: No available data

2.5 NATURAL HISTORY OF HBV INFECTION

Acute infection

HBV infection can lead to a self-limiting acute disease primarily affecting the liver which will clear within six months of infection. HBV has an incubation period of 90 to 150 days following which, markers of infection such as HBsAg and HBV DNA become detectable using the currently available commercial assays. Patients will normally have an elevated alanine aminotransferase (ALT) level. ALT is a liver enzyme released in the bloodstream during an episode of inflammation in the liver. The serological profile of acute, resolving hepatitis B infection is shown in Figure 2.5. Patients with an acute infection will show varied symptoms, ranging from nausea and vomiting to rashes and will often progress to jaundice (Previsani and Lavanchy 2002). About 1% of acute cases develop into fulminant hepatitis which is often fatal in adults (Previsani and Lavanchy 2002). There is no specified treatment for acute HBV infection unless patients are suffering from fulminant hepatitis B or protracted severe acute hepatitis B (Lok and McMahon 2009). In 95% of adults and only 10% of newborns, HBsAg will clear within three months with the development of protective antiHBs antibodies.

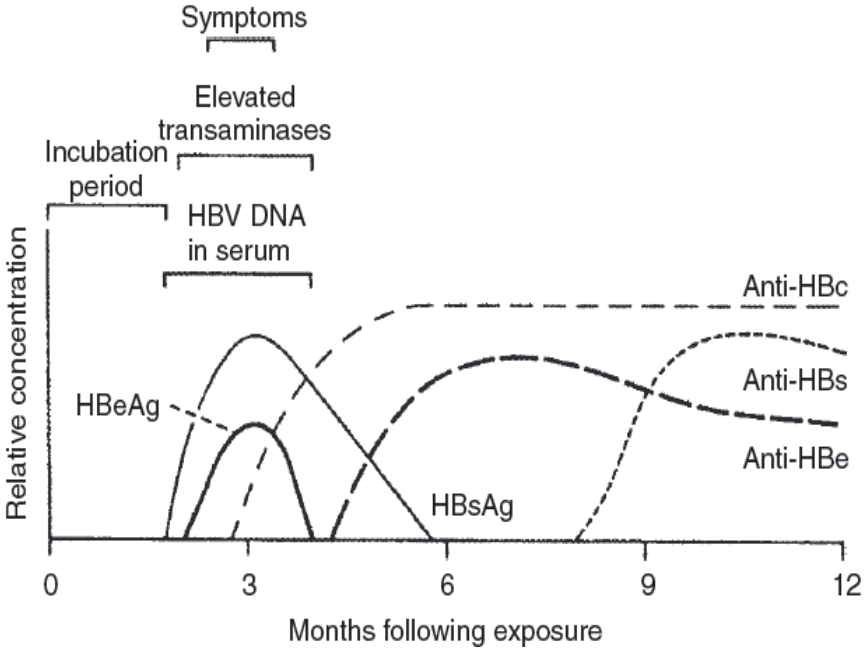

Figure 2. 5 Serological profile of acute, resolving hepatitis B infection (Source Harrison, Dusheiko and Zuckerman (2009) Reproduced with permission). HBeAg: hepatitis B e antigen; HBsAg: hepatitis B surface antigen; Anti-HBc: antibody to hepatitis B core antigen; Anti-HBs: antibody to hepatitis B surface antigen; Anti-HBe: antibody to hepatitis B e antigen.

Chronic infection

The most severe consequences of HBV infection are seen in chronically-infected patients who may develop cirrhosis and HCC decades after the initial infection. Chronic HBV infection is defined as HBsAg positivity for more than six months.

Age at infection is an important risk factor for development of chronicity. Vertical transmission from HBeAg-positive mothers has a 90% risk of resulting in a chronic infection (Beasley et al. 1977). This has been attributed to the immaturity of the infants' immune systems (Hadziyannis 2011). Horizontal transmission before the age of five years has a reduced risk of chronicity of 10-30% (Beasley et al. 1982) and in adults, the risk of chronicity is less than 5% (Lok and McMahon 2009).

Chronic HBV infection is progressive and can be divided into four phases (illustrated in Figure 2.5) although not every patient will experience all stages in a particular order (Dandri and Locarnini 2012).

Immune tolerant phase

The first is the immune tolerant phase, which occurs mostly in infants who are infected vertically and in whom it may last for decades (Fattovich et al. 2008). Older children and adults may experience the immune tolerant phase transiently (Fattovich et al. 2008; Yim and Lok, 2006). During this stage, the virus is not cleared by the immune system of the patients. As a result, the patient has high viral loads (>20 000 IU/ml), high levels of HBeAg and HBsAg (Dandri and Locarnini 2012), but normal or mildly elevated levels of liver enzymes (ALT and aspartate aminotransferase) with minimal liver damage (Fattovich et al. 2008).

Immune clearance/HBeAg-positive chronic hepatitis B

During the immune clearance phase, immune tolerance to HBV is lost and the virus is cleared by the immune system (Fattovich et al. 2008; Yim and Lok 2006). Typically in this phase,

inflammation of the liver, elevated ALT levels and fluctuating HBV DNA levels (>20 000 IU/ml) are observed (Dandri and Locarnini 2012; Fattovich et al. 2008).

Eventually, in most patients, this stage will result in the clearance of HBeAg and the appearance of antibody to hepatitis B e antigen (antiHBe) marking the transition to the next phase (Fattovich et al. 2008). Some patients however, will periodically show fluctuating levels of DNA replication and flares in liver enzyme levels (Yim and Lok 2006). The duration of this phase and the severity of the liver enzyme flares have been described as risk factors for cirrhosis and HCC (Dandri and Locarnini 2012; Yim and Lok 2006).

Immune control phase

The following phase is the inactive HBsAg carrier state characterised by the absence of HBeAg, presence of antiHBe, a persistently low or undetectable level of HBV DNA (<2000 IU/ml has been suggested) and normal ALT levels (Fattovich et al. 2008). If the patient remains in this phase, the outcome of the chronic infection is benign, with no resulting hepatic decompensation (Yim and Lok 2006).

Immune escape phase/HBeAg-negative chronic hepatitis B

However, some patients may progress to the next phase of chronic infection which is the reactivation/HBeAg-negative chronic hepatitis stage (Yim and Lok 2006). In this phase, the infection reactivates either spontaneously or due to immunosuppression of the patient with reversion to the HBeAg-positive state, or more commonly to an HBeAg-negative state due to the emergence of pre-core mutant strains of HBV which are unable to produce HBeAg (Fattovich et al. 2008). This phase is characterised by the absence of HBeAg, the presence of antiHBe along with continued varying degrees of liver inflammation and elevated but fluctuating ALT levels. HBV DNA levels may vary from 2000 to 20 million IU/ml (Fattovich et al. 2008).

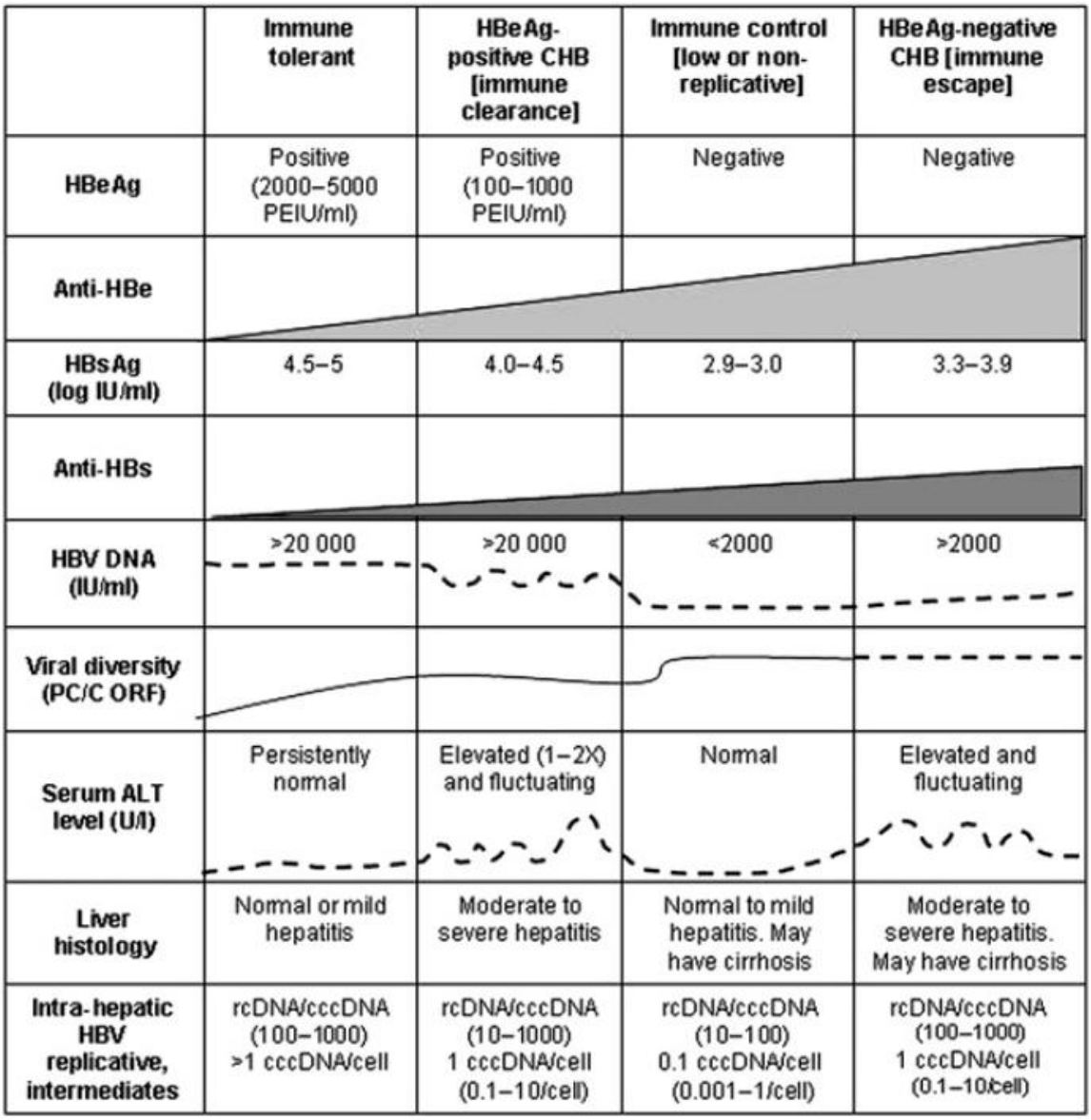

Figure 2.6 Phases of chronic hepatitis B infection: serum and liver compartment. (Source: Dandri and Locarnini (2012) Reproduced with permission)

ALT: alanine aminotransferase; cccDNA: covalently closed circular DNA; HBeAg: hepatitis B secreted e-antigen; HBsAg: hepatitis B surface antigen; HBV: hepatitis B virus; ORF: open reading frame; PC/C: precore/core; PE: Paul Ehrlich Institute; rcDNA: relaxed circular partially double-stranded DNA.

Occult infection

The detection of HBV DNA in serum in the absence of HBsAg is defined as an OBI and is thought to be due to control of the wild-type virus by the immune system rather than infection with a mutant strain of HBV (Dandri and Locarnini 2012; Hollinger and Good 2010). OBI has been associated with HBV transmission in organ transplants (Hollinger and Good 2010) and blood transfusions (Vermeulen et al. 2012) predominantly in the absence of antiHBs in the donated blood (Hollinger and Good 2010). OBI seems to be a risk factor for HCC development (Raimondo et al. 2007) although it is as yet unclear what mechanisms are involved (Zerbini et al. 2008). In an occult infection, other markers such as antiHBc may or may not be detectable.

HBV-related hepatocellular carcinoma

Chronic infection with HBV is a risk factor for the development of liver cirrhosis and HCC (Beasley 1988). In endemic countries, chronic infection is mostly due to infection in childhood and in the subSahara, this leads to early development of HCC around the age of 45 years (Yang and Roberts 2010).

The development of HBV-related HCC was originally thought to be the result of integration of the viral DNA into the host genome, but this integration does not target a specific part of the genome (Bruix and Llovet 2003) and other studies have suggested that this is not the actual mechanism behind hepatocarcinogenesis (Di Bisceglie 2009). Chronic infection may indirectly lead to HCC through continuous inflammation, scarring and repair of liver tissue leading to an increased turnover of hepatocytes and to the mutations in the genome which promote carcinogenesis (But et al. 2008). Recent studies have also directly implicated the HBx protein produced by the virus as having oncogenic properties (Bruix and Llovet 2003; Di Bisceglie 2009; Kew 2011). The truncated MHBs antigen could also be involved in HCC development as it has transactivating properties (Blum and Moradpour 2002).

Certain genotypes of HBV have been linked to higher rates of HCC and in Asia, genotype C is thought to have a higher risk of HCC development than genotype B, but further studies are needed to clarify these associations (Di Bisceglie 2009). In South Africa, studies on genotype A have shown that infection with that particular genotype had a 4.5-fold higher risk of HCC development compared to infection with other genotypes (Kew et al. 2005). Another risk factor is gender; male patients have been shown to be two to seven times more likely to develop HCC than women (Yang and Roberts 2010).

HIV-HBV co-infection

HIV co-infection is known to influence the course of HBV infection. Chronic HBV infection occurs in 5% of immunocompetent adults whereas in HIV-infected adults, 25% of HIV-HBV co-infections will become chronic (Lacombe et al. 2010). In co-infected patients not on therapy, due to the immunosuppression caused by HIV, HBV is able to replicate to high levels (Lacombe et al. 2010; Thio 2009) and in chronic infections, this has the potential to lead to early development of cirrhosis and HCC (Chen et al. 2006). Early ART for these patients is indicated (Brook et al. 2010).

MTCT of HBV in co-infected pregnant women is also an important issue. High viral loads in these patients will lead to MTCT of HBV (Burk et al. 1994) irrespective of immunization at birth (Lee et al. 1986). These women therefore need to be screened for HBsAg and HBeAg and if positive, they should be treated with a nucleos(t)ide analogue early in their third trimester to reduce their viral loads (Dusheiko 2012; Shi et al. 2010; Tran 2012).

There are several nucleos(t)ide analogues such as lamivudine or tenofovir which can be used to treat both HBV and HIV monoinfections. However, lamivudine is rarely used alone to treat either infection because of its low genetic barrier to development of resistance in either HIV or HBV. A triple *pol* (rtV173L+rtL180M+rtM204V) mutant strain arising mainly after exposure to lamivudine (Lacombe 2010) leads to corresponding mutations in the *surface* gene (sE164D+sI195M) and has been shown to result in vaccine failure in chimpanzees (Kamili et al. 2009).

The widespread use of ART to treat HIV has increased the life expectancy of HIV-infected individuals. It is therefore expected that more cases of HCC, one of the long-term consequences of HBV infection, will be seen in HIV-HBV co-infected patients (Lacombe 2010).

2.6 MODES OF TRANSMISSION

The virus is a blood-borne virus and any contact with contaminated blood or bodily fluids containing infected blood through the mucous membranes or broken skin could potentially result into transmission (Harrison, Dusheiko and Zuckerman 2009).

Vertical transmission

The virus can be transmitted from mother to child either *in utero* or during delivery and shortly after birth through the close contact between the infected mother and the neonate (perinatally).

Although *in utero* transmission is rare, its occurrence is significantly associated with high maternal viral loads (Burk et al. 1994), a history of threatened preterm labour (Tran 2012), acute infection during the third term of pregnancy (Wood and Isaacs 2012) and can occur by the infection of endothelial cells of placenta capillaries and by cellular transfer from cell to cell (Xu D et al. 2002). Polymorphisms in cytokine genes have also been correlated with a susceptibility of intra-uterine HBV infection (Jonas 2009). This mode of transmission commonly results in infection of the neonate despite vaccination at birth (Lee et al. 1986) and is associated with the mother's viral load irrespective of e-antigen status (Burk et al. 1994).

Transmission at birth is possible when the baby is exposed to the blood and the genital secretions of the mother in the birth canal (Ranger-Rogez and Denis 2004). Breast-feeding is not contra-indicated in mothers infected with HBV except if there is a possibility of exposure to maternal blood through cracked and bleeding nipples (Tran 2012). Although the virus is detected in breast milk, this has not been shown to result in HBV transmission (Hill et al. 2002; Wang et al. 2003).

Horizontal transmission in childhood

Horizontal transmission is thought to be the most common route of transmission of HBV in childhood in subSaharan Africa either from sibling to sibling or between playmates (Whittle et al. 1983). Horizontal transmission in children is transmission which does not occur vertically or through sexual or parenteral routes (Davis, Weber and Lemon 1989). The routes of horizontal transmission have not yet been clearly identified although several potential routes have been investigated.

One of the possible vehicles of transmission is saliva, which would explain how children transmit the virus from one another. Although saliva has been shown in gibbons to be infectious if injected (Bancroft et al. 1977), and high levels of HBV DNA have been found in the saliva of chronic carriers including children (Van Der Eijk et al. 2005), no studies have yet shown that oral exposure to saliva causes transmission of the virus. Transmission through bites from an infected person has been shown to occur (Stornello et al. 1991, Hui et al. 2005). This mode of transmission could be explained by the exposure of the blood of the uninfected person to the saliva of a chronic carrier known to carry a high viral load. Butler et al. (2010) showed that the mucosal surfaces and broken skin of children are commonly exposed to the saliva of their caregivers through various means including premastication of food and cleaning a cut/scrape on the children's bodies with saliva.

Other bodily fluids containing high levels of HBV DNA in infected individuals, including urine and sweat, have also been investigated but these have not been associated with transmission (Knutsson and Kidd-Ljunggren 2000; Van Der Eijk et al. 2005). A recent study showed that tears from a chronically infected baby could cause HBV infection in chimera mice (Komatsu et al. 2012).

In Africa, other modes of horizontal transmission could include ritual scarification and circumcision performed by a 'witch-doctor' or a traditional healer using unsterilized instruments (Kew et al. 1973). HBV virus is a sturdy virus capable of existing on surfaces for more than a week in the absence of visible blood, and still be infectious (Bond et al. 1981, Favero et al.

1974). Bedbugs have also been implicated as possible vehicles of transmission (Mayans et al. 1990).

Contact with infected bodily fluids

In areas of low endemicity, the virus is mostly transmitted in the adult population, between drug users sharing needles and through sexual contact with an infected person (Alter 2003).

Nosocomial infections are also possible especially among patients undergoing haemodialysis (Alter 2003). Improper sterilization of needles and reuse of disposable needles are also possible modes of transmission in hospital settings (Alter 2003).

Infections through blood transfusions in South Africa are rare because of the recent introduction of individual-donation nucleic acid testing of blood in addition to serological testing to identify any potential OBI donors. However, if a donor is still in the window period of infection and is therefore negative for the tested markers, the donated blood has the potential of infecting the recipient (Vermeulen et al. 2012).

Recently, a first report of reverse vertical transmission has been published. The authors reported that a mother was infected by her baby who had received a transfusion of contaminated blood. Phylogenetic analysis confirmed that the virus transmitted was homologous (Niederhauser et al. 2012).

2.7 PREVENTION OF HBV INFECTION IN INFANTS

Active immunisation

HBV is a vaccine-preventable disease and a plasma-derived vaccine has been available since 1980. A safe and effective second generation recombinant vaccine has also been available since 1986 (WHO 2009), although it was only introduced to the South African EPI in 1995. The latter vaccine contains yeast-derived recombinant HBsAg with the immunogenic 'a' determinant (Clements et al. 2010). Third generation vaccines containing the PreS1 and PreS2 proteins in addition to HBsAg can elicit antibody responses in non-responders of the traditional vaccine (Harrison, Dusheiko and Zuckerman 2009).

The WHO recommends the use of three doses of the vaccine in all infants: at birth, four weeks and ten weeks (WHO 2009). The efficacy of the vaccine has been demonstrated and the schedule recommended by the WHO has been shown to provide long-term immunity in healthy individuals (Viviani et al. 1999; van der Sande et al. 2006).

Lee et al. (2006) calculated that infants who were born to HBsAg positive mothers but were vaccinated at birth were 3.5 times less likely to become infected with HBV. Administration of the vaccine later than a week after birth has been associated with an increased risk of MTCT of HBV infection (Marion et al. 1994; Ruff et al. 1995).

In South Africa and in most of subSaharan Africa, the vaccine is administered at six, ten and fourteen weeks, a schedule which reflects the low risk of vertical transmission of HBV (Guidozzi et al. 1993) and the assumed predominance of horizontal transmission in infants (Botha et al. 1986; Whittle et al. 1983).

The vaccine is not contraindicated in HIV-infected infants and should be administered as soon as possible after birth with additional doses in non-responders. However, the levels of antiHBs in these infants should be monitored to ensure that they are adequately protected from HBV infection. It is recommended that immunocompromised individuals should be tested annually for

levels of antiHBs and be revaccinated if these levels fall below the protective level of 10 IU/L (Kane et al. 2000).

Hepatitis B immunoglobulin

The hepatitis B immunoglobulin (HBIg) is a human immune globulin preparation with a high titre of antibody to hepatitis B surface antigen (antiHBs) used for passive immunoprophylaxis. Since it only provides immunity for about three months, it needs to be used as a supplement to the HB vaccine in preventing MTCT of HBV.

The efficacy of using HBIg along with vaccination in infants at birth has been demonstrated. Beasley et al. (1983) showed that the combined use of HBIg and the birth dose of the vaccine was 94% effective in preventing MTCT compared to an efficacy of only 71% and 75% if HBIg and vaccine respectively were used alone.

However, the benefit of using HBIg in infants born to mothers who are infected with precore mutant strains of HBV may be limited in preventing vertical transmission of the virus (Yang et al. 2003) but may reduce the risk of fulminant hepatitis in these newborns (Chen et al. 2004).

The use of HBIg in the third trimester of pregnancy has been previously reported to significantly reduce the rate of transmission of HBV in HBeAg-positive mothers (Xiao et al. 2007). However, another study found no benefit in using HBIg before birth in HBeAg-positive mothers (Yuan et al. 2006).

In subSaharan Africa, HBIg is not available for PMTCT because of the costs involved and because vertical transmission of HBV is not considered to be a major health problem.

Role of antivirals in the prevention of MTCT

The use of either vaccine or HBIg or both will not however prevent MTCT if the mother's viral load is high (Burk et al. 1994; Lee et al. 1986). HBV infected pregnant women with high viral

loads need to be started on antiHBV therapy during pregnancy. HIV-HBV co-infected mothers with low CD4 counts are less likely to clear HBeAg (Lacombe et al. 2010; Thio 2009), and have high viral loads, making them at risk of transmitting HBV to their children.

The use of a nucleos(t)ide analogue to reduce the HB viral loads of the mothers during pregnancy in conjunction with vaccination of the baby will lower the risk of MTCT of HBV (Dusheiko 2012). Xu et al. (2009) showed that transmission of HBV was significantly decreased by the use of lamivudine when compared to the placebo group. The same has been reported by another group (van Zonneveld 2003).

However, given the rapid emergence of drug resistance mutations from lamivudine monotherapy, it is not recommended for use alone in the prevention of MTCT. In treating both mono and co-infections, the drug of choice is tenofovir. So far clinical trials have not looked at the efficacy of tenofovir in preventing MTCT of HBV. However, a retrospective study has shown that the use of tenofovir significantly reduced the viral loads in HBV-infected mothers and none of their infants were positive for HBsAg at 28-36 weeks after delivery (Pan et al. 2012).

Infants may be exposed to tenofovir through breastfeeding but the concentration secreted in breastmilk is low. There is a potential risk of bone density and renal problems in neonates who are exposed to tenofovir, but the benefits are thought to outweigh the risks (Kew et al. 2011).

CHAPTER THREE: MATERIALS AND METHODS

3.1 ETHICAL APPROVAL

Ethics Approval was received from the Health Research Ethics Committee of Stellenbosch University on the 31st of May 2011 for a period of one year (Ethics reference number: N11/05/151). This was renewed in May 2012 for an additional year.

3.2 SAMPLE AND DATA COLLECTION

Residual samples submitted to the National Health Laboratory Service (NHLS) Division of Medical Virology, Tygerberg Hospital, for HIV-1 screening PCR tests were included in this cross-sectional study. These samples were collected from HIV-exposed children between the ages of 0 and 18 months. Samples which conformed to the following criteria were included:

1. children residing in the Western Cape;
2. conclusive HIV-1 DNA PCR and HIV-1 RNA PCR results were available and
3. residual sample volume was at least 100 µl.

The chosen samples were centrifuged at 1400 relative centrifugal force (RCF) for ten minutes. The isolated plasma was transferred to a 1.5ml microcentrifuge tube before being chronologically relabeled to de-identify each sample to meet ethical requirements. Plasma samples were stored at -20 °C until further testing.

Also in accordance with ethical guidelines, personal patient information and laboratory identification numbers were stored in a password-protected digital database, which was separate from the study data. However, for demographic analytical purposes, information on the age, sex, and HIV status of the patients, where available, was documented. Since information about the race of these patients was not regularly available, this criterion was excluded from further study analyses.

3.3 SCREENING PROCESS AND FOLLOW-UP OF POSITIVE SAMPLES

The samples were initially tested for HBsAg and confirmed with an HBsAg neutralisation assay. In parallel to serological testing, the first 600 HBsAg negative samples were tested for HBV DNA. Very few OBIs (presence of HBV DNA in the absence of HBsAg) were expected. Therefore, HBsAg negative samples which had more than 150µl of plasma were pooled and extracted together. Any pooled samples found to have a detectable viral load using real-time PCR, were extracted individually and tested as individual samples. HBsAg negative samples with less than 150µl of plasma and HBsAg positive samples were extracted independently.

The names and contact details of the infants whose samples were found to be HBsAg and/or DNA positive were retrieved from the digital database. The mothers of these infants were contacted through their respective clinics and a blood sample was collected on the same day from each mother-infant pair. The blood samples were dispatched to the Division of Medical Virology, Tygerberg, where they were centrifuged and the plasma collected was stored at -20°C until further testing. The followed-up mother-child pairs are collectively called ‘patients’ henceforth. The patients were first tested for HBsAg, and the positive results confirmed by an HBsAg neutralization test using antiHBs. Confirmed HBsAg positive patients were tested for HBeAg/antiHBe and HBsAg negative patients were tested for antiHBs where possible. All followed-up patients were also tested for HBV DNA and antibody to hepatitis B core antigen (antiHBc). HBV DNA positive samples were sequenced where possible.

Patients who tested positive for HBsAg and/or HBV DNA on follow-up were referred to a hepatologist collaborating on this project from Tygerberg Hospital for further management.

3.4 SEROLOGICAL TESTING

3.4.1 SCREENING FOR HBsAg AT SCREENING STAGE AND FOLLOW UP

Principle

The Murex HBsAg Version 3 assay (Murex Biotech Ltd, Dartfort, United Kingdom (UK)) qualitatively tests for the presence of HBsAg in plasma and serum samples of human origin. The microtitre plate wells from the kit are pre-coated with mouse monoclonal antibodies which bind specifically to a primary epitope of HBsAg. Following the principle of a sandwich Enzyme Linked Immuno Sorbent Assay (ELISA), any HBsAg that was present in the sample would first bind to the pre-coated antibody. An antibody labeled with the enzyme Horse Radish Peroxidase (HRP) would then be added to the wells and would bind to a secondary epitope of the captured HBsAg, creating an antibody/antigen/antibody-enzyme complex. In the absence of HBsAg in the plasma sample, there would be no complex formation. The addition of an enzyme colorimetric substrate would cause a colour change. This substrate solution contained 3,3', 5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide which could be oxidized in a reaction catalysed by the enzyme bound in the complex, causing a colour change to purple in the positive wells. There would be a final colour change to yellow in the wells after addition of a stop solution (0.5 M H_2SO_4). A sample which was negative for HBsAg would remain colourless after the addition of the substrate and stop solutions.

Method

Samples were first diluted 1:4 with normal human plasma (NHP) provided by the Western Province Blood Transfusion Service known to be negative for all HBV markers. This dilution method has been previously validated in this laboratory (Maponga TG, MSc Thesis, Stellenbosch University, 2012) and was done because of the limited amount of plasma available for testing.

The samples were first incubated for 60 minutes in the wells at 37^{o}C with a sample diluent. A solution containing a goat anti-human antibody specific for HBsAg was then added to the wells and the microtitre plate was incubated for 30 minutes at 37^{o}C. The microtitre plate was then washed five times using an automatic plate washer. Immediately after the washing step, 100µl of

a substrate solution was added to each well. The final step in this assay was the addition of 50µl of the stop solution. The colour intensity of each well was measured spectrophotometrically using a dual wavelength Anthos HT3 Microtiter Plate Reader (Anthos Labtec Instruments GmbH, Salzburg, Austria) at 450 nm, using 650 nm as the reference wavelength, within 15 minutes of the addition of the stop solution.

Validation of results

To validate each test batch, two negative controls, as well as a positive control (provided by the manufacturer) were included in each run. An in-house working control known to be positive for HBsAg was also included in each run. For the run to be considered valid the mean absorbance value of the negative controls had to be less than 0.15 and the absorbance value of the positive control had to be greater than 0.8 above the mean of the negative controls.

Calculation of results

Once the microtitre plate was read, an average was calculated using the absorbance of the negative control wells. A value of 0.05 was added to obtain a cut off. Only samples which exceeded this threshold were considered to be positive.

3.4.2 CONFIRMATION TEST FOR HBsAg POSITIVES

Principle

Samples were confirmed as being HBsAg positive by neutralization. This in-house test was performed with the Murex HBsAg Version 3 Assay and antiHBs-positive plasma obtained from the Blood Borne Viruses Unit (BBVU), Health Protection Agency (HPA), London.

Each sample was diluted 1:2 with two different diluents. One diluent contained the antiHBs antibodies and one diluent was the NHP known to be negative for all HBV markers. These tubes were then incubated overnight and tested the following day for HBsAg. The principle of the test is that the tube containing NHP and the sample would show a certain optical density (OD) which would be above the cut off threshold making the sample positive for HBsAg. In the tube with antiHBs positive plasma and the sample however, the antibodies would bind to the HBsAg to form immune complexes. These complexes would not be detected on the Murex HBsAg assay and the wells with the antiHBs would therefore show a reduced OD.

Method

The sample itself was first diluted 1:2 using NHP. Next, 50µl of antiHBs-positive plasma and 50µl of NHP were added to two separate tubes respectively, followed by 50µl of the diluted sample. This resulted in a final sample dilution of 1:4. The tubes were vortexed, centrifuged and incubated for at least 16 hours at 37°C. After the overnight incubation, the samples were tested for HBsAg using the Murex HBsAg Version 3 Assay as described above in section 3.5.1.
One sample had a low volume of plasma left and a 1:4 dilution was first done on the sample and then 50µl of this dilution was added to 50µl of NHP and 50µl of antiHBs so that the final dilution factor was eight.

Some of the followed-up plasma samples had OD values which were above the detection range of the spectrophotometer. These samples were diluted $1:10^4$ before an observable reduction in OD could be seen.

Validation of results

To validate each test batch, two negative controls, as well as a positive control (provided by the manufacturer) were included in each run. An in-house working control known to be positive for HBsAg was also included in each run. For the run to be considered valid the mean absorbance value of the negative controls had to be less than 0.15 and the absorbance value of the positive control had to be greater than 0.8 above the mean of the negative controls.

Calculation of results

Once the microtitre plate was read, an average was calculated using the absorbance of the negative control wells. A value of 0.05 was added to obtain a cut off. Only samples which exceeded this threshold were considered to be positive.

Neutralization is defined as a reduction of at least 50% between the absorbance values obtained with and without addition of antiHBs positive plasma. Samples which were neutralized were considered to be true HBsAg positives.

3.4.3 HBeAg TESTING OF HBsAg POSITIVE SAMPLES

Principle

All followed-up patient plasmas which were confirmed HBsAg positive were tested for HBeAg. HBeAg is a marker associated with high viral loads. The ETI-EBK PLUS kit from Diasorin (Diasorin, Saluggia, Italy) qualitatively tests for the presence of HBeAg in plasma and serum samples of human origin. The microtitre wells from the kit are pre-coated with mouse monoclonal antibodies which bind specifically to HBeAg. Following the principle of a sandwich ELISA, any HBeAg that was present in the sample would first bind to the pre-coated antibody. An antibody labeled with the enzyme HRP would then be added to the wells and would bind to a secondary epitope of the captured HBeAg, creating an antibody/antigen/antibody-enzyme complex. In the absence of HBeAg in the plasma sample, there would be no complex formation. The addition of an enzyme colorimetric substrate would cause a colour change. This substrate solution contained TMB and hydrogen peroxide which could be oxidized in a reaction catalysed by the enzyme bound in the complex, causing a colour change to blue in the positive wells. There would be a final colour change to yellow in the wells after addition of a stop solution (0.4 N H_2SO_4). A sample which was negative for HBeAg would remain colourless after the addition of the substrate and stop solutions.

Method

The samples were first incubated for 120 minutes in the wells at 37^{o}C with 50µl of an incubation buffer. The plate was then washed five times to remove any excess sample using an automatic plate washer. Next, 100µl of an enzyme tracer was added to the wells and the microtitre plate was incubated for 60 minutes at 37^{o}C. The microtitre plate was once more washed five times using an automatic plate washer. Immediately after the washing step, 100µl of a substrate solution was added to each well and incubated at room temperature for 30 minutes away from direct light. The final step in this assay was the addition of 100µl of the stop solution. The colour intensity of each well was measured spectrophotometrically using a dual wavelength Anthos HT3 Microtiter Plate Reader (Anthos Labtec Instruments GmbH, Salzburg, Austria) at 450 nm, using 650 nm as the reference wavelength.

Validation of results

To validate each test batch, three calibrator controls, one negative control, as well as a positive control (provided by the manufacturer) were included in each run. An in-house working control known to be positive for HBeAg and a blank well containing only the substrate solution were also included in each run.

For a run to be considered valid, the following criteria needed to be met:

1. The absorbance value for the blank well was between 0.000 and 0.150.
2. The absorbance of each calibrator and the negative control and the mean absorbance of the 3 calibrators were greater than -0.020 and less than 0.120.
3. The absorbance of the positive control was greater than 0.500 and less than 2.500.
4. The difference between the absorbance of the positive control and the negative control was greater than 0.450.

Calculation of results

The cut off value of the run was calculated by subtracting the absorbance of the blank from the mean absorbance of the calibrator control values and adding 0.060. Samples with an absorbance of 10% or more above that of the cut off were considered to be positive.

3.4.4 AntiHBe TESTING OF HBsAg-POSITIVE SAMPLES

Principle

All HBsAg-positive samples were tested for antiHBe. AntiHBe is a marker of recovery and is present in the blood along with HBeAg when HBeAg seroconversion is imminent. The ETI-EBK AB PLUS kit from Diasorin (Diasorin, Saluggia, Italy) qualitatively tests for the presence of HBeAg in serum and plasma samples of human origin. The microtitre wells from the kit are pre-coated with mouse monoclonal antibodies which bind specifically to a primary epitope of HBeAg. A neutralization buffer containing recombinant HBeAg was added to the samples. Following the principle of a competitive ELISA, any free antiHBe in the sample would compete with the immobilized antiHBe for the HBeAg in the buffer. An antibody labeled with the enzyme HRP would then be added to the wells. In the absence of antiHBe in the samples, this antibody would bind to a secondary epitope of the captured HBeAg in the microwells, creating an antibody/antigen/antibody-enzyme complex. The amount of complex formation would be inversely proportional to the amount of antiHBe in the sample. The addition of an enzyme colorimetric substrate would cause a colour change. This substrate solution contained TMB and hydrogen peroxide which could be oxidized in a reaction catalysed by the enzyme bound in the complex, causing a colour change to blue in the wells negative for antiHBe. The addition of a stop solution (0.4N H_2SO_4) would cause a final colour change to yellow in the negative wells. A sample which was positive for antiHBe would remain colourless after the addition of the substrate and stop solutions.

Method

Fifty microlitres of each sample was first incubated for 120 minutes in the wells at 37^{o}C with 50µl of an incubation buffer and 50µl of a neutralization buffer. The plate was then washed five times to remove any excess sample and recombinant HBeAg using an automatic plate washer. Next, 100µl of an enzyme tracer containing mouse monoclonal antibody specific for HBeAg was added to the wells and the microtitre plate was incubated for 60 minutes at 37^{o}C. The microtitre plate was once more washed five times using an automatic plate washer. Immediately after the washing step, 100µl of a substrate solution was added to each well and incubated at room

temperature for 30 minutes away from direct light. The final step in this assay was the addition of 100µl of the stop solution. The colour intensity of each well was inversely proportional to the amount of antiHBe in a sample and was measured spectrophotometrically using a dual wavelength Anthos HT3 Microtiter Plate Reader (Anthos Labtec Instruments GmbH, Salzburg, Austria) at 450 nm, using 650 nm as the reference wavelength.

Validation of results

To validate each test batch, three calibrator controls, one negative control, as well as a positive control, provided by the manufacturer, were included in each run. A blank well containing only the substrate solution was also included in each run.

For a run to be considered valid, the following criteria needed to be met:

1. The absorbance value for the blank well was between 0.000 and 0.150.
2. The absorbance of each calibrator and the negative control and the mean absorbance of the 3 calibrators were greater than 0.500 and less than 2.500.
3. The absorbance of the positive control was greater than 0.050 and less than 0.300.
4. The difference between the absorbance of the negative control and the positive control was greater than 0.250.

Calculation of results

The cut off of the run was calculated by subtracting the absorbance of the blank from the mean absorbance of the calibrator values and multiplying it by 0.500. Samples with an absorbance of 10% or less below that of the cut off were considered to be positive.

3.4.5 AntiHBc TESTING OF ALL FOLLOWED-UP SAMPLES

Principle

The follow-ups were also tested for antiHBc which is a marker of past infection. The Murex antiHBc (total) assay (Murex Biotech Ltd, Dartfort, UK) qualitatively tests for the presence of total antiHBc in serum and plasma samples of human origin. The microtitre plate wells from the kit are pre-coated with recombinant hepatitis B core antigen (HBcAg). Following the principle of a competitive ELISA, any antiHBc that was present in the sample would first bind to the pre-coated recombinant HBcAg in the wells. In the absence of antiHBc in the sample, an antibody labeled with HRP would bind to the HBcAg coated on the wells, creating an antibody/antigen/antibody-enzyme complex. On the other hand, if antiHBc was present in the plasma sample, there would be no or little complex formation with the coated antigen. The addition of an enzyme colorimetric substrate would cause a colour change. This substrate solution contained TMB and hydrogen peroxide which could be oxidized in a reaction catalysed by the enzyme bound in the complex, causing a colour change to blue/green in the wells negative for antiHBc. The addition of a stop solution would cause a final colour change to yellow in the negative wells. A sample which was positive for antiHBc would remain colourless after the addition of the substrate and stop solutions.

Method

The samples were first incubated for 30 minutes in the wells at 37°C with a sample diluent. The addition of a sample to the green sample diluent changed its colour to blue. The microtitre plate was then washed five times using an automatic plate washer and immediately after washing, a solution containing antibody specific for HBcAg was added to the wells, and the microtitre plate was incubated for half an hour at 37°C. The microtitre plate was then washed another five times using an automatic plate washer and immediately after washing, 100µl of a substrate solution was added to each well and the plate was incubated for another half an hour in the dark at 37°C. The final step in this assay was the addition of 50µl of a stop solution, 0.5M H_2SO_4. The colour intensity of each well was inversely proportional to the concentration of antiHBc in the sample and was measured spectrophotometrically using a dual wavelength Anthos HT3 Microtiter Plate

Reader (Anthos Labtec Instruments GmbH, Salzburg, Austria) at 450 nm, using 650 nm as the reference wavelength, within 15 minutes of the addition of stop solution.

Validation of results

To validate each test batch, two negative controls and two positive controls, provided by the manufacturer, were included in each run. An in-house working control known to be positive for antiHBc was also included in each run. The mean absorbance of the positive controls had to be below 0.24 and the difference between the mean absorbance value of the negative controls and the mean absorbance value of the positive controls had to be between 0.5 and 2.2 for the run to be considered valid.

Calculation of results

The cut off value was calculated by adding the mean absorbance of the positive controls to the mean absorbance of the negative controls and dividing this value by two. Only samples which had an absorbance value below this threshold were considered to be positive.

3.4.6 AntiHBs TESTING OF HBsAg NEGATIVE SAMPLES

Principle

Samples which were HBsAg negative were tested for antiHBs. AntiHBs is a marker of recovery from an HBV infection and will normally be detected in the blood after HBsAg has disappeared although there is a window period between the disappearance of HBsAg and the appearance of antiHBs. AntiHBs is also produced by the immune system in response to the administration of the hepatitis B vaccine. The ETI-EBK AB PLUS kit from Diasorin (Diasorin, Saluggia, Italy) was used to qualitatively test for the presence of antiHBs in serum and plasma samples of human origin. The microtitre wells from the kit are pre-coated with human HBsAg and any antiHBs in a sample would bind to a primary epitope of the coated HBsAg. Then, HBsAg labeled with HRP would be added and the captured antiHBs would bind to a secondary epitope of the labeled HBsAg creating antigen/antibody/antigen-enzyme complexes in the microwells of the positive samples. In the absence of antiHBs in the plasma sample, there would be no complex formation. The addition of an enzyme colorimetric substrate would cause a colour change. This substrate solution contained TMB and hydrogen peroxide which could be oxidized in a reaction catalysed by the enzyme bound in the complex, causing a colour change to blue in the positive wells. Lastly, a blocking reagent (1N H_2SO_4) would be added causing a final colour change to yellow in the wells positive for antiHBs. A sample which was negative for antiHBs would remain colourless after the addition of the substrate and stop solutions.

Method

One hundred microlitres of each sample was first incubated for 120 minutes in the wells at 37^{o}C with 100µl of an incubation buffer. The plate was then washed five times to remove any excess sample. Then, 100µl of an enzyme tracer containing HBsAg was added to the wells and the microtitre plate was incubated for an hour at 37^{o}C. The microtitre plate was once more washed five times using an automatic plate washer. Immediately after the washing step, 100µl of a substrate solution was added to each well and incubated at room temperature for 30 minutes away from direct light. The final step in this assay was the addition of 200µl of a blocking reagent. The colour intensity of each well was proportional to the amount of antiHBs in a sample

and was measured spectrophotometrically using a dual wavelength Anthos HT3 Microtiter Plate Reader (Anthos Labtec Instruments GmbH, Salzburg, Austria) at 450 nm, using 650 nm as the reference wavelength.

Validation of results

To validate each test batch, three calibrator controls (two of Calibrator 1 and one of Calibrator 2) and two negative controls provided by the manufacturer, were included in each run. An in-house working control containing 10 IU/L of antiHBs and a blank well containing only the substrate solution were also included in each run.

For a run to be considered valid, the following criteria must have been met:

1. The absorbance value for the blank well must be between 0.000 and 0.150.
2. The mean absorbance of the negative control must be less than 0.100.
3. The mean absorbance of Calibrator 1 must be greater than 0.035 and less than 0.300.
4. The ratio of the mean absorbance for Calibrator 1 to the mean absorbance for the negative control must be greater than or equal to 2.0.
5. The ratio of the mean absorbance for Calibrator 2 to the mean absorbance for Calibrator 1 must be greater than or equal to 4.5.

Calculation of results

Calibrator 1 contains antiHBs in the concentration of 10 IU/L. Since a value of more than 10 IU/L is generally considered to be protective, the absorbance value observed with Calibrator 1 was taken to be the cut off. Therefore, samples which had an absorbance greater than or equal to the absorbance observed in the wells containing Calibrator 1 were considered to be positive for antiHBs.

3.4.7 QUALITY CONTROL FOR SEROLOGICAL ASSAYS

The addition of each reagent was marked by a colour change which ensured that no wells were missed.

To ensure that there was no carry-over contamination between wells, separate tips were used to pipette individual samples and reagents into the respective wells. The tubes with the plasma samples were briefly centrifuged before testing to eliminate the risk of aerosol formation from any droplets on the inside of the caps. In addition to the manufacturer's controls, an additional in-house working control which was positive for the marker under test (except for antiHBe) was included with each run to ensure that Westgard's rules of quality control were being followed.

For the neutralization in-house assay, in addition to the above, control tubes with antiHBs alone, NHP alone, antiHBs and NHP together were also incubated along with the samples. They acted as negative controls to ensure that there was no cross-reactivity between reagents which could have resulted in false positives. A working control known to be HBsAg positive was also tested in the same way as the samples to ensure the assay was working properly.

An automatic washer was used for the washing steps and was cleaned with autoclaved Milli-Q grade water and primed with washing buffer before each run. After each run, the washer was again washed with autoclaved Milli-Q grade water to prevent blocking of the system with salts from the washing buffer.

In any cases where a run did not meet the manufacturer's validation criteria, the results were considered to be invalid and the test was repeated for those samples.

3.5 MOLECULAR TESTING

3.5.1 DNA EXTRACTION OF SAMPLES

3.5.1.1 INDIVIDUAL VIRAL DNA EXTRACTIONS

The HBsAg positive samples were first diluted 1:4 with NHP known to be negative for HBV markers due to limited sample volumes. The QIAamp® Min Elute® Virus Spin Kit (QIAGEN, Germany) was used to extract DNA from the samples. The minimum amount of plasma required for the test using this kit was 200µl.

A lysis buffer containing 1µg/µl of carrier RNA (provided with the kit) and an internal control [400 copies/µl of murine cytomegalovirus (mCMV)] was freshly prepared before each extraction. The mCMV was obtained from the BBVU, HPA (Colindale, UK). The extraction was then performed according to the manufacturer's instructions with some minor modifications described by Garson et al. (2005) and the DNA eluted in 65µl of elution buffer provided with the kit.

Ten-fold dilutions were made of a patient sample with a known viral load of 10^8 IU/ml. The kit was then used to extract dilutions of 10^7 IU/ml to 10^1 IU/ml and eluted in 65µl of elution buffer. These dilutions (henceforth referred to as real-time PCR standards) were subsequently used to construct a standard curve.

3.5.1.2 VALIDATION OF POOLING ASSAY FOR VIRAL DNA EXTRACTIONS

The limit of detection of the real-time PCR assay, D_L IU/ml, was determined first. A patient sample of known HBV viral load (10^8 IU/ml) was diluted to 250 IU/ml and 100 IU/ml of HBV. One-fold dilutions were made from these two stock concentrations as shown in Table 3.1 and termed 'working dilutions'. These working dilutions were extracted and run in duplicate on three different days using a real-time PCR protocol described elsewhere (in section 3.5.2). The viral loads corresponding to the lowest dilution detectable in all three runs were thus deduced to be D_L IU/ml.

Table 3. 1Dilutions of stock solutions of 250 IU/ml, 100IU/ml and PL IU/ml

	Neat	1:2	1:3	1:4	1:5	1:6	1:8	1:10	1:20	1:25
NHP/ µl	0	100	133.3	150	160	166.7	175	180	190	192
Neat/ µl	200	100	66.7	50	40	33.3	25	20	10	8

From the stock solution of 100 IU/ml, working dilutions until 1:10 were made and extracted and from the stock solution of P_L IU/ml, dilutions until 1:6 were made and extracted.

The real-time assay would be used to detect the viral load in a batch of four pooled samples of 50µl each. The viral load in any positive sample in a batch would thus be diluted by a factor of four. D_L IU/ml was therefore multiplied by four to theoretically determine the limit of detection, P_L IU/ml, for a pooling assay. To confirm this, the 10^8 IU/ml sample was diluted to obtain a stock solution containing P_L IU/ml of HBV. One-fold dilutions were made from the working solution containing P_L IU/ml as shown in Table 3.1 and were tested in duplicate on three different days using a real-time PCR protocol described elsewhere (section 3.5.2). The viral load corresponding to a dilution of 1:4 needed to be detectable in all three runs for P_L IU/ml to be confirmed as the detection limit of the pooling assay. The pooling protocol was then employed to screen batches of four, pooled, 50µl, HBsAg negative samples for HBV DNA as described elsewhere (sections 3.5.1.3 and 3.5.2).

3.5.1.3 POOLED VIRAL DNA EXTRACTIONS

DNA was extracted from the pooled samples, as described for the individual extractions in section 3.5.2. The only change to the protocol was in the first step where 50µl of plasma from four different samples (giving a total volume of 200µl) was added to 25µl of Proteinase K in a 1.5ml microcentrifuge tube followed by 200µl of the lysis buffer.

Furthermore, if HBV DNA was detected in a batch of pooled samples, the four samples making up the positive pool were extracted individually as described under section 3.5.1.1 and tested for HBV DNA by real time PCR as described under section 3.5.2.

3.5.2 VIRAL LOAD TESTING USING REAL-TIME PCR

A multiplex, hydrolysis probe-based, real-time PCR assay, with mCMV as an internal control, described by Garson et al. (2005) was used to calculate the viral loads of the samples. The assay was validated for DNA quantification using a final reaction volume of 25µl on the RotorGene™ 6000 (Corbett Life Science, Australia) in this laboratory (Maponga TG, MSc Thesis, Stellenbosch University, 2012), and is based on the in-house real-time HBV assay used at the BBVU, HPA London (http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1194947340684).

The HBV primers and probe used in this assay target highly conserved sequences in the HBsAg gene. The mCMV primers and probe target regions specific to mCMV and were not expected to cross-react with sequences from other species. The probes used a TAMRA quencher and VIC and FAM as the reporter dyes. FAM has an emission wavelength of 517nm which can be detected by the green channel of the real-time thermocycler and was used for the detection of HBV. VIC was detected by the yellow channel of the real-time thermocycler as its emission wavelength is 554nm and was used to detect mCMV. As described in section 3.5.1.1, previously extracted real-time PCR standards were used to construct a standard curve against which the viral loads of the samples were calculated.

The primer and probe sequences are listed in Table 3.2.

Table 3. 2 List of primer and probe sequences used for HBsAg gene real-time PCR assay

Target	Primer sequence
HBV_Forward	5′-GTG TCT GCG GCG TTT TAT CA-3′
HBV_Reverse	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	5′-AAC CCG GCA AGA TTT CTA ACG-3′
mCMV_Reverse	5′-ATT CTG TGG GTC TGC GAC TCA -3′
mCMV_Probe	5′-VIC-CTA GTC ATC GAC GGT GCA CAT CGG C-3′TAMRA

Method

The master mix was prepared for the appropriate number of samples, controls and standards (in duplicate), using the volumes and concentrations for each reagent given in Table 3.3 including a 2x SensiMix Probe Kit II buffer from Bioline (Bioline, GmbH, Germany). Next, 15µl of the master mix was distributed in individual 0.2ml PCR tubes and ten µl of DNA extract from each sample or standard was added to their respective tube so that the final reaction volume was 25µl. The cycling parameters of the PCR reaction given in Table 3.4 were used.

Table 3. 3 Master mix composition for surface gene real-time PCR assay

Reagent	Starting Concentration	Volume/µl x1	Final Concentration
SensiMix Buffer	2x	12.50	1x
HBV_Forward	100 µM	0.10	400 nM
HBV_Reverse	100 µM	0.10	400 nM
HBV_Probe	100 µM	0.05	200 nM
mCMV_Forward	100 µM	0.10	400 nM
mCMV_Reverse	100 µM	0.10	400 nM
mCMV_Probe	100 µM	0.05	200 nM
Water	n/a	2.00	n/a
Total volume	n/a	15.00	n/a

n/a: not applicable

Table 3. 4 Cycling parameters for surface gene real-time PCR assay

Cycling parameter	Cycles	Temperature	Time
Initial denaturation	1 hold	95°C	15 minutes
Denaturation	45 cycles	95°C	15 seconds
Annealing/Extension		60°C	60 seconds

After the completion of each run, results were analysed using the Rotor-Gene 6000 Series Software 1.7 (Corbett Life Sciences, Australia).

Validity of results

For a run to be considered valid, the following criteria needed to be met:

1. There was no detectable viral load in the negative controls (NHP) and the NTC.
2. The standard curve used to calculate viral loads was valid. This was given by:
    a. An R^2 value of between 0.9 and 1.1. The R^2 is a measure of how reliable the results are.
    b. An M value of between 3.1 and 3.4. The M is used to calculate the efficiency of the reaction,
    c. The cycle threshold (Ct) values of the standards and the samples (the point where the amplification curve crosses the threshold and which was used to calculate the viral load in the sample from the standard curve) was less than the value of B. B was the highest cycle number at which the viral load obtained from the graph was considered to be reliable. If a sample crossed the detection threshold after cycle number B, its viral load was not calculated by the software.

The values of R^2, M and B were automatically calculated by the software and displayed next to the standard curve. The threshold was manually adjusted in certain cases to obtain satisfactory values for the M and R^2.

Calculation of results

Since each sample was diluted four times before being extracted, the viral load given by the above method was multiplied four fold to calculate the viral load in the original sample.

3.5.3 SEQUENCING OF *POL/SURFACE* GENE REGION OF HBV DNA POSITIVE SAMPLES

Principle

The *pol/surface* genomic region of the HBV strains was sequenced in DNA-positive samples using a protocol established by the BBVU, HPA (Colindale, London) which has since been established in this laboratory (http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1194947340684).

The steps followed for the sequencing can be summarized as follows: Two rounds of conventional PCR were performed on the DNA positive samples. The PCR products were then electrophoresed on an agarose gel to ensure that sufficient quantities of the target DNA fragment had been amplified to be sequenced. This would be indicated by the presence of a crisp thick band of the expected size for each sample. The PCR products were purified and subjected to a cycle sequencing reaction. The sequencing reaction product was cleaned and loaded on a capillary sequencer to be analysed.

Method

Pre-nested PCR

A pre-nested PCR was performed on the DNA extracts of the HBV DNA-positive samples. The primers shown in Table 3.5 were used and the master mix for the PCR reaction was prepared using the reagents listed in Table 3.6.

Table 3. 5 Primers used for pre-nested PCR of *pol/surface* region

Name	Sequence	Binding position from *Eco* R1 site
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. 6 Master mix for pre-nested PCR of *pol/surface* region

Reagents	Starting Concentration	Volume/µl x1	Final Concentration
PCR Buffer	10x	2.50	1x
$MgCl_2$	50mM	0.75	1.5mM
dNTPs*	10mM	0.50	0.2mM
Taq Polymerase		0.10	
HBV 3	20pmol/µl	0.50	0.4pmol/µl
HBV Z	20pmol/µl	0.50	0.4pmol/µl
dH_2O	n/a	15.15	n/a
Total volume	n/a	**20.00**	n/a

*deoxynucleoside triphosphate; n/a: not applicable

The master-mix was distributed in volumes of 20µl in 0.2ml PCR tubes to which five µl of DNA extract was added. The thermocycling parameters given in Table 3.7 were used for the PCR reaction.

Table 3. 7 Cycling parameters for pre-nested PCR of *pol/surface* region

Cycling parameter	Cycles	Temperature	Time
Initial denaturation	1 hold	95°C	5 minutes
Denaturation	34 cycles	94°C	30 seconds
Annealing		55°C	30 seconds
Extension		72°C	1 minute
Final Extension	1 hold	72°C	2 minutes

Nested PCR

A second round of PCR amplification was performed on the pre-nested samples to increase the specificity of the reaction and to increase the product yield. The primers used are shown in Table 3.8 and the master mix was prepared using the reagents listed in Table 3.9.

Table 3. 8 Primers used for nested PCR of *pol/surface* region

Name	Sequence	Binding position from *Eco* R1 site
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. 9 Master mix for nested PCR of *pol/surface* region

Reagents	Starting Concentration	Volume/µl x1	Final Concentration
PCR Buffer	10x	5.0	1x
$MgCl_2$	50mM	1.5	1.5mM
dNTPS	10mM	1.0	0.2mM
Taq		0.2	
HBV 3	20pmol/µl	1.0	0.4pmol/µl
HBV Z	20pmol/µl	1.0	0.4pmol/µl
dH_2O	n/a	39.3	n/a
Total volume	n/a	**49.0**	n/a

pmol/µl: picomole per microliter; n/a: not applicable

The master-mix was aliquoted in volumes of 49µl into 0.2ml PCR tubes to which one µl of the pre-nested product was added. The cycling parameters given in Table 3.10 were used for the nested PCR reaction.

Table 3. 10 Cycling parameters for nested PCR of *pol/surface* region

Cycling parameter	Cycles	Temperature	Time
Initial denaturation	1 hold	95°C	5 minutes
Denaturation	34 cycles	94°C	30 seconds
Annealing		50°C	30 seconds
Extension		72°C	1 minute
Final Extension	1 hold	72°C	7 minutes

Gel electrophoresis and visualization

A 1.8% agarose gel was prepared by melting 1.8 grams of agarose powder (SeaKem® LE Agarose, LONZA, Rockland, ME, USA) in 100ml of 1x Tris Acetate Buffer. Novel Juice (GeneDireX, Taiwan), a non-mutagenic fluorescent dye supplied in a 6x loading buffer was used to visualize the DNA bands. One microlitre of Novel Juice was mixed with five microlitres of each nested PCR product before the mixture was loaded into separate wells on the gel. The samples were separated alongside a 1kb DNA ladder (GeneRuler™ 1 kb DNA Ladder, Fermentas, ThermoFisher Scientific, USA) for at least 30 minutes at 60 volts. The gel was then viewed under ultraviolet light using the Platinum HD Gel Documentation System (UVItec Limited, Cambridge, UK) and the image acquired using the UVIband-1D gel analysis software. The expected product size was around 1kb. Samples which were judged to have enough amplified DNA to be sequenced (based on the visibility of crisp single bands of the correct size) were then selected for downstream processing.

Clean-up and analysis of PCR product

The PCR products were purified using the QIAGEN® QIAquick PCR Purification Kit by following manufacturer's instructions.

The purified DNA was analysed 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. If the purified DNA product had a concentration above 20ng/µl, it was diluted until it was within the required range (5-20ng/µl) for the sequencing PCR reaction.

Sequencing PCR reaction

The sequencing PCR reaction was carried out in a 96-well plate using four different primers to sequence the region of interest. These primers (Table 3.11) were diluted to 2pmol/µl before being used. A master mix consisting of the ready-to-use reagents shown in Table 3.12 was prepared and distributed in volumes of 8µl into the appropriate number of wells. Next, 1µl of each primer and 1µl of purified template DNA from each sample was added to the appropriate wells. The cycling conditions shown in Table 3.13 were used.

Table 3. 11 Sequencing primers targeting the *pol/surface* region

Name	Sequence	Binding position from *Eco* R1 site
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. 12 Master mix for sequencing reaction of *pol/surface* region

Reagents	Volume/µl x1
Terminator ready reaction mix	1
ABI sequencing buffer	3
Water	4
Total volume	**8**

Table 3. 13 Cycling parameters for sequencing reaction of *pol/surface* region

Cycling parameter	Cycles	Temperature	Time
Denaturation	30 cycles	96°C	20 seconds
Annealing		50°C	20 seconds
Extension		60°C	4 minutes

Sequencing clean-up

The sequencing PCR reactions were cleaned up using the BigDye® Xterminator Purification Kit (Applied Biosystems, Foster City, California, USA) consisting of SAM™ and XTerminator® solutions. These two reagents were brought to room temperature and vortexed for ten seconds at maximum speed before use.

The sequencing 96-well plate was centrifuged for one minute at 1000 RCF in a swing-bucket centrifuge. Next, 45µl of SAM solution and 10µl of Xterminator® solution were added to each well before the wells were sealed with a clear, adhesive film. The 96-well plate was then vortexed for 30 minutes at 2000 RCF and centrifuged for one minute at 1000 RCF.

Sequence Analysis

Capillary electrophoresis was performed on the samples using the ABI Prism 3130XL Genetic Analyzer (Applied Biosystems, Foster City, California, USA). The information obtained was converted to raw data files using DNA sequencing analysis software from Applied Biosystems, (USA). The length of the capillaries allowed sequence reads of around 600 base pairs (bp) to be obtained.

The raw trace files were further analysed using Sequencher 4.8 (Gene Codes Corporation, Michigan, USA). The quality of each sequence was improved individually by looking at the chromatograms and trimming the ends where necessary. The four sequences for each patient were then aligned to create a consensus sequence. Mismatches or ambiguities were rectified manually. The consensus sequence was renamed and saved in FASTA format before being blasted against the Stanford University HBVSeq sequence database. (http://hivdb.stanford.edu/HBV/HBVseq/development/HBVseq.html).

This allowed the genotype identification of the HBV strains and the detection of any clinically-relevant mutations in the sequences.

3.5.4 WHOLE GENOME SEQUENCING OF HBV DNA POSITIVE SAMPLES

Whole genome sequencing was attempted on samples which were HBV DNA positive. Only samples which had more than 10^8 IU/ml of viral DNA could be sequenced. Firstly, the whole genome was first amplified in two separate reactions resulting in two large fragments which were then sequenced.

Both PCR reactions used MyFi™ Mix from Bioline (Bioline, GmbH, Germany) which utilizes a high-fidelity DNA polymerase and requires only the addition of specific primers and template for amplification.

Two sets of primers were selected to amplify a fragment of around ~2.5kb and a smaller fragment of ~1.2kb respectively. The reactions were prepared using the reagents listed in Table 3.14 and Table 3.15 and the thermocycling parameters are given in Table 3.16.

Table 3. 14 Master mix for PCR of 2.5kb fragment of HBV genome

Reagents	Starting Concentration	Volume/µl x1	Final Concentration
MyFi Mix	2x	25	1x
UBC7_Forward	20pmol/µl	1	0.4pmol/µl
UBC6_Reverse	20pmol/µl	1	0.4pmol/µl
dH_2O	n/a	18	n/a
Total volume	n/a	**45**	n/a

n/a: not applicable

Table 3. 15 Master mix for PCR of 1.2kb fragment of HBV genome

Reagents	Starting Concentration	Volume/µl x1	Final Concentration
MyFi Mix	2x	25	1x
H4072_Forward	20pmol/µl	1	0.4pmol/µl
91DMas_Reverse	20pmol/µl	1	0.4pmol/µl
dH_2O	n/a	18	n/a
Total volume	n/a	**45**	n/a

n/a: not applicable

Table 3. 16 Cycling parameters for PCR of two fragments of HBV genome

Cycling parameter	Cycles	Temperature	Time
Initial denaturation	1 hold	95°C	3 minutes
Denaturation	15 cycles	94°C	25 seconds
Annealing		50°C	30 seconds
Elongation		68°C	3.5 minutes +30s/5cycles
Denaturation	25 cycles	94°C	25 seconds
Annealing		60°C	30 seconds
Elongation		68°C	5 minutes +9s/cycle
Final Elongation	1 hold	68°C	7 minutes

The two PCR products were then purified as described previously, and a sequencing reaction plate was set up using the master mix given in Table 3.12.

Sequencing of the 2.5kb fragment was executed using a set of nine different primers (given in Table 3.17). Another set of five primers (given in Table 3.18) were used to amplify different overlapping sections of the 1.2kb fragment. The combination of these primers and fragments allowed for the sequencing of the entire 3.2kb genome of HBV.

The thermocycling conditions given in Table 3.13 were used on all the primers for the sequencing reaction except for the 0.7F_New primer reaction. This primer had a melting temperature of 37°C and therefore in the sequencing PCR an annealing temperature of 37°C was used for 20 seconds.

The sequencing reaction products were cleaned as previously described before being subjected to capillary electrophoresis using the ABI Prism 3130XL Genetic Analyzer (Applied Biosystems, Foster City, California, USA) and the sequences were analysed using DNA sequencing analysis software from Applied Biosystems (USA).

Table 3. 17 Sequencing primers targeting the 2.5kb fragment

Name	Sequence	Binding position from *Eco* R1 site
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
HBV H_Forward*	5′- TATCAAGGAATTCTGCCCGTTTGTCCT -3′	628 – 655
HBV P_Forward*	5′- TCA TCC TCA GGC CAT GCA GT -3′	3247 – 3266
UBC7_Forward[#]	5′- CTT TTT CAC CTC TGC CTA RTC A -3′	1828 – 1850
UBC6_Reverse[#]	5′- AAA AAG TTR CAT GRT GMT GG -3′	1813 – 1832
0.7F_New_Forward[#]	5′- GCT AGR TTY TAT CC -3′	2694 – 2707
B2as_Reverse[$]	5′- GGCAGCACASCCGAGCAGCCATGG -3′	1402 – 1379
0.7R_Reverse[#]	5′- CAATTTATGCCTACAGCCTCC -3′	1803 – 1783

[#] Primer sequences were obtained from Samreen Ijaz, HPA (personal communication); ***** Primer sequences were obtained from the HPA website: http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1194947340684; [$] Primer sequence was obtained from Guillaume Fallot, INSERM (personal communication)

Table 3. 18 Sequencing primers targeting the 1.5kb fragment

Name	Sequence	Binding position from *Eco* R1 site
CSeqQR_Reverse[#]	5′- TCT TGC CCA AGG TCT TAC AT -3′	1747 – 1766
Core_RT_Forward*	5′- CCT GGG TGG GAA GTA ATT TGG -3′	
91DMas_Reverse[$]	5′- CCC AAG AAT ATG GTG ACC C -3′	3028 – 3010
UBC6_Reverse[#]	5′- CTT TTT CAC CTC TGC CTA RTC A -3′	1932 – 1954
0.7new_Forward[#]	5′- GCT AGR TTY TAT CC -3′	2698 – 2712

[#] Primer sequences were obtained from Samreen Ijaz, HPA (personal communication); [$] Primer sequence was obtained from Guillaume Fallot, INSERM (personal communication); * Primer sequence was obtained from Qian et al. (2005)

3.5.5 PHYLOGENETIC ANALYSIS OF SEQUENCES OBTAINED FROM SEQUENCING OF *POL/SURFACE* REGION AND WHOLE GENOME

Phylogenetic trees comparing sequences obtained in sections 3.5.3 and 3.5.4 to each other and to reference sequences of HBV genotype A from South Africa (downloaded from GenBank) were constructed in MEGA5 (Tamura et al. 2011). These reference sequences were obtained from online searches on GenBank and from Maponga TG, MSc Thesis, Stellenbosch University (2012).

For the phylogenetic analysis, the evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) and the evolutionary distances were calculated using the Kimura 2-parameter method (Kimura 1980). The Neighbour-Joining method was used as it is able to process large volumes of data quickly. The Kimura 2-parameter generates evolutionary distance between sequences and is able to differentiate between base transitions and transversions.

3.5.6 QUALITY CONTROL FOR MOLECULAR ASSAYS

DNA Extraction

The DNA extractions were performed in a laminar flow cabinet to minimize the risk of contamination. The microcentrifuge tubes containing the samples were briefly centrifuged before being opened, to eliminate the risk of aerosol formation from any droplets on the inside of the tube caps. During the extraction process, gloves were changed when dealing with sterile consumables. Reagents were aliquoted into 50ml conical centrifuge tubes before being used so that the stock solutions were not exposed to a possible source of contamination by repeated pipetting from the original bottles containing the reagents. With each extraction batch, NHP was used as a negative control to ensure that there was no crossover contamination either from the reagents used or from the operator. A positive control was not included in the extraction process as an internal control was added to every sample including the negative control. In the subsequent real time PCR which was performed on the extracted samples, the detection of the internal control in all the extracts ensured that the extraction process was working.

PCR

Reagent preparation and amplicon addition for the PCR was done in separate rooms to prevent contamination. The master mix was prepared in a room designated as a ‘clean area’ which is exclusively used for the preparation of master mixes. Before setting up a reaction, the bench top and the micropipettes were wiped, first with 0.35% bleach and then with 70% ethanol. DNA addition was done in a separate room and the thermocyclers were housed in a room designated as a ‘dirty area’. Under no circumstances was the operator allowed to move from a ‘dirty area’ into a ‘clean area’.

The different reagents were thawed on ice to prevent degradation. Primers and probes were aliquoted into volumes of five and three microlitres respectively to prevent degradation through several freeze-thaw events. The DNA extracts were allowed to thaw at 4°C while the master mix was prepared.

Conventional PCR

An appropriate positive control was included with each conventional PCR and sequencing reaction to ensure the validity of the results. A negative control consisting of the master mix and nuclease-free water was included with each PCR run. This ensured that the reagents were not contaminated and would not cause the detection of false positives.

Real-time PCR

The standards used in the real-time PCR were run in duplicate to reduce error and to increase the confidence in the interpreted results. No positive control was included in the runs as the amplification of the internal control in every individual tube was judged to be sufficient to ensure that amplification conditions were optimal. A no template control consisting of the master mix and nuclease-free water was included with each real-time PCR run. This ensured that the reagents were not contaminated and would not cause the detection of false positives. To ensure that consistency between each separate run, a sample of known viral load, termed the working control, was included with each real-time PCR run.

CHAPTER FOUR: RESULTS

4.1 SAMPLE AND DATA COLLECTION

A total of 1468 samples were received by the NHLS, Division of Medical Virology, Tygerberg Hospital between the 8th of June 2011 and the 18th of August 2011. From these, 604 samples with more than 100µl residual plasma were selected for the current study. An additional 396 samples were selected from a total of 1114 samples received from the 6th of October 2011 to the 13th of February 2012. The study population made up 39% of the sample population.

The demographics of the tested population are summarised in Table 4.1 below.

Table 4. 1 Demographics of study population

Characteristic	Total sample population* (n=2582)	Study population (n=1000)
Age		
Median (range), days	45 (0-730)	46 (0-540)
Sex		
Male, %	41.2	48.7
Unknown gender, %	15.7	2.9
HIV status		
HIV-infected, %	4.6	6.1

* Total number of samples received for HIV-1 PCR testing by the NHLS during the sample collection period

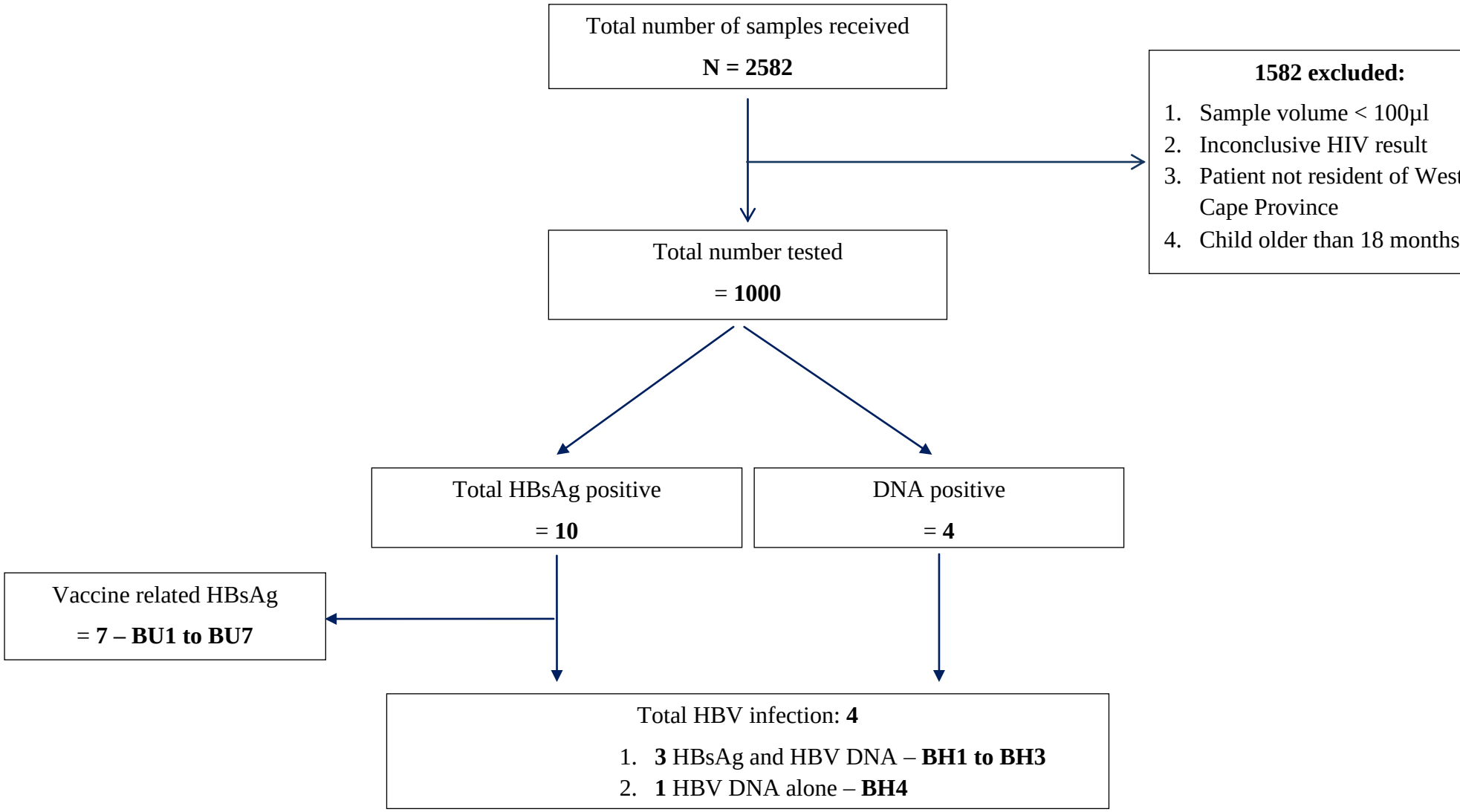

Figure 4. 1 Categorisation of samples at screening

4.2 FOLLOW-UP OF HBsAg AND/OR DNA POSITIVE PATIENTS

From the screening tests described elsewhere (section 4.3.1 and 4.4.3), 11 samples were found to be HBsAg and/or HBV DNA positive. Using each positive sample's code, the name of the corresponding infant was retrieved from a digital database and the mother of the infant was contacted through their clinic. Ten of eleven infants and 11/11 mothers were successfully traced. Where the term 'patients' is used henceforth, it encompasses both tested infants and mothers.

One infant had been sent back to his hometown and was lost to follow-up. Insufficient blood was received from another infant who could then only be tested for HBsAg and HBV DNA at follow-up. Although a request was made to re-bleed this infant, by the time of submission, no blood sample had been received for further testing. The demographics of the followed-up infants and their mothers are shown in Table 4.2.

Table 4. 2 Demographics of the followed up infants and their mothers

Characteristic	Infants (n=10)	Mothers (n=11)
Age		
Median at screening (range), days	45 (41-120)	-
Median at follow up (range)	227.5 days (143-327 days)	35 years (24-43 yrs)
Sex		
Male, %	90	-
HIV status		
HIV-infected, %	0	100

SEROLOGY RESULTS

4.3.1 PREVALENCE OF HBsAg AT SCREENING STAGE

Out of the 1000 infants screened using the Murex HBsAg Version 3 kit, 11 were found to be reactive for HBsAg. An HBsAg neutralization test was performed on these 11 HBsAg-positive samples. Following neutralization, one sample was found to be negative. Of the remaining ten samples, eight were confirmed to be true HBsAg positives as a reduction of at least 50% was observed in the OD between antiHBs positive and negative wells.

One sample displayed an OD which was above the linear detection range (0–3.3) of the spectrophotometer in both wells (with and without antiHBs). This was attributed to a very high level of HBsAg in the patient's plasma. Another sample had insufficient plasma for the neutralization test and was thus diluted 1:8 in NHP. Although a substantial reduction in OD between antiHBs positive and negative wells was observed for this sample (0.090 to 0.052), the absorbance from the antiHBs-negative well was below the threshold of positivity (0.102). The low OD seen in the antiHBs-positive well was attributed to the high dilution factor and the sample was therefore considered to be HBsAg positive. This patient was subsequently shown to be HBV uninfected.

These results are summarised in Table 4.3 below.

Table 4. 3 HBsAg screening and confirmation results

Samples initially positive for HBsAg	11/1000 (1.1%)
Samples confirmed positive for HBsAg by neutralization	10/1000 (1.0%)

The detailed results observed for the neutralization test are shown in Table 4.4.

Table 4. 4 Absorbance values for screening HBsAg neutralization test

Patient Number	Absorbance / NHP Well	Absorbance / AntiHBs Well	Cut-Off	% Reduction in Absorbance
1	0.332	0.052	0.102	84
2	0.090[#]	0.052[#]	0.102	42
3	> 3.300*	> 3.300*	0.102	N/A
4	0.151	0.062	0.102	59
5	0.154	0.059	0.101	62
6	0.879	0.057	0.101	94
7	0.122	0.041	0.101	66
8	0.045	0.052	0.101	NEG
9	0.270	0.046	0.101	83
10	0.139	0.049	0.101	65
11	0.319	0.071	0.101	78

%: Percentage; [#]absorbance value in NHP just below cut-off, dilution of 1:8 done on this sample; [*] Absorbance value out of range; absorbance values too high for neutralization to be possible without further dilution; N/A not applicable; NEG: Negative

4.3.2 PREVALENCE OF HBsAg AT FOLLOW-UP

The follow-up data of the 11 infants included the dates on which they had received their vaccinations against HBV. It was confirmed that the blood sample for the HIV-1 test had been taken from these infants on the same day or very close to the date they had received one of three doses of the HB vaccine. The infants and their mothers were therefore divided into two groups. One group encompassed the four HBV DNA-positive infants and their mothers and was termed the ‘infected group’. These infants and their mothers were code-named BH1 to BH4 and MH1 to MH4 respectively. The other group was made up of the seven infants who were positive for HBsAg alone and their mothers and was termed the ‘uninfected group’. They were code-named BU1 to BU7 and MU1 to MU7 respectively.

Additional information on the mothers from the infected group was available and it was seen that these four mothers had CD4 counts above 350 cells/mm^3 and that they were therefore not on ART.

The followed-up patients were first tested for HBsAg. After testing, 66.7% (2/3) of infants (BH1 to BH3) and 100% (4/4) of mothers (MH1 to MH4) belonging to the infected group were found to be positive for HBsAg. None of the tested infants (7/7) and mothers (7/7) from the uninfected group was positive for HBsAg on follow-up.

These six HBsAg positive patients were further tested by the HBsAg neutralization assay. Two out of two infants and all four mothers were confirmed to be HBsAg positive on neutralisation. The absorbance values observed upon neutralization are shown in Table 4.5.

Table 4. 5 Absorbance values for HBsAg neutralization test of infected group at follow-up

Patient	Absorbance/ NHP Well	Absorbance/ AntiHBs Well	% reduction in absorbance
MH1	>3.300	0.122	>50
BH2	NT	NT	NT
MH2	0.814	0.071	91
BH2	>3.300	0.093	>50
MH3	2.179	0.044	98
BH3	0.525	0.060	89
MH4	>3.300	0.052	>50
BH4*	ND	ND	ND

* Patient was lost to follow-up; %: Percentage; BH: infant from infected group; MH: mother from infected group; NT: Not tested as was HBsAg negative; ND: Not Done

4.3.3 PREVALENCE OF HBeAg AND AntiHBe IN HBsAg POSITIVE SAMPLES

In the infected group, two HBsAg positive infants and the four HBsAg positive mothers were tested and were all positive for HBeAg. The absorbance values observed in these infants and mothers were higher than the detection limit of the spectrophotometer except for MH2.

The six HBeAg-positive patients were also tested for antiHBe. The two tested infants were negative for antiHBe. Out of the four tested mothers, only MH2 was positive for antiHBe.

4.3.4 PREVALENCE OF AntiHBc IN ALL FOLLOWED-UP SAMPLES

The followed up patients with sufficient plasma (9/10 infants and 11/11 mothers) were tested for antiHBc. In the infected group, two (BH2 and BH3) out of three tested infants and all four tested mothers were positive for antiHBc. In the uninfected group, none of the tested infants (0/6) and three out of seven mothers were positive for antiHBc.

A summary of the serological results of the infants and mothers from the infected and uninfected groups at screening and follow-up are shown in Table 4.6 and Table 4.7 respectively.

Table 4. 6 Serological results of mother-child pairs in infected group at follow-up

PATIENT	HBsAg	HBeAg	AntiHBc	AntiHBe
MH1	+	+	+	−
BH1	−	−	−	ND
MH2	+	+	+	+
BH2	+	+	+	−
MH3	+	+	+	−
BH3	+	+	+	−
MH4	+	+	+	−
BH4*	ND	ND	ND	ND

* Patient was lost to follow-up; HBsAg: hepatitis B surface antigen; HBeAg: hepatitis B e antigen;
AntiHBc: antibody to hepatitis B core antigen; AntiHBe: antibody to hepatitis B e antigen;
BH: Infant from infected group; MH: Mother from infected group; − : Negative; + : Positive; ND: Not done

Table 4. 7 Serological results of mother-child pairs in uninfected group at follow-up

PATIENT	HBsAg	AntiHBc
MU1	−	+
BU1	−	−
MU2	−	+
BU2	−	−
MU3	−	−
BU3	−	−
MU4	−	+
BU4	−	NT[#]
MU5	−	−
BU5	−	−
MU6	−	−
BU6	−	−
MU7	−	−
BU7	−	−

HBsAg: hepatitis B surface antigen; AntiHBc: antibody to hepatitis B core antigen; BU: Infant from uninfected group; MU: Mother from uninfected group; − : Negative; + : Positive; NT[#] Not tested because of insufficient sample volume

4.3.5 PREVALENCE OF AntiHBs IN HBsAg NEGATIVE SAMPLES

Infants who were negative for HBsAg were tested for antiHBs. However, due to the small volume of plasma, only one out of seven infants from the uninfected group could be tested for this marker. The only HBsAg negative infant from the infected group was also tested for antiHBs. These two infants were found to have protective levels of antiHBs (greater than 10 IU/L).

MU7, who was positive for antiHBe, was tested for antiHBs and was found to have levels of antiHBs of greater than 10 IU/L.

4.3 MOLECULAR TESTING

4.4.1 VALIDATION OF POOLING ASSAY

The validation test results (Table 4.8) showed that the lowest detectable viral load using the real-time PCR assay was at the dilution of 1:20 for the sample with 250 IU/ml and at the dilution of 1:10 for the sample with 100 IU/ml. The 1:20 dilution of the 250 IU/ml working solution gave a final viral load of 12.5 IU/ml while the 1:10 dilution of the 100 IU/ml working solution gave a final viral load of 10 IU/ml. The higher value was chosen as the detection limit and therefore D_L was determined to be 12.5 IU/ml. Since four samples were pooled, the limit of detection of the pooled assay, P_L, was calculated to be 50 IU/ml. A working solution containing 50 IU/ml was prepared and one-fold dilutions were run in duplicate on three different days. The results obtained (Table 4.8) confirmed that P_L was 50 IU/ml.

Table 4. 8 Validation results of pooling assay. Samples were tested in duplicate on three different days

Stock solution	Working solution	Ct_1	VL_1	Ct_2	VL_2	Ct_3	VL_3
250 IU/ml	Neat	34.99	323	39.53	65	ND	ND
	Neat	44.5	1	38.79	108	35.03	300
	1:2	36.22	147	39.81	54	39.73	9
	1:2	35.39	250	38.11	170	36.15	131
	1:3	41.91	4	40.62	32	35.56	202
	1:3	40.83	8	40.63	31	36.9	75
	1:4	40.03	13	39.24	80	39.77	9
	1:4	37.11	83	40.54	33	37.16	62
	1:5	42.14	3	39.69	59	38.45	24
	1:5	38.29	39	39.35	74	38.57	22
	1:6	37.72	56	40.57	33	36.6	94
	1:6	39.95	14	42.05	12	38.25	28
	1:8	ND	ND	40.44	36	38.16	29
	1:8	40.92	7	42.81	7	38.82	18
	1:10	38.2	41	41.4	19	40.97	4
	1:10	39.28	21	38.8	107	38.65	20
	1:20	**ND**	**ND**	**40.9**	**26**	**40.29**	**6**
	1:20	**39.65**	**16**	**40.91**	**26**	**39.27**	**13**
	1:25	39.22	22	ND	ND	ND	ND
	1:25	41.19	6	ND	ND	ND	ND
100 IU/ml	Neat	35.77	197	37.27	104	37.54	59
	Neat	35.87	183	35.74	278	36.93	88
	1:2	38.82	25	38.73	41	38	43
	1:2	36.09	158	37.94	68	37.68	54
	1:3	38.04	42	40.01	18	40.01	11
	1:3	ND	ND	ND	ND	41.28	5
	1:4	38.87	24	ND	ND	40	11
	1:4	37.73	52	38.94	36	40.39	9
	1:5	38.1	41	38.87	37	39.61	15
	1:5	38.51	31	39.57	24	37.75	51
	1:6	38.86	24	38.78	40	ND	ND
	1:6	37.74	52	ND	ND	41.81	3
	1:8	**39.5**	**16**	**38.21**	**57**	**43.58**	**1**
	1:8	**39.25**	**19**	**39.98**	**18**	**ND**	**ND**
	1:10	39.64	14	38.15	59	ND	ND
	1:10	37.29	70	39.33	28	40.7	7

Stock solution	Working solution	Ct_1	VL_1	Ct_2	VL_2	Ct_3	VL_3
50 IU/ml	Neat	38.65	42	34.55	97	38.34	34
	Neat	36.99	125	35.09	68	41.32	5
	1:2	39.63	22	32.81	294	38.43	32
	1:2	38.25	54	37.66	13	ND	ND
	1:3	37.23	107	37.3	17	40.61	8
	1:3	38.01	64	37.69	13	ND	ND
	1:4	**37.69**	**78**	**ND**	**ND**	**40.21**	**10**
	1:4	**38.94**	**34**	**34.87**	**79**	**ND**	**ND**
	1:5	39.81	19	ND	ND	40.53	8
	1:5	38.92	35	ND	ND	ND	ND
	1:6	39.67	21	ND	ND	39.38	17
	1:6	38.14	58	37.38	16	ND	ND

Ct: Cycle at which the fluorescence observed in the sample crossed the detection threshold; VL: Viral load; ND: Not Detected

4.4.2 INDIVIDUAL AND POOLED VIRAL DNA EXTRACTIONS

Out of the 1000 samples screened for HBsAg, 989 were serologically negative and therefore not further tested by the neutralization assay. DNA was only extracted from the first 600 serologically negative samples and the 11 samples which were tested by neutralization.

Of the 600 samples, 228 had less than 150µl of plasma available and were extracted individually. The remaining 372 HBsAg negative samples, which had more than 150µl of plasma available, were pooled and extracted in 93 batches of four each. Individual DNA extractions were also done on the 11 samples which were tested using the HBsAg neutralization assay.

4.4.3 VIRAL LOAD TESTING AT SCREENING STAGE

The 611/1000 extracted samples were tested for the presence of HBV DNA by real-time PCR. None of the 372 pooled and none of the 228 individually-extracted HBsAg negative samples had a detectable viral load.

Of the ten confirmed HBsAg positive samples, three were positive for HBV DNA. Two samples had viral loads of 10^2 IU/ml and 5 x 10^4 IU/ml respectively. The third sample which had previously shown OD values greater than the detection range of the spectrophotometer for the HBsAg neutralization test, had a viral load of $>10^8$ IU/ml. One sample which was HBsAg negative was HBV DNA-positive with a low viral load of 5 x 10^1 IU/ml. These four samples made up the infected group.

The remaining HBsAg positive samples were negative for HBV DNA and were categorized as the uninfected group.

Based on the HBsAg and HBV DNA results, the prevalence of HBV infection in this cohort of 1000 infants was calculated to be 0.4% (4/1000).

4.4.4 VIRAL LOAD TESTING AT FOLLOW-UP

In the infected group, a positive viral load was detected in 2/3 followed-up infants and 4/4 mothers. The two infants (BH2 and BH3) had a HBV viral load of greater than 10^8 IU/ml. Their mothers (MH2 and MH3) had a viral load of 10^3 IU/ml and 10^8 IU/ml respectively. The other two mothers MH1 and MH4 had viral loads of greater than 10^8 IU/ml.

In the uninfected group, none of the tested infants (0/7) and mothers (0/7) was positive for HBV DNA.

A summary of the serological and viral load results obtained for the infected group at screening and follow-up is given in Table 4.9.

Table 4. 9 Serological and molecular results of mother-child pairs in infected group at screening and at follow-up

SCREENING			FOLLOW-UP				
PATIENT	**HBsAg**	**HBV DNA IU/ml**	**HBsAg**	**AntiHBe**	**HBeAg**	**AntiHBc**	**HBV DNA IU/ml**
MH1	ND	ND	+	−	+	+	1×10^8
BH1	+	1×10^2	−	ND	ND	−	−
MH2	ND	ND	+	+	+	+	1×10^3
BH2	+	2×10^8	+	−	+	+	6×10^8
MH3	ND	ND	+	−	+	+	4×10^8
BH3	+	5×10^4	+	−	+	+	2×10^9
MH4	ND	ND	+	−	+	+	4×10^7
BH4*	−	5×10^1	ND	ND	ND	+	ND

*Patient was lost to follow-up; IU/ml: International Units per millilitre; HBsAg: hepatitis B surface antigen; HBeAg: hepatitis B e antigen; AntiHBe: antibody to hepatitis B e antigen; AntiHBc: antibody to hepatitis B core antigen; BH: Infant from infected group; MH: Mother from infected group; − : Negative; + : Positive; ND: Not done

4.4.5 SEQUENCING OF *POL/SURFACE* REGION RESULTS

The *pol/surface* gene of the HBV strains was sequenced in the samples from the two infants and four mothers who were positive for HBV DNA. A typical nested PCR yielded bands as shown in Figure 4.2.

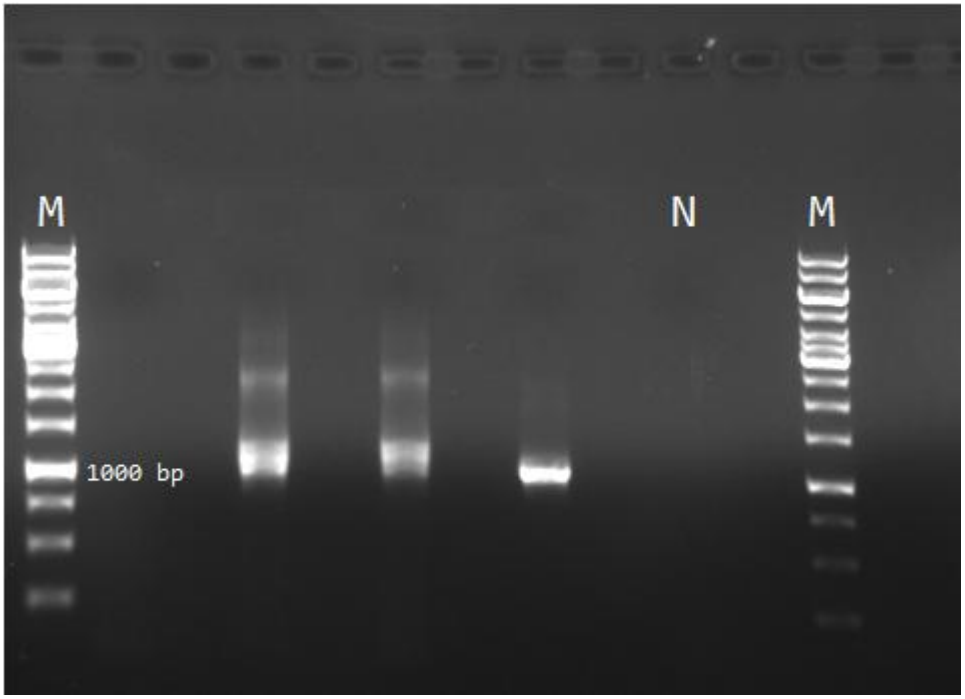

Figure 4. 2 Gel of typical bands obtained in 3/6 patients after nested PCR of *pol/surface* region M: 1kb Molecular Weight marker; N: No-template control

After the clean-up step, the products were analysed spectrophotometrically. The concentration and the purity of the DNA obtained by readings at A_{260nm} and $A_{260/280nm}$ respectively are shown in Table 4.10.

Table 4. 10 Concentration and Purity of DNA after clean-up

Sample ID	ng/µl	$A_{260/280nm}$
MH1	21.50	2.29
MH2	19.92	1.97
BH2	16.20	2.10
MH3	63.45	1.93
BH3	59.72	1.86
MH4	59.71	1.84

The DNA from BH3, MH3 and MH4 was diluted since it was higher than the recommended range (5-20ng/µl) for the sequencing PCR reaction. The samples from these patients were diluted with buffer EB from the QIAquick purification kit so that 1µl of each sample could be used for the sequencing reaction.

After the sequences were analysed, the ends were trimmed and the sequences amplified by the four different primers. The sequences were then aligned, a contiguous sequence was obtained which was approximately 900 bp long for all six patients.

Analysis on the HepSeq website of the sequences of the two infants and the four mothers showed they belonged to genotype A. No mutation associated with drug-resistance or vaccine-escape was identified in the *pol/surface* region of HBV strains in these patients.

The sequences from the mother-child pairs were used to construct a phylogenetic tree (figure 4.3). The HBV strains from the two mother-child pairs (MH2/BH2 and MH3/BH3) were found to be identical.

A separate tree was drawn to compare the four maternal sequences with other South African HBV strains (Figure 4.4).

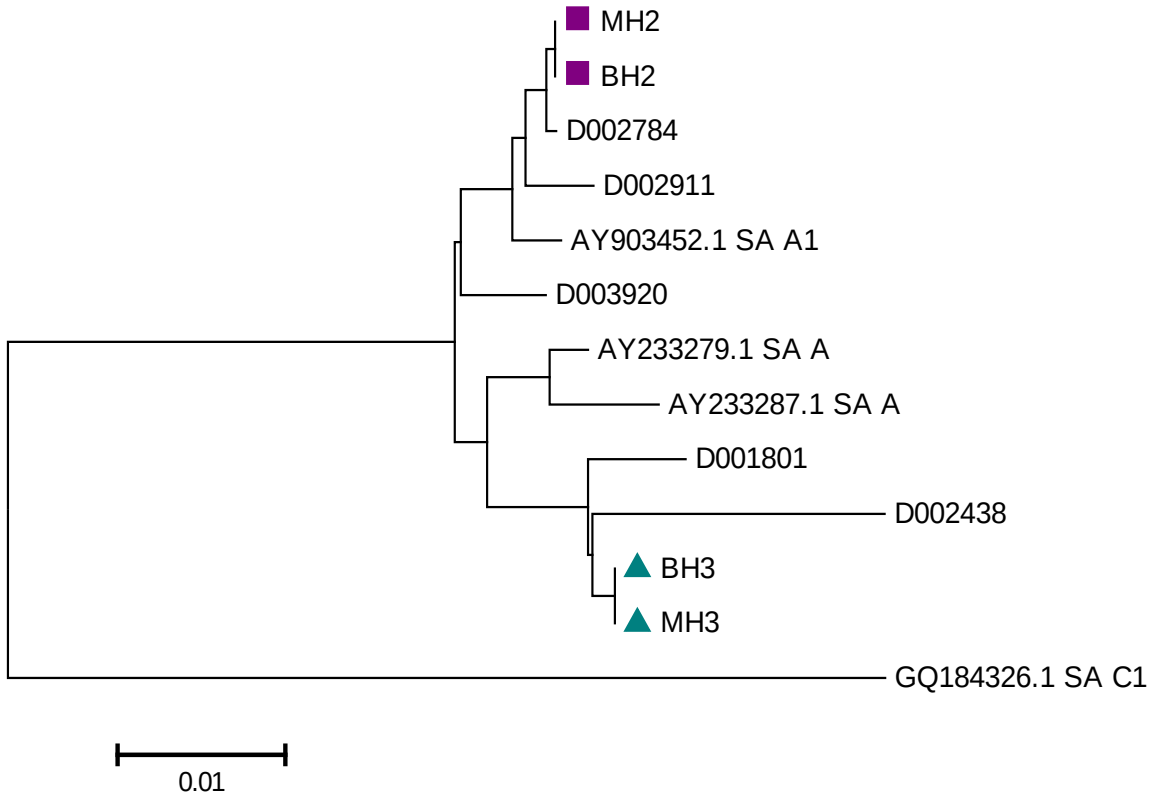

Figure 4. 3 Phylogenetic tree of mother-child pairs with HBV strains belonging to subgenotype A1 based on *pol/surface* region

BH: infant from infected group; MH: mother from infected group; Sequences D002438, D001801, D002911 and D002784 were sequenced from patients from the Western Cape (Maponga TG, MSc Thesis, Stellenbosch University, 2012). Sequences with accession numbers AY233279.1, AY233287.1, AY903452.1, AY233280.1, Z72478.1 and GQ184326.1 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 = 0.16052268 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 14 nucleotide sequences. There were a total of 917 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011).

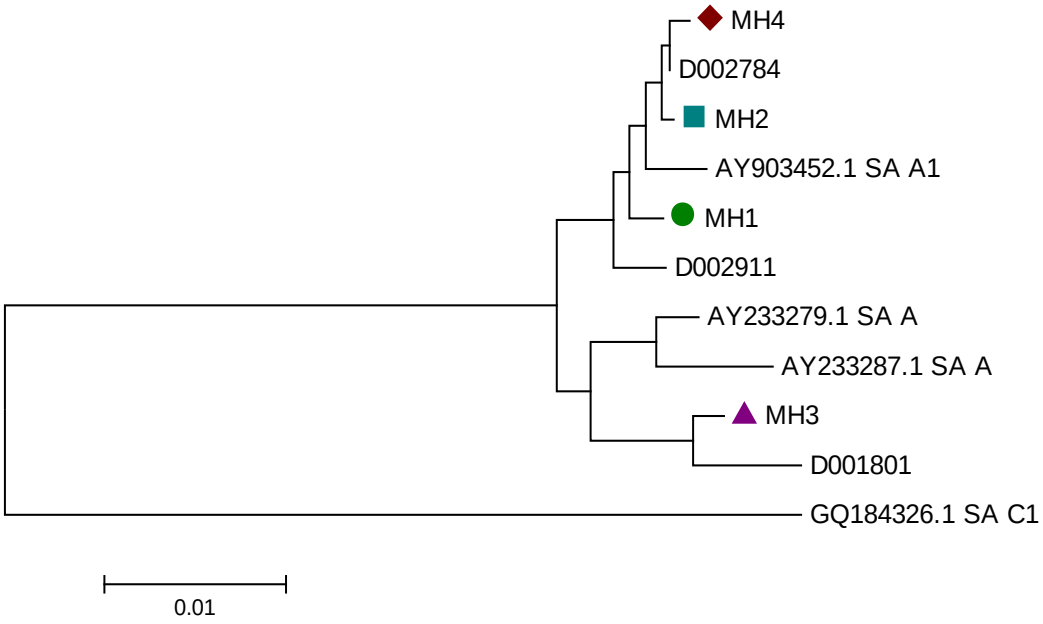

Figure 4. 4 Phylogenetic tree comparing maternal HBV strains to South African sequences
MH: mother from infected group; Sequences D001801, D002911 and D002784 were sequenced from patients from the Western Cape (Maponga TG, MSc Thesis, Stellenbosch University, 2012). Sequences with accession numbers AY233279.1, AY233287.1, AY903452.1 and GQ184326.1 were downloaded from GenBank. All sequences belong to subgenotype A1 except for GQ184326.1 which belongs to genotype C.
The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). 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 14 nucleotide sequences. There were a total of 917 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011).

4.4.6 WHOLE GENOME SEQUENCING RESULTS

In the infected group, the whole genome in 5/6 samples could be sequenced. The viral load from MH2 was too low for whole genome sequencing using the assay described in section 3.5.4.
A gel with typical bands obtained with the PCR on the two fragments for each sample is shown in figure 4.6.

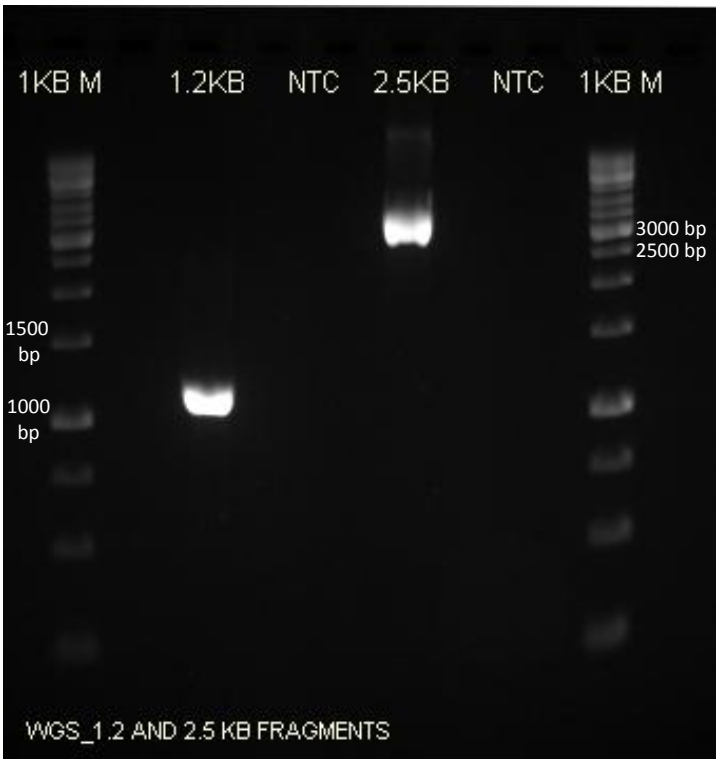

Figure 4. 5 Typical bands (1.2 and 2.5 kb) obtained with PCR for whole-genome sequencing

After the clean-up step, the products were analysed spectrophotometrically. The concentration and the purity of the DNA obtained by readings at A_{260nm} and $A_{260/280nm}$ respectively for both fragments are shown in Table 4.10 and Table 4.11.

Table 4. 11 Concentration and Purity of DNA after clean-up of 2.5kb fragment

Sample ID	ng/µl	$A_{260/280nm}$
MH1	33.86	2.1
BH2	59.08	1.87
MH3	25.93	2.14
BH3	61.5	2.1
MH4	43.03	2.26

Table 4. 12 Concentration and Purity of DNA after clean-up of 1.2kb fragment

Sample ID	ng/µl	$A_{260/280nm}$
MH1	73.64	2.05
BH2	134.77	1.95
MH3	24.4	2.05
BH3	137.59	2.01
MH4	32.87	1.86

None of the amplified PCR products for the 2.5kb fragment were diluted since the DNA concentration required for the sequencing reaction was within the recommended range (20-50ng/µl). However, for the 1.2 kb fragment, samples MH1, BH2 and BH3 were diluted with buffer EB from the QIAquick purification kit so their DNA concentrations were within the recommended range (10-40ng/µl).

The sequences were analysed, the ends were trimmed and the different sections amplified by the 14 primers were aligned to obtain a contiguous sequence. The contiguous sequence was then aligned with whole genome sequences from GenBank and trimmed to obtain the 3.2kb sequence in the five patients.

The five sequences were used to construct a phylogenetic tree (figure 4.5). The HBV strains from the mother-child pair MH3/BH3 were identical.

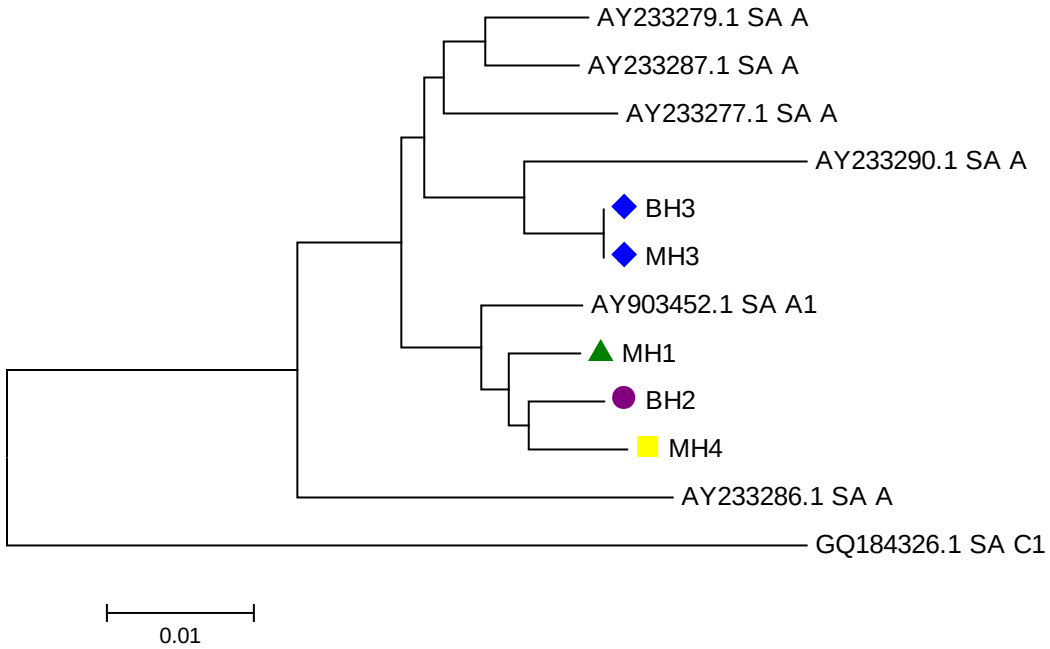

Figure 4. 6 Phylogenetic tree of five patients with HBV strains belonging to subgenotype A1 based on whole genome sequences. Sequences with accession numbers AY233277.1, AY233279.1, AY233286.1, AY233287.1, AY903452.1, AY233290.1 and GQ184326.1 were downloaded from GenBank. All sequences belong to subgenotype A1 except for GQ184326.1 which belongs to genotype C.
The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 0.21123244 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 12 nucleotide sequences. There were a total of 3209 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011).

CHAPTER FIVE: DISCUSSION

This study has shown the prevalence of HBsAg and HBV infection in a cohort of HIV-exposed infants to be 0.3% (3/1000) and 0.4% (4/1000) respectively. Two of the three HBsAg positive infants were HBeAg positive. On follow-up three months later, two of three HBV-infected infants remained positive for HBsAg and HBV DNA. These two infants were both male and HIV-uninfected. All four mothers who transmitted HBV to their infants were HBeAg positive and not on ART. The two mother infant pairs had identical sequences. This is the first study showing vertical transmission of HBV in HIV-exposed infants in South Africa in the context of HBV vaccination.

Prevalence of HBsAg

In comparison to a study by Simani et al. (2009), the rate of HBsAg positivity observed in this study is lower. They found an HBsAg carriage of 0.9% (3/303) in vaccinated children between the ages of 5 and 24 months, two of whom were HIV-infected. However, the Simani study looked at a much smaller cohort attending one clinic and one hospital serving mainly a peri-urban and urban population in Gauteng of whom 36% were HIV-infected. In contrast, in the current study, a larger cohort of 1000 HIV-exposed infants was tested of whom only 6% were HIV-infected. This is a more accurate representation of the prevalence in the community given that the number of HIV-exposed uninfected (HEU) infants in South Africa far exceeds the number of HIV-infected infants.

In the context of the HIV epidemic, it is expected that the prevalence of infection found in this study should be higher. However, it was observed that none of the mothers who transmitted the virus to their infants in the current study were on ART and all had CD4 counts greater than 350 cells/mm^3. It is therefore possible that a proportion of mothers of the tested infants in this cohort would have been on ART during pregnancy and therefore exposed to tenofovir/lamivudine. These drugs would have reduced the maternal HBV DNA levels thus inadvertently protecting the HIV-exposed infants in this cohort from perinatal infection with HBV and would explain the low prevalence of vertical transmission of HBV in HIV-exposed infants.

This study has clearly demonstrated that HEU infants are at risk of infection with HBV. Two South African studies carried out in 1995-1999 by Tsebe et al. (2001) and in 1996-1997 by Hino et al. (2001) found no transmission of HBV in 598 and 1213 vaccinated children respectively. The cohort in both studies consisted of older children (8-72 months for the Tsebe study and 12-24 months for the Hino study) of unspecified HIV status. In the Tsebe study, these children were recruited from various primary health care clinics and hospitals from the Northern Province and the Hino study tested children from the Eastern Cape. The current study tested children between the ages of 0 and 18 months but the median age of the cohort was 1.5 months making this study reliable for looking at vertical transmission of HBV. Furthermore, the tested samples came from various clinics, making this the first community-based study looking at the prevalence of HBV in HIV-exposed infants in the Western Cape.

Extrapolating from the paediatric HBsAg prevalence before the introduction of HBV vaccination (Abdool Karim et al. 1988; Prozesky et al. 1983; Vardas et al. 1999), it is clear that this intervention has been successful in decreasing the prevalence of HBsAg in the paediatric population. However, this study has shown that transmission of HBV is occurring from HIV-infected women who are not on combination antiretroviral therapy, to their infants. The data also shows that this transmission is vertical and not horizontal as was postulated in the pre-HIV era (Whittle et al. 1983).

Vertical transmission

Maternal plasma samples were unavailable at the screening stage and it was therefore not possible to determine the rate of HBsAg and HBeAg positivity in the mothers of the infants who were HBsAg negative. However, an antenatal community-based seroprevalence study of 1543 HIV-infected and 1546 HIV-uninfected pregnant women from the Western Cape carried out in 2012 (Maponga TG, MSc Thesis, Stellenbosch University, 2012) showed the prevalence of HBsAg and HBeAg in HIV-infected mothers to be 3.4% (95% CI: 2.5%, 4.3%) and 18% respectively.

Another study conducted in the Northern Province by Tsebe et al. (2001) showed that the prevalence of HBsAg in mothers of vaccinated children was 3.2%, although the HIV status of these mothers was not reported and only one mother was found to be HBeAg-positive.

The prevalence of HBsAg observed in the community based study of HIV-infected women from the Western Cape (Maponga TG, MSc Thesis, Stellenbosch University, 2012) was considered to reflect the maternal HBV prevalence of this cohort. This figure was used to calculate the rate of vertical transmission. Using the prevalence of HBV infection of 0.4% in this cohort of HIV-exposed infants and HBsAg positivity of 3.4% (53/1543, 95% CI 2.5% to 4.3%) in HIV-infected women, the rate of MTCT of HBV was calculated to be 12% (Figure 5.1).

Prevalence of HBsAg in HIV-infected mothers: **3.4%**
Prevalence of HBsAg in HIV-exposed infants: **0.4%**

Rate of transmission = **0.4/3.4 * 100 = 12%**

Figure 5. 1 Calculations for rate of transmission of HBV in HIV-exposed infants

In comparison, in neighbouring Namibia, a study conducted by Botha et al. (1984) showed the prevalence of HBsAg in a group of mothers to be 11% but their HBeAg status was unknown. Cord blood testing showed that 27% of the unvaccinated newborns of the HBsAg positive mothers were positive for HBsAg. However, contamination of cord blood with maternal blood was a possibility and these infants were not retested to verify their status (Botha et al. 1984). No post-vaccine studies looking specifically at the rates of MTCT of HBV have been previously conducted in South Africa.

The Tsebe study also showed that one out of four tested HBsAg positive mothers was positive for HBeAg and the remaining three mothers were antiHBe positive/HBeAg negative. None of the mothers transmitted the virus to their children. In comparison, the mothers of the four infected infants identified in this study were all HBeAg positive and at follow-up, three still had a high viral load which is a risk factor for MTCT of HBV (Burk et al. 1994). All four mothers had CD4 counts above 350 cells/mm^3 and were therefore not on ART. First line ART for

pregnant women with CD4 counts below 350 cells/mm^3 includes tenofovir and lamivudine (National Department of Health, South Africa and South African National AIDS Council 2010). The use of these drugs will decrease HBV viral load (Pan et al. 2012; Shi et al. 2010; van Zonneveld et al. 2003; Xu et al. 2009). This is the likely explanation for why transmission only occurred in mothers who were not on ART.

Sequencing analysis and significance

The HBV strains sequenced from the two infants and the four mothers belong to subgenotype A1. This is known to be the most prevalent circulating strain of HBV in South Africa (Kramvis and Kew 2007). HBV strains circulating in South Africa have been previously characterised (Bowyer et al. 1997; Kimbi et al. 2004; Kramvis et al. 2002; Owiredu et al. 2001a, Owiredu et al. 2001b) but very few have been sequenced from patients residing in the Western Cape. This data will therefore contribute to the existing data on circulating strains in the Western Cape.

Phylogenetic analysis based on the surface gene showed that the two mother-child pairs were infected with identical strains of HBV giving further proof that the infection was of maternal origin given the young age of these infants. This is the first study looking at HBV sequences obtained from mother and child pairs in South Africa.

No drug-resistance mutations were observed in this cohort. This is not surprising given that the mothers were not on ART, although mutations have previously been observed in the YMDD motif (where mutations leading to lamivudine resistance commonly occur) in treatment-naïve patients (Selabe et al. 2007). The roll-out of tenofovir as part of first-line ART will further reduce the risk of these mutations emerging.

All the six HBV strains characterised in this study clustered with South African strains belonging to subgenotype A1, but they were genetically closer to the Western Cape sequences compared to the sequences from other provinces.

Infection with HBV strains of genotype A has a higher likelihood of leading to chronicity compared to other genotypes (Lin and Kao 2011). A South African study also showed that

infection with subgenotype A1 had a 4.5-fold risk of HCC development compared to infection with non-A genotypes (Kew et al. 2005). In subSaharan Africa, chronic carriers of HBV progress to HCC early, with the highest rates of cancer developing at around 45 years (Yang and Roberts 2010). Male patients have been shown to be two to seven times more likely to develop HCC than women (Yang and Roberts 2010).

The two infants who were found to still be infected on follow-up are highly likely to become chronic carriers and are therefore at high risk of developing HCC during the most productive years of their lives. Perinatally-infected chronic carriers normally experience an immunotolerant phase where they are highly infectious but show no symptoms of infection. They can therefore transmit the virus, not only to their playmates who may not have been vaccinated, but also to their care-givers. The ways in which the virus is transmitted horizontally are not clearly understood (Alter 2003) but contact of broken skin or mucous membranes with even small amounts of blood and other bodily fluids from an infected person (Alter 2003) are the most likely modes of transmission. In addition, the virus can survive on surfaces for at least seven days (Bond et al. 1981) and has been found on various household objects touched frequently by chronically infected infants (Petersen et al. 1976). Transmission of HBV through bites has also been demonstrated (Stornello et al. 1991, Hui et al. 2005). Therefore, unvaccinated children and adults who are in close contact with the persistently-infected infants from this study are at high risk of being infected with HBV.

Prevalence of HBeAg/AntiHBe

Two out of three followed-up infected infants in this study were HBeAg-positive. In contrast, Botha et al. (1984) showed that in unvaccinated Namibian infants younger than one year, the prevalence of HBsAg was 2.7% and 29% of the HBsAg positives were positive for HBeAg. However, only 1% of the infected infants in that age group was younger than six months. A study by Abdool Karim et al. in 1988 showed that in a cohort of 136 urban unvaccinated children less than two years old, only two of the tested children were positive for HBsAg and one out of the two children was positive for HBeAg. However, compared to the current study, none of the

HBsAg positive urban infants were under one year of age. The same study found that 25% of institutionalized infants younger than two years were HBsAg positive and 40% of the HBsAg positives were positive for HBeAg. However, only 20 participants were enrolled in that particular age group and the rates of infection observed would not reflect the prevalence in the general population.

Although the prevalence of HBeAg in the HBsAg positive infants in the current study was higher than seen in the Botha and Abdool Karim studies, the number of infected infants was too low to draw any definite conclusions and more studies are needed to evaluate the differences in HBeAg-positivity between HIV-exposed and HIV-unexposed infants.

HBV DNA levels

A viral load of $>10^8$ IU/ml was observed in two out of three tested HBV infected infants at follow-up in the current study. Simani et al. (2009) reported 'a low level viraemia' in 1.3% of their cohort of 303 HIV-infected and uninfected infants although they did not use a quantitative assay for DNA testing. A study by Hino et al. (2001) reported a prevalence of HBsAg of 7.8% in an unvaccinated cohort of children and showed a decrease from 6.5% to 0.3% in HBV DNA detection in vaccinated children compared to unvaccinated children from the Eastern Cape. Tsebe et al. (2001) did not detect HBV DNA in their cohort of vaccinated children. The HIV status of the children tested in the Tsebe and Hino studies was not stated.

This is therefore the first report persistently high hepatitis B viral loads in vaccinated South African HIV-exposed infants.

Significance of the study

Four infants were found to be infected with HBV in this study. These infants were first tested in the first three months of life, where they are mostly in contact with their mothers who were HBeAg-positive and would have minimal interactions with other individuals. These infants were therefore considered to be vertically infected. The first blood sample was taken from these infants either on the same day or very close to the first day they were vaccinated against HBV eliminating the possibility that the infections observed were breakthrough infections. In addition, phylogenetic analysis showed two mother-child paired sequences were identical confirming that the transmission was maternal in origin.

This transmission occurred despite complete and timely vaccination of the infected infants at six weeks, ten weeks and fourteen weeks. Two of the three followed up infected infants were still positive for HBV infection three months later. It is therefore clear that the current immunization schedule has failed in its role to protect these infants from HBV infection.

This schedule was based on studies showing that transmission of HBV occurred horizontally mainly in childhood and that vertical transmission was minimal. However, in the context of the HIV epidemic, where HIV-infected mothers show less HBeAg seroconversion (Thio 2009), the risk of vertical transmission is much higher. However, administering a birth dose of the HB vaccine will reduce the risk of transmission of HBV from HBeAg-positive mothers to their infants by approximately 75% (Beasley et al. 1983).

HBV vaccination at birth alone however will not prevent all vertical infections. Mothers with high viral loads ($>10^5$ IU/ml) are at high risk of infecting their infants (Burk et al. 1994) despite the administration of the HB vaccine to the neonates at birth (Lee et al. 1986). In the current study, three of the four mothers who transmitted HBV to their infants had a viral load of greater than 10^7 IU/ml. The fourth mother had a lower viral load of 10^3 IU/ml but since she showed HBeAg seroconversion at follow-up, it is likely that her viral load was much higher at the time of delivery.

Treating women with high viral loads with nucleos(t)ides will reduce the risk of transmission. When done in conjunction with the administration of a birth dose of the HB vaccine, the risks of MTCT are further reduced. Clinical trials have shown the efficacy of lamivudine in reducing the risks of MTCT in HBV-infected women and did not report any serious side effects (van Zonneveld et al. 2003; Xu et al. 2009). However, the use of lamivudine in co-infected patients is not recommended because of its low genetic barrier. A retrospective study has shown that the use of tenofovir significantly reduced the viral loads in HBV-infected mothers and none of their infants were positive for HBsAg at 28-36 weeks after delivery (Pan et al. 2012). However, in these studies, HBIg and the birth dose of the vaccine were administered to the neonates and it is therefore not known what the efficacy would be in the absence of the use of HBIg. Nonetheless, knowing that the use of the birth dose of the vaccine will reduce transmission by 75% (Beasley et al. 1983), it is expected that the concomitant administration of the vaccine at birth and the use of nucleos(t)ide analogues will decrease MTCT of HBV considerably.

Since this study only recruited HIV-exposed babies, the rate of transmission observed in this study cannot be extrapolated to the HIV-unexposed paediatric population. The antenatal study from the Western Cape showed the prevalence of HBeAg in HIV-uninfected women to be 15% (Maponga TG, MSc Thesis, Stellenbosch University, 2012). Given this HBeAg prevalence, and knowing that none of the HIV-infected women would be on antiHBV therapy, it is highly likely that vertical transmission of HBV is occurring in that group. Further studies are required to investigate the prevalence of HBV in HIV-uninfected infants.

Proposals to address the problem of HBV MTCT

This study has shown that vertical transmission of HBV is occurring in South Africa and that the current immunization schedule does not protect infants from vertical transmission of HBV. It is therefore proposed that the HB vaccine be administered at birth. The HB vaccine is safe and can be administered at birth with no serious adverse effects reported except for transient fever and pain at the site of injection (Romano et al. 2011).

Most HIV-exposed infants born in South Africa are uninfected at birth but still show immune impairments which are associated with their exposure to the HIV virus making them more prone to infections with more severe outcomes (Filteau 2009). However, Jones et al. (2011) showed that although HEU infants receive lower levels of protective antibodies from their mothers they mount robust responses to vaccination. As all the HBV-infected infants in this cohort were HIV-uninfected, it is likely therefore that administration of the vaccine at birth will benefit those infants.

Currently, a single-antigen HB vaccine is given to infants at six weeks. For women who deliver in clinics and hospitals, the same vaccine can be administered concomitantly with the BCG vaccine, another vaccine which should be given at or near delivery. It has been pointed out that there are some logistical problems associated with coverage and timeliness of a birth dose of the vaccine in the South African context (Kramvis and Clements 2010). An unknown proportion of women, still give birth at home in South Africa and their infants would therefore not have access to the vaccine within 24 hours of delivery as recommended by the WHO (Kramvis and Clements 2010). A community outreach approach as described in an Indonesian study (Ruff et al. 1995) could be used to target those infants and the single-antigen vaccine could be administered within seven days of birth by health workers from the nearest clinics catering to the mothers who deliver at home (Marion et al. 1994; Ruff et al. 1995). A study in Indonesia, a country with a rate of vertical transmission of HBV of 25% (Ruff et al. 1995), showed that administering the HB vaccine within seven days of delivery decreased the prevalence of HBsAg from 6.2% to 1.4% in infants (Ruff et al. 1995).

For long-term purposes, the use of Uniject™, a single-dose, auto-disable injection device, prefilled with hepatitis B vaccine (HB-Uniject) could be considered for use on the infants who are born at home. The use of HB-Uniject was shown to be cost-effective and to increase coverage of the birth dose of the HB vaccine among infants who are born at home in another Indonesian study (Levin et al. 2005). However, 79% of women deliver at home in Indonesia (Levin et al. 2005) and this may not reflect the situation in Africa. To determine the practicality of this approach in the African setting, studies are needed to find out what proportion of women give birth at home and whether the use of the HB-Uniject would be cost-effective.

Secondly, in order to identify those mothers with high viral loads, at risk of transmitting infection to their infants, pregnant women should be screened at booking for HBsAg and if positive, they should be further tested for HBeAg. Once identified, these women should be treated with a nucleos(t)ide analogue to reduce their HBV viral loads (Dusheiko 2012; Shi et al. 2010; Tran 2012) and therefore decrease the risks of MTCT.

LIMITATIONS

The results of this study should be interpreted in the context of the limitations in the study design.

It is likely that ART reduced the rate of HBV transmission in this cohort, but since we did not know what proportion of mothers who were HBsAg positive were on ART, nor what the viral loads were at delivery we were not able to quantify this.

Furthermore, the HBV DNA levels at the time of delivery of the mothers who transmitted HBV to their infants are not known and could only be estimated based on the viral loads observed at follow-up. However, given that they were not on ART during pregnancy, it is highly likely that the viral loads at delivery were similar to the ones at follow-up.

This study only looked at the infants of mothers who have regular follow-ups at their local clinics. Infants whose mothers do not come for regular follow-ups were therefore missed in this cohort. These could have included mothers who were too sick to come to the clinics.

Lastly, only samples which had a minimum of 100µl of plasma were selected for testing and therefore, there is a risk of sampling bias in the collection procedure as possibly samples from a certain clinic could have been favoured over others. However, this is negated by the fact the number of samples collected from all the clinics were proportional to the number of samples received from those same clinics by the NHLS.

CHAPTER SIX: CONCLUSION

This study has described a HBV prevalence of 0.4% (4/1000) in a large HIV-exposed paediatric population. This study has also reported persistence of HBV infection and elevated viral loads at follow up in two of the four infected infants. It has been shown that the likely route of infection in these two infants was vertical transmission.

The mothers of the four infected children were not on ART, all were positive for HBeAg and three of four mothers had viral loads of $>10^8$ IU/ml. These three mothers would be at risk of transmitting HBV to their infants (Burk et al. 1994), even if HB vaccine had been administered at the time of birth (Lee et al. 1986). These mothers should be identified by screening for HBsAg and then HBeAg, and where possible HBV viral load. The institution of nucleos(t)ide therapy early in the third trimester will reduce the risk of HBV transmission, together with a change in the timing of HBV vaccination to around the time of birth.

The prevalence of HBV transmission in HIV-uninfected women is not known, but it is proposed that in the absence of antiviral therapy and a prevalence of HBeAg positivity of 15% in HIV-uninfected women in the Western Cape (Maponga TG, MSc Thesis, Stellenbosch University, 2012), the risk of transmission may be even higher in HIV-uninfected women than that described here. This too supports the introduction of antenatal screening for HBsAg with the view to administer tenofovir to those at high risk of HBV transmission and early vaccination of the infant.

This study has described an important problem in current HBV public health policy and has shown that there is a serious need to re-examine the current immunization schedule in South Africa and to change the timing of HBV vaccination to include a birth dose of the vaccine.

Further studies are needed to determine the risk of HBV transmission in HIV-unexposed infants, to perform a cost-benefit analysis of screening women for HBsAg in pregnancy and to determine the operational problems associated with screening and the introduction of a birth dose of HBV vaccination.

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ADDENDUM A

UNIVERSITEIT•STELLENBOSCH•UNIVERSITY

01 June 2011 **MAILED**

Ms BN Chotun
C/O Dr M Andersson
Medical Virology
8th Floor
Clinical Building

Dear Ms Chotun

A cross-sectional study of the prevalence of Hepatitis B infection in HIV exposed infants who are tested for HIV at Tygerberg Hospital, Cape Town.

ETHICS REFERENCE NO: N11/05/151

RE : APPROVAL

A panel of the Health Research Ethics Committee reviewed this project on 17 May 2011; the above project was approved on condition that further information is submitted.

This information was supplied and the project was finally approved on 31 May 2011 for a period of one year from this date. This project is therefore now registered and you can proceed with the work.

Please quote the above-mentioned project number in ALL future correspondence.

Please note that a progress report (obtainable on the website of our Division: www.sun.ac.za/rds should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly and subjected to an external audit.
Translations of the consent document in the languages applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239
The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Hélène Visser at City Health (Helene.Visser@capetown.gov.za Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

Verbind tot Optimale Gesondheid · Committed to Optimal Health
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ADDENDUM B

50X TAE (recipe makes 1L of 50X)

Reagents needed

242g Tris base

57.1mL Galatial acetic acid

37.2g $Na_2EDTA.2H_2O$

pH ~8.5

Making the Stock

To make 50X, add 600mL H_2O to a flask. Add Tris base and let dissolve. Add EDTA and acetic acid and stir until all components are in solution. Finally, transfer mixture to a 1L graduated cylinder and add water to make total volume 1L.

Making the Working Solution

To make the working solution, dilute TAE to 1X. (e.g., Add 20mL 50X TAE into 980mL H_2O.)