

**An investigation of hepatitis B virus in antenatal women tested for human immunodeficiency virus, in the Western Cape Province of South Africa**

by

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



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March 2012

## Declaration

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

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## Summary

Hepatitis B virus (HBV) immunisation protocols in much of Africa are based on data from the pre-human immunodeficiency virus (pre-HIV) era that indicated that HBV transmission occurs predominantly horizontally between siblings and play-mates rather than vertically from mother to child. The immunosuppression associated with HIV infection however may release HBV from immune control resulting in higher HBV viral loads, which may increase the risk of perinatal mother to child transmission of HBV. The aim of this study was to determine the prevalence and characteristics of chronic HBV infection in HIV-infected pregnant women compared to HIV-uninfected pregnant women in the Western Cape province of South Africa.

Ethical approval was obtained to conduct a retrospective, matched case-control, unlinked anonymous study using residual plasma samples from the 9355 pregnant women included in the Western Cape's 2008 National HIV and Syphilis Antenatal Survey. Samples were tested for HBsAg on the AxSYM (Abbott, Chicago, IL) and confirmed by neutralization. Confirmed HBsAg-positive samples were tested for HBeAg, anti-HBe and anti-HD (Diasorin, Saluggia, Italy) and had HBV viral load and genotyping done. In addition, HBsAg-negative samples were tested for anti-HBc.

Samples from 1549 HIV-infected pregnant women were included and matched to the same number of samples from age- and race-matched HIV-uninfected women. Median age of 26 years, parity and education were similar in the two groups. The prevalence of HBsAg was 3.4% for the HIV-infected group and 2.9% for the HIV-uninfected group. HBV DNA loads of greater than  $10^4$  IU/ml were detected in 32.1% of HBsAg-positive, HIV/HBV co-infected women, and in 14.3% HBsAg positive, HBV mono-infected women. Among the HIV-infected group 18.9% of HBsAg-positive were HBeAg positive, with a median viral load of  $7.93 \log_{10}$  IU/ml; whilst 15.5% HIV-uninfected women were positive for HBeAg with a median viral load of  $6.07 \log_{10}$  IU/ml. Genotype A was seen in 92.6% of the isolates while 7.4% of the isolates were genotype D. Serum total anti-HBc antibodies that are a marker of past infection were detected in 42.2% of HIV-infected and in 24.1% of HIV-uninfected women that were negative for HBsAg. No positive sample for anti-HD was seen among all HBsAg-positive samples. This data indicates that there is increased exposure to HBV in HIV-infected pregnant women than in HIV-uninfected women and that a greater proportion of HIV-infected pregnant women compared to HBV mono-infected pregnant women may be at

increased risk of transmitting HBV to their infants. Further studies are needed to determine the rate of vertical transmission of HBV in the HIV era.

## Opsomming

Hepatitis B virus (HBV) immunisasie protokolle vir meeste dele van Afrika is gebaseer op data versamel in die era voor MIV. Die data dui aan dat HBV oordrag hoofsaaklik deur horisontale transmissie tussen broers, susters en speelmaats eerder as vertikale transmissie van moeder na kind plaasvind. Die onderdrukking van die immuunstelsel as gevolg van MIV infeksie kan egter lei tot 'n verhoogde risiko van perinatale HBV oordrag van moeder na kind. Die doel van hierdie studie was om die voorkoms en karakter van chroniese HBV infeksie in MIV-positiewe swanger vroue te vergelyk met die van MIV-negatiewe swanger vroue.

Etiese goedkeuring is verkry om 'n retrospektiewe, deursnee-, ongekoppelde anonieme studie uit te voer wat gebruik maak van oorblywende plasma monsters van 9355 swanger vroue wat ingesluit is in die Wes-Kaap 2008 Nasionale MIV en Sifilis Voorgeboortelike Opname. Die monsters was getoets vir HBsAg antiliggame (AxSYM, Abbott, Chicago, IL) en bevestig deur neutralisasie toetse. Positiewe monsters was getoets vir HBeAg en anti-HBe (Diasorin, Saluggia, Italië). HBV viruslading en genotipering was ook op HBsAg positiewe monsters gedoen. Die HBsAg negatiewe monsters was getoets vir die teenwoordigheid van anti-HBc.

Monsters van 1549 MIV-positiewe swanger vroue was ingesluit in die studie. Dieselfde aantal monsters van MIV-negatiewe vroue, met ooreenstemende ouderdom en etnisiteit, was ingesluit as kontroles. Die gemiddelde ouderdom van albei groepe was 26 jaar. Pariteit en opvoeding was dieselfde in albei groepe. Die voorkomssyfer van HbsAg was 3.4% in die MIV-positiewe groep en 2.8% in die MIV-negatiewe groep. HBV DNS ladings van meer as  $10^4$  IU/ml was waargeneem in 32.1% van die MIV-mede-geïnfekteerde vroue en in 14.3% van die MIV-negatiewe groep. In die MIV-positiewe groep was 18.9% vroue HBeAg positief, met 'n gemiddelde virale lading van  $7.93 \log_{10}$  IU/ml, terwyl 15.5% MIV-negatiewe vroue positief was vir HBeAg met 'n gemiddelde virale lading van  $6.07 \log_{10}$  IU/ml. In ons studie was 92.6% van die monsters genotipe A en 7.4% genotipe D. Toatale anti-HBc antiliggame, 'n merker van vorige infeksie, was gevind in 42.2% van MIV-mede-geïnfekteerde vroue en 24.1% van MIV-negatiewe vroue wat negatief was vir HBsAg antiliggame.

Data van ons studie dui op 'n verhoogde risiko vir vertikale HBV transmissie van MIV-positiewe moeders na hul babas. Verdere studies word benodig om vas te stel of vertikale transmissie van HBV van MIV-positiewe vroue na hul babas plaasvind.

## **Acknowledgements**

I would like to express my sincere gratitude to the following people and organizations who all made completion of this thesis possible.

Dr. Monique I. Andersson, my supervisor and mentor who provided invaluable guidance and inspiration throughout this project. Thank you for being more than just a supervisor; you are a mother away from home.

Prof. Wolfgang Preiser for being a good co-supervisor and for the insightful guidance. Your wisdom at every moment is greatly appreciated.

Prof Richard Tedder and Dr. Samreen Ijaz, the collaborators at the Health Protection Agency for the standard operating procedures, reagents and always giving the needed advice when experiments did not work.

The Wellcome Trust and the Poliomyelitis Research Foundation for providing funding for research and bursaries.

The Western Cape Provincial Department of Health for allowing use of the samples.

Colleagues from the diagnostic section in the Division of Virology, Tygerberg Hospital for helping with some of the testing.

Fellow students and senior researchers in the Division of Medical Virology for providing support.

My parents, Mr and Mrs Maponga and my siblings for always being a constant pillar of support.

To my wife, Vimbai for encouraging me to study and the prayers.

And finally to my Lord and Savior Jesus Christ without whom I would not be the person I am.

“There are two possible outcomes: if the result confirms the hypothesis, then you've made a measurement. If the result is contrary to the hypothesis, then you've made a discovery.”

Enrico Fermi

## Table of Contents

Declaration .....	ii
Summary .....	iii
Opsomming .....	v
Acknowledgements .....	vi
Table of Contents .....	viii
List of abbreviations and symbols.....	xi
List of Figures .....	xiii
List of Tables.....	xiv
CHAPTER ONE .....	1
1. Introduction .....	1
1.1. Global HBV epidemiology .....	1
1.2. HBV epidemiology in Africa .....	1
1.3. Aims and Objectives.....	4
CHAPTER TWO.....	5
2. Literature review .....	5
2.1. HBV virology .....	5
2.1. HBV replication.....	7
2.2. Molecular epidemiology of HBV .....	8
2.3. Natural history and pathogenesis of chronic HBV .....	10
2.4. HBV transmission.....	11
2.5. HBV diagnosis.....	13
2.6. HBV treatment.....	14
2.7. HBV vaccination .....	15
2.8. Effects of HBV/HIV co-infection.....	16
2.9. Lamivudine resistance and vaccine escape mutants .....	17
2.10. HBV vaccine in HIV era.....	18



CHAPTER THREE.....	19
3. Methods and materials .....	19
3.1. Ethical approval.....	19
3.2. Study population and samples .....	19
3.3. Serology tests.....	19
3.3.1. HBsAg testing .....	19
3.3.2. HBsAg confirmatory testing .....	20
3.3.3. HBeAg and anti-HBe testing.....	22
3.3.4. Anti-HBc(total) testing.....	24
3.3.5. Anti-HDV testing .....	26
3.3.6. Quality control for serology assays.....	27
3.4. Molecular tests.....	27
3.4.1. HBV viral load testing.....	27
3.4.2. Direct Nucleotide Sequencing of HBV Surface Antigen Region .....	30
3.4.3. Quality control for molecular assays.....	35
3.5. Sequencing data and phylogenetic analysis.....	36
3.6. Statistical analysis.....	36
CHAPTER FOUR.....	37
4. Results .....	37
4.1. Demographic data.....	37
4.2. Serology results .....	38
4.3. HBV DNA load test results .....	40
4.4. Occult HBV testing .....	44
4.5. Sequencing results .....	44
4.6. Phylogenetic analysis .....	47
CHAPTER FIVE.....	49
5. Discussion .....	49
5.1. Serology results .....	49

5.1.1.	HBsAg prevalence rate.....	49
5.1.2.	HBeAg and anti-HBe prevalence .....	50
5.1.3.	Total anti-HBc prevalence .....	51
5.1.4.	Anti-HDV prevalence .....	52
5.2.	Molecular results .....	53
5.2.1.	HBV DNA in HBeAg positive only.....	53
5.2.2.	HBV DNA in anti-HBe positive only .....	54
5.2.3.	HBV DNA in anti-HBc positive samples and occult infections .....	54
5.2.4.	HBV genotyping .....	55
5.2.5.	Mutation analysis .....	56
5.3.	Strengths and limitations of study .....	57
CHAPTER SIX .....		59
6.	Conclusion.....	59
References .....		61
Addendum A .....		73
Addendum B .....		75

## List of abbreviations and symbols

AIDS	-	acquired immunodeficiency syndrome
ALT	-	alanine transaminase
Anti-HBc	-	antibodies to HBV core antigen
Anti-HBe	-	antibodies to HBeAg
Anti-HBs	-	antibodies to HBV surface antigen
Anti-HD	-	antibodies to hepatitis delta
ART	-	antiretroviral therapy
bp	-	base pair
cccDNA	-	covalently closed circular DNA
CHB	-	chronic hepatitis B virus infection
DNA	-	deoxyribonucleic acid
dNTP	-	deoxynucleotide triphosphate
FBS	-	fetal bovine serum
FDA	-	Food and Drug Administration Agency
HBcAg	-	HBV core protein
HBeAg	-	hepatitis B virus e antigen
HBsAg	-	hepatitis B virus surface antigen
HBV	-	hepatitis B virus
HCV	-	hepatitis C virus
HDV	-	hepatitis delta virus
HIV	-	human immunodeficiency virus
HRP	-	horse-radish peroxidase
IU/ml	-	International units per ml
kb	-	kilobase
LAM	-	lamuvudine
mCMV	-	murine cytomegalovirus virus
MgCl <sub>2</sub>	-	Magnesium chloride
mM	-	millimolar
mRNA	-	messenger RNA
NIBSC	-	National Institute of Biological Standards and Controls
ORF	-	open reading frame
PBS	-	phosphate buffered saline

PCR	-	polymerase chain reaction
qPCR	-	quantitative PCR
RNA	-	ribonucleic acid
Taq	-	<i>Thermus aquaticus</i>
TDF	-	tenofovir
TMB	-	Tetramethylbenzidine
UNAIDS	-	Joint United Nations Programme on HIV/AIDS
USA	-	United States of America
v/v	-	volume/volume
WHO	-	World Health Organization

## List of Figures

Figure 1.1 Map of CHB prevalence according to geographical distribution. ....	2
Figure 2.1 Schematic diagram of a complete HBV virion. ....	5
Figure 2.2 Genome organisation of HBV and its transcripts. ....	7
Figure 2.3 Illustration of the replication process of HBV. ....	9
Figure 3.1 Example of an electrophoresis migration pattern. ....	32
Figure 4.1 Log <sub>10</sub> HBV viral loads according to HIV status. ....	42
Figure 4.2 Log <sub>10</sub> HBV Viral loads for HBeAg and anti-HBe positive samples. ....	43
Figure 4.3 Frequency and location of amino acid substitutions within HBsAg, comparing HIV-infected and HIV-uninfected women. ....	45
Figure 4.4 Frequency and location of mutations within the different codons of the polymerase region according to HIV status. ....	46
Figure 4.5 Phylogenetic analyses of HBV genomes. ....	48

## List of Tables

Table 3.1 Oligonucleotide primers used for quantitative detection of HBV DNA.....	29
Table 3.2 Reagent components of the quantitative HBV PCR reaction mix .....	29
Table 3.3 Oligonucleotide primers used for pre-nested PCR amplification of the HBV polymerase gene.....	30
Table 3.4 Primers used for sequencing of the polymerase region .....	34
Table 3.5 PCR Master Mix for HBV Sequencing Reaction .....	34
Table 4.1 Demographic data of patients sampled .....	37
Table 4.2 HBsAg testing results.....	38
Table 4.3 Results of HBeAg and anti-HBe testing of confirmed HBsAg positive samples ....	39
Table 4.4 Total anti-HBc testing on HBsAg negative samples.....	39
Table 4.5 HBV DNA detection/quantification on HBsAg positive samples .....	41
Table 4.6 Characteristics of the genotypes in isolated sequences.....	47

# CHAPTER ONE

## 1. Introduction

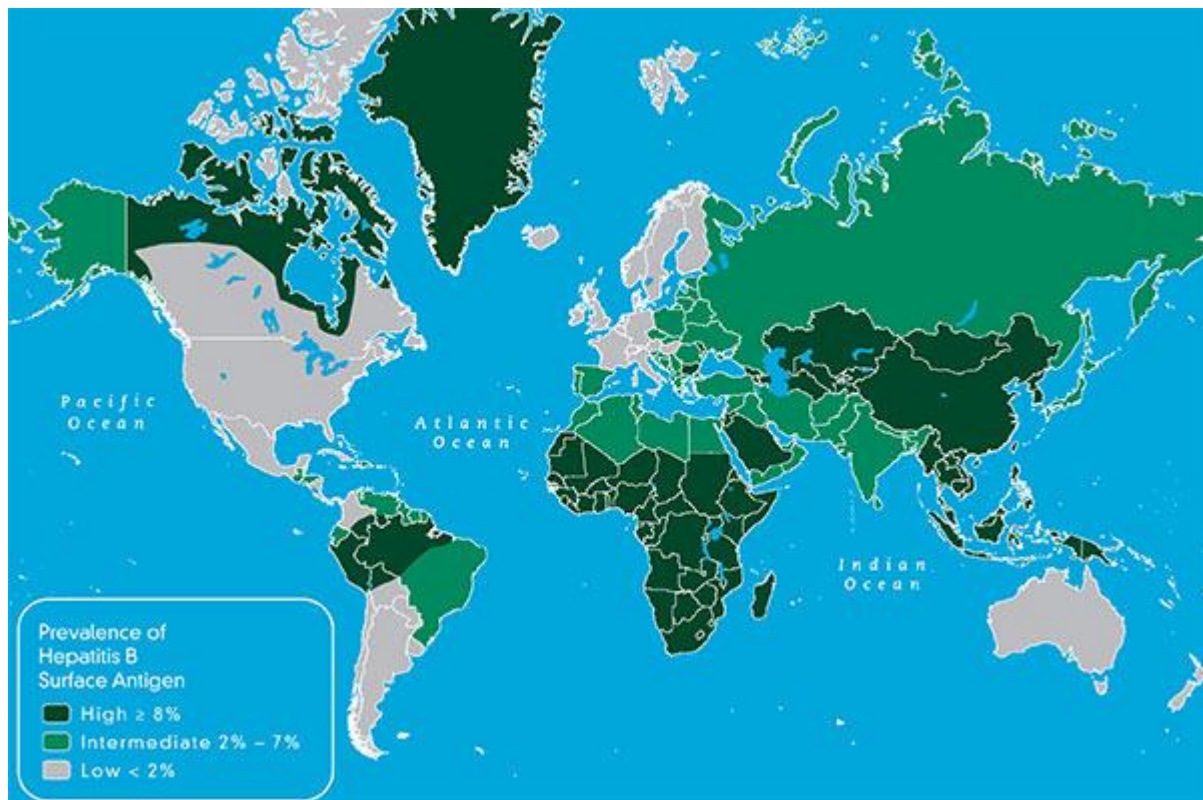
### 1.1. Global HBV epidemiology

Infection with HBV poses a major public health concern and is rated among the ten leading causes of death worldwide despite availability of a safe and effective vaccine (Mphahlele and Francois, 2008; World Health Organization [WHO], 2008; Lavanchy, 2004; Kiire, 1996). It is estimated that two billion people worldwide have been infected with HBV, with more than 350 million becoming chronically infected (WHO, 2008). It is also estimated that globally, about 10-30 million new infections occur annually and that over 600 000 people die each year from diseases related to HBV infection (WHO, 2008; Cooley and Sasadeusz, 2003). Chronic HBV (CHB) infection is particularly common in Asia and Africa where the virus is usually acquired either perinatally or in early childhood respectively (Hoffmann and Thio, 2007). CHB may be defined as continuous detection of HBV surface antigen (HBsAg) in a patient's sample for over six months (Soriano et al., 2010; Hoffmann and Thio, 2007; Shepard et al., 2006).

### 1.2. HBV epidemiology in Africa

Most African countries are classified as areas of high HBV endemicity. High endemicity for HBV is defined as CHB prevalence of  $\geq 8\%$ , intermediate endemicity is 2-7% and low endemicity refers to a prevalence of less than 2% (Heathcote, 2008; Hou et al., 2005). However, even within highly-endemic countries there is some variation in the distribution of HBV, with pockets of low and intermediate endemicity being seen.

The global differences in prevalence rates of CHB have been associated with differences in the age at acquisition of the virus (Heathcote, 2008; Hou et al., 2005). There is an inverse relationship between the risk of becoming a chronic HBV carrier and the age at acquisition of infection with about 90% of infants who get infected within the first year of birth progressing to chronic infection (Tran, 2009; WHO, 2008). This is in contrast to more than 90% of healthy adults that get infected and recover (Tran, 2009; WHO, 2008). Statistics estimate that about 18% of the global population of chronic carriers of HBV reside in Africa with sub-Saharan Africa being home to a greater number of this population (Kramvis and Kew, 2007a).



**Figure 1.1** Map of CHB prevalence according to geographical distribution. [Source: CDC, 2006]

It is estimated that in South Africa alone, there are between three to four million black people that suffer from CHB (Kew, 2008; Kew, 1996). Prevalence of HBV among pregnant women varies in different parts of the world with rates between 0.1% - 20% (Sinha and Kumar, 2010). In sub-Saharan Africa, there is a trend of higher prevalence rates of CHB in males than in females (Kew, 2008). In addition to infection by HBV, sub-Saharan Africa is also the region worst affected by the HIV pandemic being home to about 70% of the global population of 33 million people who are HIV-infected (UNAIDS, 2009).

HBV and HIV share transmission routes and it has been estimated that 80% of HIV-infected persons are exposed to HBV and that 4%-16% of the same population are chronically infected with HBV (Trevino et al., 2009; Mphahlele et al., 2006; Thimme et al., 2005). Co-infection with HIV alters the natural history of HBV infections, delaying the seroconversion to anti-HBe, causing higher replication rate and reactivation of infection despite the presence of anti-HBs (Hoffmann and Thio, 2007; Cooley and Sasadeusz, 2003; Puoti et al., 2002). Results from a Zambian study showed that pregnant women with dual HIV and HBV infections were twice as likely to have detectable HBeAg in their sera compared to those who were infected with HBV only (Oshitani et al., 1996). A study from Côte d'Ivoire found that the detection of plasma HBV DNA was more frequent in the co-infected HBV carrier than in HBV mono-



infected carriers (Rouet et al., 2004). Also, a study from Burkina Faso which is a country of moderate HIV prevalence revealed that HIV infection is a factor that is significantly associated with increased mother-to-child transmission of HBV (Sangare et al., 2009). Earlier data indicates that occult HBV infection may also be more common in HIV-infected patients (Kew, 1996). The findings by Kew are supported by a more recent study in SA which found that occult HBV infection is more prevalent in HIV-infected patients than in HIV-uninfected individuals although the study also reported a lower carriage of HBsAg in HIV seropositive patients (Mphahlele et al., 2006). The same study however found a statistically significant higher carriage of HBsAg in the HIV seronegative patients than in HIV-infected patients. Burnett et al. reported HBsAg prevalence of 7.4% in HIV-infected and 8.2% in HIV-uninfected pregnant women in South Africa (Burnett et al., 2007). The study by Burnett et al. was performed on samples from the national HIV antenatal surveys covering the period 1999-2001 collected from women who attended antenatal clinics in the Limpopo Province and North West Province of South Africa. However, a study by Firnhaber et al. in the Gauteng Province of South Africa reported an increased prevalence of HBV carriage in HIV-infected patients than in HIV-uninfected individuals (Firnhaber et al., 2009). These observed differences between HIV patients and pregnant women is likely to be explained by the differences in their immune status. Firnhaber et al. utilised samples from patients who were just about to start ART and had CD4 cell counts below 200 cells/ml. Also, the cohort comprised of both male and female gender. Perinatal transmission of occult HBV has not been well described although one study reported of a mother who had occult infection and transmitted the virus to her daughter (Saito et al., 1999). However, high HBV DNA levels as much as  $10^8$  IU/ml that were observed in some HIV-infected patients with occult infection may facilitate vertical transmission (Lukhwareni et al., 2009; Mphahlele et al., 2006). In addition, vertical transmission of occult infection with woodchuck hepatitis virus (WHV) has been observed in woodchucks which are considered as the natural model of human HBV infection (Mulrooney-Cousins and Michalak, 2007). Taken together these findings suggest that the epidemiology of HBV in the antenatal setting may be changing, although the situation in South Africa is largely unknown.

### **1.3. Rationale for the study**

As the immune suppression associated with progression of HIV becomes evident, it is apparent that there is increased HBV replication and infectivity that have been reported elsewhere. Previous findings of higher rates of HBV DNA and occult HBV infection that

have been seen on hospitalised or AIDS patients cannot be extrapolated onto HIV-infected pregnant women (Lukhwareni et al., 2009; Mphahlele et al., 2006). HIV-infected pregnant women are likely to be healthier than AIDS and hospitalised patients. Previous studies done in other provinces of South Africa using serology and HBV DNA testing have suggested that vertical transmission of HBV from HIV-infected mothers may not be a problem (Burnett et al., 2007). Thus this study is needed because only one province in South Africa has been studied, and we need to know what the situation is in other provinces as well. It is agreed that prevalence of HBV within a particular country may not be uniform across all region. Importantly, there are no data on HBV viral loads in pregnant women in South Africa, and this data is needed to see if HBV viral loads in the co-infected are higher than in the mono-infected.

#### **1.4. Significance of study**

The data from this study is important for determining evidence of exacerbation of HBV infection in HIV/HBV co-infected pregnant women. When combined together with data on the current rates of perinatal HBV infection, the study is useful for determining whether there may be a need to alter immunisation schedules for HBV to take account of HIV/HBV co-infection in South Africa.

#### **1.5. Aims and Objectives**

This study was conducted to determine the prevalence and characteristics of HBV infection among HIV-uninfected and HIV-infected pregnant women in the Western Cape province of South Africa.

The objectives of the study were- (i) to measure and compare the prevalence of HBsAg, HBeAg, anti-HBe, anti-HBc, anti-HD and HBV DNA in HIV-infected and HIV-uninfected pregnant women, (ii) to measure and compare HBV viral loads in HIV-infected and HIV-uninfected pregnant women, (iii) to determine HBV genotypes in HIV-infected and HIV-uninfected pregnant women, and (iv) to identify mutations in the overlapping polymerase and surface antigen regions of the HBV genome in HIV-infected and HIV-uninfected pregnant women.

## CHAPTER TWO

### 2. Literature review

#### 2.1. HBV virology

HBV has a deoxyribonucleic acid (DNA) genome and belongs to the *Hepadnaviridae* family under the subgroup human hepatitis virus (Ganem and Prince, 2004; Cooley and Sasadeusz, 2003). HBV exhibits tropism for hepatic cells although small amounts have also been found in other cells from the kidneys, pancreas and mononuclear cells (Ganem and Prince, 2004). The virus has a 3.2kb genome composed of circular DNA that is partially double-stranded because of an incomplete positive sense strand (Howard, 1986).

The complete and infectious HBV virion, which is also known as the Dane particle, has a 42 nm diameter and is composed of an icosahedral nucleocapsid core of 27 nm diameter that is surrounded by a lipoprotein envelope (Francois et al., 2001; Howard, 1986). The nucleocapsid core is made up of viral DNA bound with the polymerase enzyme and surrounded by the HBV core antigen. The lipid component of the lipoprotein envelope is derived from the host internal membranes during budding while the glycoprotein is a product of the preS-S gene of the virus (Harrison et al., 2008b). The lipoprotein envelope containing surface antigen is approximately 4 nm thick (Chen et al., 2008). A schematic diagram of the HBV virion is shown in Figure 2.1.

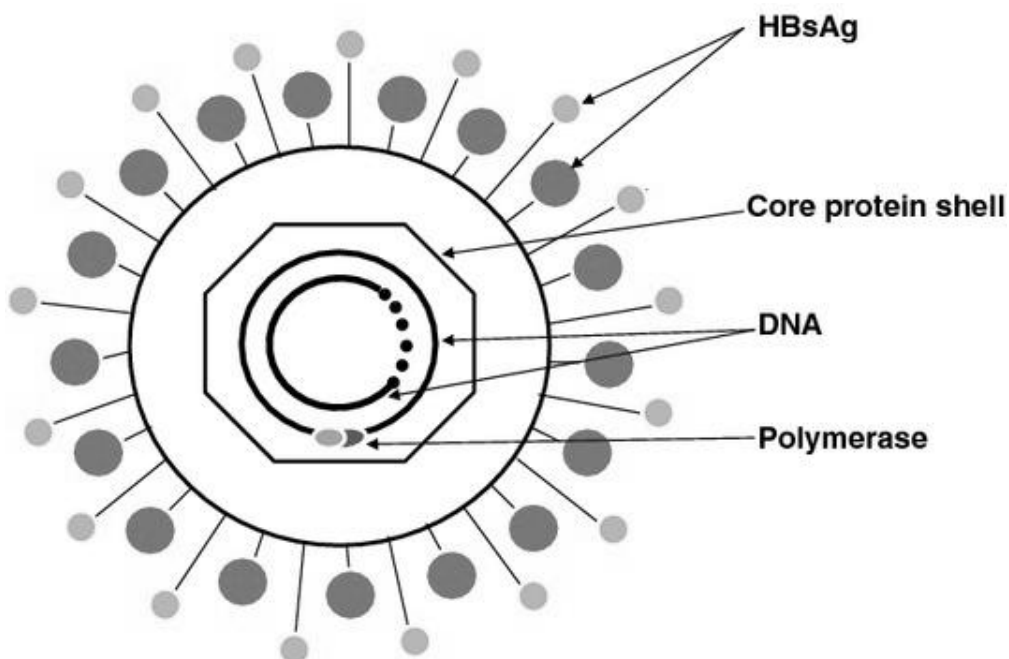


Figure 2.1 Schematic diagram of a complete HBV virion. [Source: Chen et al., 2008]

The HBV genome contains four open reading frames (ORFs) that overlap each other in a frame-shifted manner to produce seven identified proteins. These four open reading frames are C, P, S and X (Lüsebrink, 2009). ORF-C (core/precore) codes for the HBV core protein (HBc) and the HBV e antigen (HBeAg). The core protein is vital for viral assembly and makes the nucleocapsid that encapsulates the viral DNA and the polymerase while HBeAg has no role in viral assembly but is known to induce immunotolerance. Although HBeAg does not have a role in viral assembly and is secreted protein, its detection in blood serves to show active replication of HBV and is used as a marker of high infectivity (Harrison et al., 2008). ORF-P codes for the polymerase enzyme that is involved in replication and also has transcriptase as well as RNase H activity. ORF-S (preS-S) contains genetic information for the three polypeptides of the surface antigen (preS1, preS2 and S). Surface antigen proteins of different lengths are produced depending on which translation site has been read for initiation within the gene but all share the same C-terminus (Lüsebrink, 2009; Harrison et al., 2008b; Ganem and Prince, 2004). Antibodies against the surface antigen (anti-HBs) confer immunity to HBV (Harrison et al., 2008b). ORF-X codes for a transactivator of viral transcription (Lüsebrink, 2009; Pawlotsky, 2006). The X protein may modulate expression of both viral and host genes and is vital for viral replication and transmission (Ganem and Prince, 2004). Figure 2.2 depicts the genome organization of HBV.

Besides the Dane particles, HBV also occurs in two other distinct physical forms which are; (i) small, spherical, non-infectious particles, containing HBsAg, that on average measure 22 nm in diameter and (ii) tubular, filamentous forms of various lengths, but with same diameter as the spherical forms and also contain HBsAg (Lüsebrink, 2009; Bruss, 2007). The spherical and filamentous forms are however non-infectious as they are devoid of nucleic acids but are found in excess of the infectious particles by factors of up to 10 000 fold (Bruss, 2007). These excess subviral particles are thought to subvert the immunological response to surface proteins by mopping up low level anti-HBs thereby allowing infectious particles to escape from the immune system (Harrison et al., 2008b; Maruyama et al., 1993). The subviral or HBsAg particles share same antigens as the complete virions, although the protein composition is different (Bruss, 2007). Complete virions have the highest content of large HBsAg (L) protein, the filamentous forms have lesser and the spherical subviral particles have the least relative amount of L within their structure.

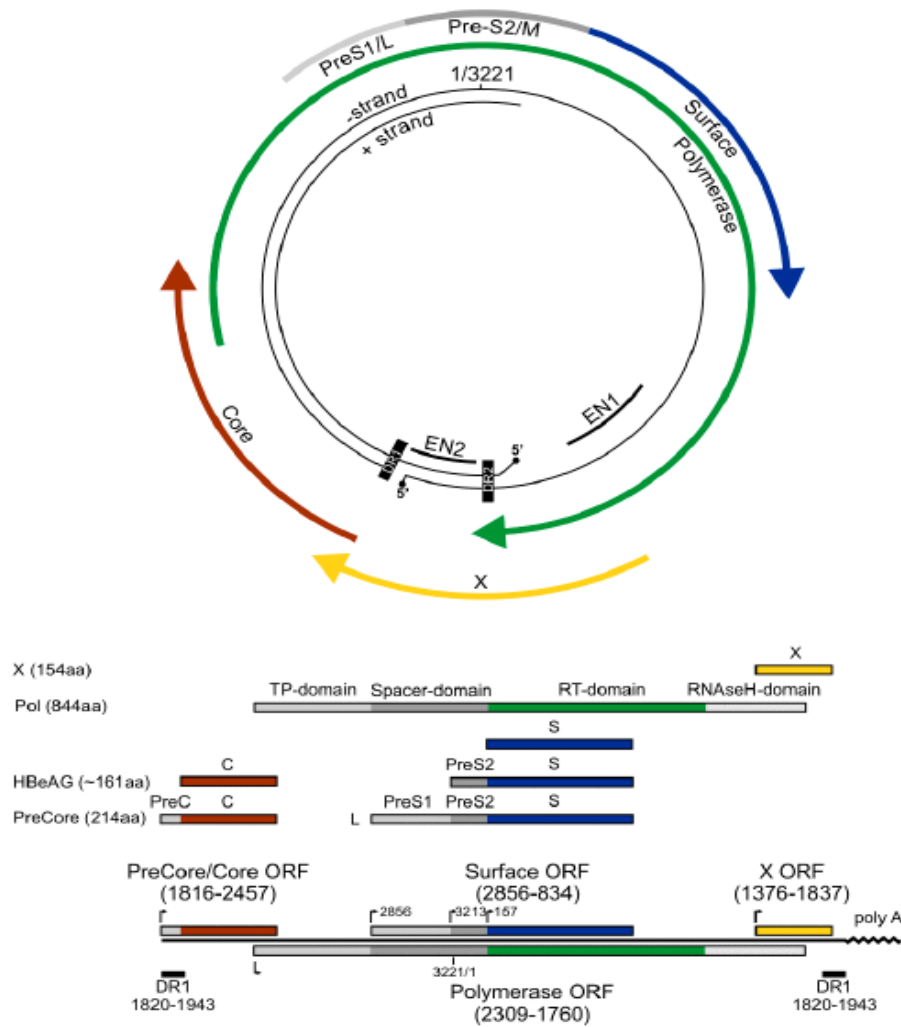


Figure 2.2 Genome organisation of HBV and its transcripts. [Source: Lüsebrink *et al*, 2009]

## 2.1. HBV replication

The receptors required for hepatocyte infection by HBV are as yet unknown although it is believed that first contact involves a domain located near to the N-terminus of L (absent in M or S), and other interactions involving S may also be important (Harrison *et al.*, 2008b). It is thought that when pre-S1 is expressed, L is translocated into the lumen of the endoplasmic reticulum where it may then be exposed on the exterior of the viral particle and then presents as the receptor-binding domain (Harrison *et al.*, 2008b). Virus entry is posited to be through the endosomal route and results in the delivery of the genome to the nucleus (Harrison *et al.*, 2008b). When the genome has been delivered to the nucleus, there is repair of the incomplete positive strand and subsequent formation of covalently closed circular DNA (cccDNA) (Ganem and Prince, 2004). Repair of the incomplete strand is thought to be due to a host enzyme (Harrison *et al.*, 2008b). The cccDNA serves as the transcriptional template for host

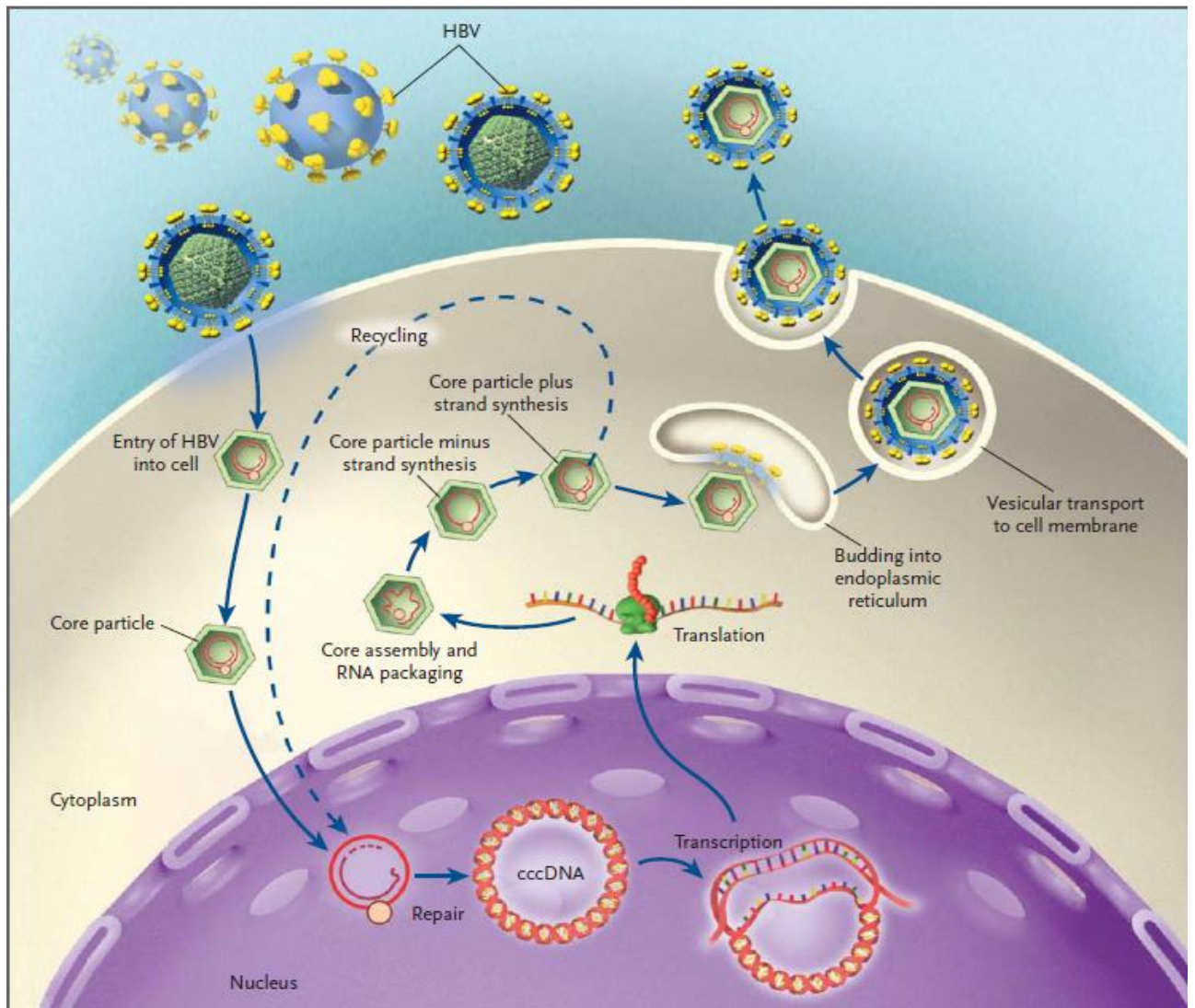
RNA polymerase II which leads formation of 3.5-kb pregenomic and precore (pre-C) RNAs (Quasdorff and Protzer, 2010). Core and polymerase/reverse transcriptase proteins are transcribed from pregenomic RNA (Quasdorff and Protzer, 2010). HBeAg is translated from pre-C mRNA (Quasdorff and Protzer, 2010). One molecule of genomic ribonucleic acid (RNA) is enclosed in the viral capsid as the nucleocapsid is constructed in the cytoplasm (Ganem and Prince, 2004). Once encapsidation has occurred, reverse transcription of the genomic RNA to DNA by the viral polymerase begins (Ganem and Prince, 2004). Use of a reverse transcriptase is a feature that HBV shares with retroviruses although the former is a DNA virus. A total of 240 subunits of core protein are combined to make the viral capsid which in some way directs the arrangement of the envelope proteins (Quasdorff and Protzer, 2010).

The viral envelope, which is composed of a lipid bilayer that is densely packed with the large (L), middle (M) and predominantly the small (S) envelope proteins, is gained when the capsids bud into the intracellular membranes (endoplasmic reticulum) into which the surface proteins (HBsAg) are embedded (Harrison et al., 2008a; Ganem and Prince, 2004). The complete viral particles are then transported out the cell. However, some capsids that contain the normal HBV genome shuttle back to the nucleus. Once in the nucleus, the HBV DNA is again repaired to form cccDNA. The cccDNA maintains a pool of HBV mRNAs inside the nucleus of host cells (Ganem and Prince, 2004). Figure 2.3 summarizes the process of HBV replication.

## **2.2. Molecular epidemiology of HBV**

HBV is widely classified into eight genotypes (A-H) which have distinctive geographical distribution (Audsley et al., 2010; Yu et al., 2010; Tatematsu et al., 2009). However, two additional genotypes I and J, have recently been described in Asian patients (Yu et al., 2010; Olinger et al., 2008). Genotype I is described as having evolved from recombination events (Yu et al., 2010; Olinger et al., 2008). It may therefore be questionable if I and J are true genotypes. HBV genotype classification is based on sequence heterogeneity either in the entire genome length or in the S gene (Kramvis et al., 2005; Kramvis and Kew, 2005). HBV genotypes are defined by divergence in the entire HBV genomic sequence of more than 8% or more than 4% at the level of the S gene (Hou et al., 2005; Kramvis et al., 2005; Kao and Chen, 2002). Some researchers have advocated using the entire genome sequence to allow detection of recombination of different genotypes that may arise as a result of co-infection by more than one genotype (Hou et al., 2005; Kramvis et al., 2005).





**Figure 2.3** Illustration of the replication process of HBV. [Source: Ganem and Prince, 2004]

HBV genotype A is endemic to Europe, North America and Africa, genotypes B and C are predominant in Asian populations, whereas D has a worldwide distribution but is more common in the Mediterranean area, genotype E is restricted to Africans, F is found in the aboriginal populations of South America, H is confined to the indigenous populations of Central America and G has been isolated in carriers in developed countries (Hou et al., 2005; Kramvis et al., 2005; Kao and Chen, 2002). Knowledge of the various genotypes is important because of the clinical and epidemiological implications of individual genotypes (Kramvis and Kew, 2007a; Kramvis et al., 2005). Different genotypes have also been linked to perinatal transmission of HBV (Sinha and Kumar, 2010; Hou et al., 2005; Kao and Chen, 2002). Genotypes B and C are prevalent in Asian countries that are mostly highly endemic and where

mother-to-child transmission is particularly responsible for the spread of the virus. It is thought that vertical transmission may be responsible for conservation of these genotypes in the population (Hou et al., 2005). In contrast, the other genotypes (that is excluding B and C) are prevalent in areas where horizontal transmission is the main route of infection because these are associated with early seroconversion to anti-HBe (Kao and Chen, 2002).

### **2.3. Natural history and pathogenesis of chronic HBV**

The natural history of chronic HBV infection (CHB) falls into four phases (Heathcote, 2008; Hoffmann and Thio, 2007). The phases are- immunotolerant, immunoactive, inactive carrier, and reactivation (Heathcote, 2008; Hoffmann and Thio, 2007). An increasingly-recognized category of occult HBV infection is also acknowledged and increasingly gaining importance (Hoffmann and Thio, 2007). The immunotolerant phase is characterized by active viral replication, punctuated by high plasma HBV DNA, presence of HBeAg, normal alanine aminotransferase (ALT), and little histologic activity. The immunotolerant phase is observed almost exclusively in persons infected neonatally or in early childhood and may last for several decades (Heathcote, 2008; Hoffmann and Thio, 2007). The immunotolerant phase is usually absent in persons who acquire HBV in adulthood (Hoffmann and Thio, 2007). The immune clearance or “immunoactive” period follows as the immune system attempts to remove the virus by attacking infected host liver cells (Heathcote, 2008; Hoffmann and Thio, 2007). HBV replication gradually declines, although HBeAg is still secreted during immune clearance (Heathcote, 2008). Some individuals in the immunoactive phase may develop mutation(s) in either the pre-core or core region causing reduced or non-expression of HBeAg (Hoffmann and Thio, 2007). The mutant virus in such individuals remains competent to replicate and may cause high HBV DNA loads hence infectivity even in the absence of HBeAg (Hoffmann and Thio, 2007). The third phase is the inactive carrier state where antibodies to HBeAg (anti-HBe) are detected, HBV DNA is not found in blood, and this phase may continue indefinitely (Heathcote, 2008; Hoffmann and Thio, 2007). Serum ALT normalizes and liver disease becomes inactive and some degree of histologic regression may take place in the inactive carrier state (Heathcote, 2008). The reactivation phase occurs in persons who, despite testing negative for HBeAg, have elevated HBV DNA levels and ongoing intermittent hepatitis (Heathcote, 2008).

Intracellular HBV is not considered to be directly cytopathogenic to host cells in immunocompetent persons and this is a good strategy for its own survival (Lüsebrink, 2009; Grob, 1998). This notion is supported by the fact that many HBV carriers do not show



symptoms and there is little liver injury, regardless of continued replication of the virus inside the hepatocytes (Pungpapong et al., 2007). However, presentation of processed HBV peptides to cytotoxic CD8<sup>+</sup> lymphocytes through human leukocyte antigen (HLA) class 1 molecules causes immune-mediated deletion of infected hepatocytes (Grob, 1998). Liver pathology arises from these efforts of the immune system to clear the virus and this may in turn cause acute hepatitis (Heathcote, 2008; Ganem and Prince, 2004; Grob, 1998). However, direct cytopathogenicity of HBV has been described in AIDS patients in a condition called fibrosing cholestatic hepatitis (FCH) where prolonged immunosuppression and excessive replication are the hallmarks (Kao and Chen, 2002).

The high rates of chronic carriage in childhood acquired HBV is partly explained by the immaturity of the immune systems in children (Pungpapong et al., 2007). An immature immune system means that children cannot mount an effective immune response to clear the virus. However, the explanation that children have an immature/underdeveloped immune system has been partly refuted by researches that have shown that the infants respond well to the HBV vaccine. The inability of children to clear HBV stands in contrast to some patients that experience fulminant hepatitis for a brief duration because of a strong immune response that is mounted against the virus resulting in viral clearance (Pungpapong et al., 2007). Fulminant hepatitis results from an unintended attack on hepatocytes as immune cells fight against the virus.

The worst outcomes of CHB include the development of cirrhosis and hepatocellular carcinoma. The highest rates of hepatocellular carcinoma (HCC) are observed in regions that are highly endemic for HBV, sub-Saharan Africa being one of the three regions of high HCC incidence (Kew, 2010). The prevalence of HCC in sub-Saharan Africa is high in males and it tends to affect younger ages than is seen in other geographical locations (Kew, 2010).

#### **2.4. HBV transmission**

HBV may be transmitted vertically or horizontally but in all cases, direct/indirect contact with infected body fluids is a requirement (Lüsebrink, 2009). The different modes of transmission for HBV include;

- Vertical which refers to transmission from a mother to her infant and this may occur in utero, at the time of birth, or after birth. In utero transmission is rare. Vertical transmission is high when the mother is HBeAg seropositive. Up to as much as 90%

of infants born to HBeAg seropositive mothers may become chronically infected compared to only 10-30% of infants that are born to mothers who are only positive for HBsAg (Hoffmann and Thio, 2007; Hou et al., 2005). Vertical transmission is thought to play a minor role in sub-Saharan Africa but predominates in Asia.

- Horizontal infection in childhood which is defined as transmission that is unrelated to known sexual, perinatal or parenteral exposure (Davis et al., 1989). This may be facilitated by via minor breaks in the skin or mucous membranes or close bodily contact with other children or close family members. Most infections in sub-Saharan African children are attributed to horizontally acquired HBV. Horizontal transmission may occur through other close community members although intrafamilial spread is more common than interfamilial. Horizontal transmission may occur through contaminated inanimate objects such as toothbrushes, razors, and even toys because HBV can survive for long periods outside the human body (Wasmuth, 2009). Children who acquire HBV infection horizontally when they are still under 5-years of age are likely to become chronic carriers and are susceptible to adverse long-term effects (Davis et al., 1989).
- Sexual transmission from unprotected intercourse. In the USA, it is estimated that about 40% of new HBV infections are attributed to heterosexual transmission while 25% of new infections occur in men who have sex with men (Wasmuth, 2009).
- Percutaneous transmission that is associated with intravenous drug use when drug users share a contaminated needle/syringe. Statistics from developed countries, such as the USA and Europe where intravenous drug use is more common, indicate that 15% of new infections may be due to percutaneous transmission (Wasmuth, 2009).
- Transfusion-associated infection that is linked to use of contaminated blood and blood products. Mathematical models that have been used in Sub-Saharan Africa estimate that transfusions from contaminated blood would potentially be responsible for almost 29 000 HBV infections if the required 6.65 million units of blood that are required for the region's population were to be issued (Jayaraman et al., 2010).
- Nosocomial infections which are hospital-acquired and may occur between patient and patient or between patient and health care worker in either direction. It also includes needle-stick injuries to healthcare workers from syringes and apparatus that are contaminated with HBV. In a recent study in the USA, health-care associated transmission accounted for 18.6% of new infections (Daniels et al., 2010). Nosocomial infections also occur in the hospital-setting due to unsafe injection

practices when the same needle is used on multiple patients without being sterilized (Simonsen et al., 1999).

- Organ transplantations including liver, kidney and cornea transplants (Wasmuth, 2009).

CHB is common in many countries in Southern Africa and is maintained because of ongoing networks of transmissions in infancy (horizontal transmission) (Kramvis and Kew, 2007a; Kew, 1996; Kiire, 1996). Perinatal transmission is considered uncommon in sub-Saharan Africa because of the low infectivity of mothers. Studies have shown that very few pregnant HBsAg carriers in southern Africa carry HBeAg when compared to Asian women and this may account for relatively low rates of perinatal infection in sub-Saharan Africa (Burnett et al., 2005; Madzime et al., 1999; Kiire, 1996).

HBeAg is generally accepted as the marker of high infectivity (Gilbert et al., 2002; Francois et al., 2001; Kiire, 1996). However, the absence of HBeAg is not synonymous with non-infectivity as some HBV variants exist which will not shed HBeAg even in the most infectious phases. These variants have a mutation in the pre-C region which results in introduction of a premature stop codon that prevents further synthesis of HBeAg. Studies from African countries have found the prevalence of HBeAg to be <2-24% among HBsAg positive pregnant women (Sinha and Kumar, 2010; Kiire, 1996; Guidozi et al., 1993; Prozesky et al., 1983).

## **2.5. HBV diagnosis**

The diagnosis of HBV infection is made using a combination of serologic, virologic, biochemical, and histologic tests. Immunological tests are the primary diagnostic utility for HBV (Dény and Zoulim, 2010). The HBsAg test is the mainstay for screening of both acute and chronic infection (Gish, 2008) together with detection of antibodies against the capsid/core antigen (Dény and Zoulim, 2010). HBsAg is detected early during acute infection, on average 6–10 weeks after exposure and several weeks before symptoms are observed (Chevaliez and Pawlotsky, 2008). When immunological tests for anti-HBc and HBsAg are negative, it is highly unlikely that the patient is infected with HBV. When both HBsAg and total anti-HBc are detected in the serum, acute or chronic infection can be differentiated by clinical history and anti-HBc IgM detection (Dény and Zoulim, 2010). It is noteworthy that a positive result for anti-HBc IgM does not always point to the presence of an acute infection and may be due to low levels of IgM arising from reactivation of CHB (Dény and Zoulim,

2010). It is advised that a first positive test for HBsAg be confirmed using an HBsAg confirmatory assay to exclude false positive results (Chevaliez and Pawlotsky, 2008). Detection of HBsAg for more than six months defines a chronic infection. Nevertheless, HBsAg may fail to be detected in some patients even though they are infected. Such results may be observed when: (i) there is low viral replication in asymptomatic carriers, (ii) the carrier harbours HBV mutants whose surface antigen does not bind to antibodies used in commercial assays, (iii) when infection clears spontaneously or after successful antiviral therapy in chronic HBV-infected patients and, (iv) in HDV/HBV co-infected patients, where hepatitis delta virus may prevent HBV replication and expression (Chevaliez and Pawlotsky, 2008).

In addition to the primary serologic diagnostic markers, additional markers are employed in HBV prognosis. Presence of antibodies to the surface antigen (anti-HBs) normally represents immunity to HBV either from vaccination (anti-HBs alone) or from a resolved infection (anti-HBs together with anti-HBc) (Chevaliez and Pawlotsky, 2008). However, simultaneous presence of anti-HBs and HBsAg has been described and is thought to occur in chronic carriers who develop immune-escape mutants (Colson et al., 2007). HBeAg and anti-HBe are used to determine the infectivity status of an HBV-infected patient although these may be affected by the presence of pre-core mutants which do not result in secretion of HBeAg. In addition, HBV DNA detection and quantification are also employed to determine the need for treatment and response to therapy as well as exploring viral reactivation (Dény and Zoulim, 2010). HBV DNA detection has also become a useful marker for detection of occult infections where surface antigen is not detected while genotyping is gaining widespread importance for predicting HBV disease progression and treatment response (Soriano et al., 2008).

## **2.6. HBV treatment**

There is no specific treatment for acute HBV infection and the goal is usually to maintain comfort and adequate nutritional balance, including replacement of fluids that are lost from vomiting and diarrhoea (WHO, 2008). In contrast, there are currently seven Food and Drug Administration Agency (FDA)-approved drugs, including interferon for treating CHB (Soriano et al., 2008). The desired targets for treating CHB are prevention of the development of progressive disease, particularly cirrhosis and liver failure, as well as hepatocellular carcinoma (Dény and Zoulim, 2010; Hoffmann and Thio, 2007; Ganem and Prince, 2004). There are two classes of drugs used to treat chronic HBV namely, immunomodulators and

antivirals. This classification is rather variable as the immunomodulators also exert direct antiviral activity. The immunomodulators widely used are natural interferon- $\alpha$ -2b and pegylated interferon- $\alpha$ -2a (Dény and Zoulim, 2010; Chevaliez and Pawlotsky, 2008). The FDA-approved antivirals are nucleos(t)ide analogues that target the reverse transcriptase activity of the polymerase enzyme in a similar fashion to anti-HIV drugs. The nucleos(t)ide analogues include lamivudine (LAM), tenofovir (TDF), entecavir (ETV), telbivudine (LdT) and adefovir dipivoxil (ADV) (Dény and Zoulim, 2010). However, the efficacy of antivirals in HBV treatment is dampened by the development of drug-resistance mutations within the viral genome (Ohkawa et al., 2008).

## **2.7. HBV vaccination and post-exposure prophylaxis**

HBV used to be frequently transmitted by transfusion during the 1970s before screening of donated blood could be performed (Wedermeyer, 2009). With the advent of improved diagnostic tests and screening of blood products for transfusion transmissible infections, vertical transmission or horizontal transmission and sexual exposure have become the most frequent routes of HBV infection (Wedermeyer, 2009; Lavanchy, 2004). To curb increased horizontal and vertical transmission, WHO in 1991 recommended general vaccination against HBV in all countries (Wedermeyer, 2009; Lavanchy, 2004). The first plasma-derived HBV vaccine was approved by the FDA in 1981 followed later by recombinant vaccines produced in yeast cells in 1986 (Wedermeyer, 2009; Lavanchy, 2004). The first national universal vaccination programme that was introduced in Taiwan in 1984 to vaccinate all infants led to a reduction of the overall HBsAg prevalence from 9.8% in 1984 to 1.3% in 1994 among children <15 years of age (Wedermeyer, 2009; Lavanchy, 2004). Improved maternal screening for HBV carriers also led to a reduction of the HBV carrier population in Taiwan (Chen et al., 1996). In addition to vaccination, high-titre hepatitis B immunoglobulin (HBIG) is also available for post-exposure prophylaxis of non-immune subjects. Unlike vaccination, HBIG only offers short term protection in recipients. HBIG is not widely available in most resource-poor countries because of its cost.

Immunisation protocols in most sub-Saharan African countries are aimed at preventing infant infection through horizontal transmissions rather than preventing mother-to-child transmission at birth (Hoffmann and Thio, 2007). Thus the first doses of vaccine are given later in the postnatal period. The timings for the three doses of vaccine range from 4, 8 and 12 weeks in Tanzania; 6, 10 and 14 weeks in South Africa and Kenya; to 3, 4 and 9 months in Zimbabwe (Mphahlele, 2008). However, these immunisation protocols leave the neonate

susceptible to exposure at birth should the mother's HBV infection pose a risk of transmission at that time. A study conducted in the Eastern Cape province of SA found that neonatal transmission was occurring at higher rates than previously reported (Vardas et al., 1999). Vardas et al. reported HBsAg prevalence rates of 8.1% and 8.9% in the 0-6- and 7-12-months age groups, respectively (Vardas et al., 1999). The HBsAg carriage rates reported by Vardas et al. are higher than the rate of 2.5% in newborns to 6 years upon which the current South African vaccine schedules were made (Robson and Kirsch, 1991). The current immunisation schedules in most sub-Saharan states, including South Africa, are based on studies that were conducted before HIV had reached the current pandemic levels. There is little data on how HIV co-infection affects transmissibility of HBV from pregnant women (Burnett et al., 2005).

## **2.8. Effects of HBV/HIV co-infection**

SA has the largest number of HIV infections in the world with nearly 5.3 million people living with HIV/AIDS (Joint United Nations Programme on HIV/AIDS [UNAIDS], 2009; Mphahlele, 2008; Burnett et al., 2005). The antenatal HIV prevalence rates approach one in three in some areas of South Africa. Antiretroviral therapy (ART) for HIV is increasingly becoming available in SA and the benefits for those who are HIV-infected are indisputable. Most adult and paediatric ART regimens used in South Africa include LAM which also has potent HBV antiviral activity (Thimme et al., 2005; Da Silva et al., 2001). LAM is accepted as an effective and safe drug for treatment of both HIV and HBV (Clements et al., 2010; Sinha and Kumar, 2010; Agarwal and Tiwari, 2009). LAM suppresses HBV and HIV replication by inhibition of the RNA-dependent DNA polymerase of both viruses (Coates et al., 1992). Unwittingly, use of LAM as monotherapy for HBV as provided for in most of the current ART regimens in SA, is associated with the emergence of HBV strains that are LAM-resistant leading to worsening of liver disease (Papatheodoridis et al., 2005; Liaw et al., 2004). These drug-resistant viruses carry specific mutations and may be present in more than 90% of HIV/HBV co-infected patients who have received four years of therapy and their development may be potentiated by HIV-related immunosuppression which up regulates HBV replication (Matthews et al., 2006; Benhamou et al., 1999).

With the availability of highly active antiretroviral therapy (HAART) for HIV treatment, the incidence of HBV-related liver disease and mortality has also increased (Kew, 2010). It is thought that HIV-infected persons who have previously been exposed to HBV are now living longer allowing the development of cirrhosis and HCC (Kew, 2010). Even without HIV co-infection, statistics estimate that 25% of patients with CHB develop HCC (Burnett et al.,

2005). Data emerging from different studies suggests that HIV hastens the progression of chronic HBV to HCC although this is not conclusive. Other studies have suggested that there is less liver damage due to the dampened HBV-specific response as a result of HIV-induced immunosuppression (Herrero-Martinez, 2001). Also, the use of HAART in HIV/HBV co-infected patients poses a greater risk of liver disease due to the effects of the immune reconstitution syndrome (Burnett et al., 2005).

The immunosuppression caused by HIV may permit reactivation of latent HBV infections to active CHB particularly in patients who have progressed to acquired immunodeficiency syndrome (AIDS) (Horvath and Raffanti, 1994; Hudson, 1990). Immunosuppression due to HIV may also lead to susceptibility to re-infections in patients previously exposed to HBV because such individuals may lose their protective antibodies as they progress to AIDS (Horvath and Raffanti, 1994).

### **2.9. Lamivudine resistance and vaccine escape mutants**

The primary mutation associated with resistance to LAM is the methionine to valine or isoleucine change that is seen on amino acid 204 (M204V/I) which occurs in the catalytic domain of the HBV RNA-dependant DNA polymerase. On rare occasions a methionine to serine change (M204S) may also be seen. A range of other early and late mutations associated with LAM resistance have also been reported, some of which are thought to act as compensatory mutations contributing to a recovery of replication efficiency in the face of antiviral resistance mutations (Bartholomeusz and Locarnini, 2006). LAM resistance mutations in the P gene of HBV have the capacity to also induce changes in the S gene which codes for HBsAg. This is because the S gene encoding for HBsAg is completely overlapped by the longer P gene (Torresi, 2002). Subsequently, some mutations in the P gene following nucleos(t)ide analogue therapy may result in structural changes in the HBsAg protein and a subsequent reduction in antigenicity (Torresi et al., 2002). HBsAg is the viral constituent in all current hepatitis B vaccines. It has been shown that recombinant HBsAgs bearing these changes behave as vaccine escape mutants (Carman et al., 1990).

A recent investigation reported that the triple mutant, rtV173L/rtL180M/rtM204V, was more common in persons with HIV/HBV co-infection than in those with HBV infection only (Sheldon et al., 2007) indicating a potential selective advantage for this strain in immunosuppressed hosts, perhaps reflecting HBsAg epitope deletion. The triple mutant has been shown to produce a variant HBsAg which does not bind to vaccine-induced antibodies in



vitro and may promote vaccine failure (Torresi et al., 2002). The roll-out of ART gives ample opportunity for HIV therapy to induce drug resistant HBV and subsequent vaccine escape variants in co-infected patients. This is in addition to LAM-resistant variants that have already been identified in some therapy naive patients (Selabe et al., 2007). Use of TDF would circumvent the risk of HBV resistance that is associated with LAM use. TDF has a higher potency and genetic barrier compared to LAM but it unfortunately is not widely available in developing countries (Soriano et al., 2008).

#### **2.10. HBV vaccine in HIV era**

A study performed before the widespread availability of ART reported no transmission of HBV from mother to child in 598 babies although in fact only six children were born to known HBV-infected mothers, only one of whose serum contained HBeAg (Tsebe et al., 2001). The findings provide encouraging data on the success of the HBV immunization program in the first five years of implementation even though HIV prevalence was not reported. It would appear that the child born to the HBeAg carrying mother was not tested for HBV DNA since the baby was negative for both anti-HBc and HBsAg. It would have been conclusive to test for HBV DNA because occult infection has been reported to occur in the absence of any serological markers (Lukhwareni et al., 2009; Hino et al., 2001).

The rationale behind current immunization schedules is that the first dose of vaccine administered at six weeks will protect the infant by preventing subsequent horizontal HBV transmission in infancy which historically has been the usual route of infant transmission in SA. The fact that these babies remain at risk of perinatal mother to child transmission of HBV in the first 6 weeks of life is irrelevant if the HBV infected mother is of low HBV infectivity. It is also thought that maternal antibodies that are passively transferred will protect the infant during the period before it makes its own antibodies through active immunization (Ayoola, 1988). However, HIV co-infection is likely to increase HBV replication in HIV/HBV co-infected mothers and they will become more likely to transmit HBV perinatally, thereby altering the epidemiology of early infections (Burnett et al., 2005).



## CHAPTER THREE

### **3. Methods and materials**

#### **3.1. Ethical approval**

The study was approved by the University of Stellenbosch's Health Research Ethics Committee (ethics clearance number N09/11/319) (Addendum A). Permission to use the patient serum was granted by the Western Cape Province Department of Health and South African National Department of Health (Addendum B).

#### **3.2. Study population and samples**

The samples tested in this study were drawn from a cohort of 9354 pregnant women from the Western Cape who were part of the 2008 South African National HIV and Syphilis Annual Antenatal Sentinel Survey. Their residual serum samples, following HIV and Syphilis testing were stored at -20°C in the Division of Virology at Tygerberg Hospital. From the 9354 pregnant women, all HIV-infected women were identified and their samples were selected.

Each sample from an HIV-infected subject was then matched to a single HIV negative sample according to race and age. This resulted in a total of 3099 women being selected for the study.

#### **3.3. Serology tests**

Serology tests were performed to detect markers that are associated with HBV diagnosis and prognosis. Serology tests were performed for HBsAg, HBeAg, anti-HBe and anti-HBc (total) and anti-HD (total).

##### **3.3.1. HBsAg testing**

All suitable (sufficient volume and non-haemolysed) samples from the cohort of 3099 pregnant women were tested for HBsAg using the Abbott AxSYM (Abbott Diagnostics, Chicago, IL) according to the manufacturer's protocol. Four samples from the HIV-uninfected group and six from the HIV-infected group could not be tested because they were either haemolysed or had insufficient volume to be tested. As a result, 1546 HIV positive and 1543 HIV positive samples were tested on the AxSYM.

The AxSYM is an automated immunoassay analyzer that uses the technology of micro particle enzyme immunoassays (MEIA) for qualitative detection of HBsAg in a patient's serum or plasma (Abbott AxSYM HBsAg (v2) kit insert). The principle of the AxSYM HBsAg (v2) assay is that there is direct binding of the HBsAg in the sample to anti-HBs coated micro particles and indirect detection of the HBsAg by biotinylated anti-HBs followed by anti-biotin-alkaline phosphatase conjugate (Abbott AxSYM HBsAg (v2) package insert). Substrate solution is then added and the fluorescent product formed is measured by the MEIA optical assembly. The reactions are carried out on a matrix cell instead of the conventional microwell plate. A negative or positive result for HBsAg is determined by comparing the rate of formation of fluorescent product from a patient's sample to the cut off rate of the index calibrator (Abbott AxSYM HBsAg (v2) package insert). To preserve volume for further tests, samples were diluted in 50% volume/volume (v/v) phosphate buffered saline: fetal bovine serum (PBS: FBS). All positive samples were re-tested on the Abbott AxSYM. This was done to rule out any false-positive results before neutralization was done. Quality of results from the AxSYM was ensured by testing calibrators and controls each day when the samples were tested.

Dilution of samples was validated by testing two replicates of serial dilutions of an HBsAg standard from the National Institute of Biological Standards and Controls (NIBSC, Hertfordshire, UK) using the AxSYM and Murex HBsAg assays. The HBsAg standard was procured at a concentration of 33 IU/ml and was serially diluted in 50% PBS:FBS. The dilutions were tested on the AxSYM and the minimum detectable limit was determined to be 0.04 IU/ml of HBsAg. In addition, known HBsAg positive samples were also diluted and tested.

### **3.3.2. HBsAg confirmatory testing**

All samples that were repeatedly reactive for HBsAg on the AxSYM were confirmed using an in-house HBsAg neutralization assay. Sixty-one HIV positive and fifty HIV negative samples were subjected to the neutralization assay. The neutralization assay was performed using the Abbott Murex HBsAg Version 3 immunoassay kit (Murex Biotech, Kent, England) and anti-HBs at a concentration of 10 IU/ml. Anti-HBs was provided by the Health Protection Agency (HPA) at Colindale, London. A sample volume of 50 µl was pipetted into each of two microcentrifuge tubes. To one tube, 50 µl of negative human plasma was added to the sample while 50 µl of anti-HBs positive plasma was added to the second microcentrifuge tube. The

tubes were then mixed by vortexing and left to incubate at 4°C overnight. HBsAg testing was performed after 16 hours using the Murex HBsAg kit according to the manufacturer's instructions.

The principle of the HBV neutralization assay is that the anti-HBs antibodies added to the patient plasma/serum bind to and reduce the amount of HBsAg that will then bind to the anti-HBs that is coated onto the microwell plates. In contrast, the negative human plasma does not contain any anti-HBs and therefore a greater amount of HBsAg should bind to the anti-HBs coated on the microwell plates during testing. The difference in the amount of surface antigen binding onto the microwell plates is measured spectrophotometrically in the immunoassay. If the sample is truly reactive for HBsAg, a decrease in colour intensity of at least 50% should be seen in the tube that was incubated with anti-HBs.

The neutralisation assay is validated and is in use at the Health Protection Agency (Colindale, UK). Before being used in this study, the assay was verified for local conditions by performing an initial neutralization assay using an in-house positive control. Serial dilutions of the control were made using negative human plasma and these were then subjected to the neutralization test. The validation exercise showed that a sample with an HBsAg concentration of 0.04 IU/ml could be neutralized using the in-house neutralisation assay.

### **3.3.2.1. Confirmatory HBsAg testing using the Abbott Murex HBsAg version 3 immunoassay kit**

Each well is pre-coated with mouse monoclonal antibody to HBsAg that capture any HBsAg in a sample/control (Abbott Murex version 3 kit insert). A volume of 25 µl of sample diluent was added to each well of the microplate, followed by addition of 75 µl of the patient's sample that had been incubated with either negative human plasma or anti-HBs as mentioned before. The plate was covered with a lid and left to incubate for 60 minutes at 37°C.

After the one hour incubation, 50 µl of conjugate was added to each well. The conjugate is composed of horseradish-peroxidase labelled goat antibody to HBsAg. The sides of the microplate were tapped gently for 10 seconds to release any air bubbles from the wells. The plates were covered with a lid again and incubated for 30 minutes at 37°C. At the end of the incubation time, the plate was washed five times on a Bio-Rad PW40 microplate washer (Bio-Rad, Hercules, CA) using a wash fluid volume of 500 µl in each well. The wash step serves to remove excess or unbound HBsAg and conjugate from the well.

After washing was completed, the plate was inverted and tapped onto absorbent paper to remove any residual wash fluid. Substrate solution of 100 µl was immediately added to each well, the plate was covered with a lid and incubated for 30 minutes at 37°C to allow for colour development. The substrate solution contains hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine (TMB). The TMB turns a purple colour when oxidised by the breakdown of hydrogen peroxide horseradish-peroxidase and antibody conjugate in the positive samples. Stop solution made of 50 µl of 1M sulphuric acid was then added to each well. Colour intensity for each well was measured on a microplate reader at 450 nm using 650 nm as the reference wavelength on the dual wavelength Anthos HT3 Microtiter Plate Reader (Anthos Labtec Instruments GmbH, Salzburg, Austria).

The HBsAg reactivity in each well was then measured by comparing its absorbance to the cut-off value. The cut-off value for each run was calculated using the following formula that is provided by the kit manufacturer (Abbott Murex version 3 kit insert): Cut-off value = Mean of the Negative Control replicates + 0.05.

### **3.3.3. HBeAg and anti-HBe testing**

Samples that were confirmed positive for HBsAg by the neutralization assay were tested for HBeAg and anti-HBe. Samples with insufficient volume after the neutralization assay were not tested for HBeAg and anti-HBe. Testing for HBeAg and anti-HBe was performed using the DiaSorin ETI-EBK PLUS and ETI-AB-EBK PLUS immunoassay kits (DiaSorin S.pA, Salugia, Italy) respectively.

#### **3.3.3.1. HBeAg Testing**

The DiaSorin ETI-EBK PLUS assay is a direct, non-competitive assay and is based on the use of polystyrene microwells coated with mouse monoclonal antibodies to HBeAg (DiaSorin ETI-EBK PLUS kit insert). In the test, 50 µl of patient specimen/controls/calibrator was incubated with 50 µl of incubation buffer in antibody-coated microwells. The plates were sealed using a cardboard cover and left to incubate for two hours in a 37°C incubator. If HBeAg is present in a specimen or control, it binds to the anti-HBe antibody coated on the microwell. Excess sample was removed by a wash step on a Bio-Rad PW40 microplate washer, composed of five wash cycles using 400 µl of wash buffer.

A volume of 100 µl of enzyme tracer was then added to the microwells and allowed to incubate for one hour in a thermostatically-controlled 37°C incubator. The enzyme tracer contains antibodies to HBeAg conjugated to horseradish peroxidase and binds to any antigen-antibody complexes present in the microwells. Excess enzyme tracer was removed by a wash step as previously described above. A 100 µl volume of tetramethylbenzidine/hydrogen peroxide (chromogen/substrate) solution was added to the microwells and allowed to incubate for 30 minutes at ambient temperature in the dark. Wells containing HBeAg bind to the antibody-enzyme conjugate whose enzyme then reduces the hydrogen peroxide, which then oxidizes the chromogen to a blue colour (DiaSorin ETI-EBK PLUS kit insert). The blue colour of oxidised TMB was converted to a more stable yellow by adding 100 µl of 0.4N aqueous sulphuric acid stop solution into all wells maintaining the order and rate in which chromogen/substrate had been added. The wells of samples without HBeAg remained colourless after addition of both the hydrogen peroxide/TMB solution and aqueous sulphuric acid (stop solution).

Colour intensity of each well was measured spectrophotometrically using the Anthos HT3 Microtiter Plate Reader at 450 nm, using 650 nm as the reference wavelength, within 15 minutes of the addition of stop solution. The intensity of the yellow colour indicates carriage of e antigen in the patient's sample (DiaSorin ETI-EBK PLUS kit insert). Optical density values for study samples were compared to a cut-off value derived from the average optical density of the calibrator. The cut-off value was calculated by adding 0.060 to the average absorbance for the calibrator values after subtraction of the substrate blank absorbance value (DiaSorin ETI-EBK PLUS kit insert).

### **3.3.3.2. Anti-HBe Testing**

The Diasorin ETI-AB-EBK PLUS assay is a competitive test based on the use of polystyrene microwells coated with mouse monoclonal antibodies to HBeAg (Diasorin ETI-AB-EBK PLUS kit insert). In the procedure, 50 µl of incubation buffer was added into all wells except for the blank well. Calibrator, negative and positive controls and samples at volume of 50 µl were pipetted into their respective wells followed by addition of 50 µl of neutralizing solution into all wells except for the blank well. The neutralization solution has, among other components, recombinant HBeAg (produced in transfected *Escherichia coli* bacteria) that provides the basis for the competitive assay (Diasorin ETI-AB-EBK PLUS kit insert). A cardboard sealer was then used to cover the plate in order to prevent evaporation followed by

gentle tapping of the reaction wells to release any air bubbles trapped in the liquid. The plates were incubated for two hours in a 37°C thermostatically-controlled incubator. Excess sample was removed by a wash step on the Bio-Rad PW40 microplate washer, composed of five wash cycles using 400 µl of wash buffer for each well.

Following the wash step, 100 µl of working enzyme tracer solution was added into all wells except for the blank well. The enzyme tracer solution is a conjugate of antibody to HBeAg (mouse monoclonal) and horseradish peroxidase (HRP) and attaches to unbound HBeAg that is coated on the microwell plate (Diasorin ETI-AB-EBK PLUS kit insert). A cardboard sealer was applied onto the plate to prevent evaporation followed by gentle tapping of the reaction wells to release any air bubbles trapped in the liquid. The plate was incubated for one hour at 37°C in a thermostatically-controlled incubator. After lapsing of the incubation period, the plates were washed again as previously described.

Thereafter, 100 µl of tetramethylbenzidine/hydrogen peroxide chromogen/substrate solution was pipetted into all wells followed by incubation of the plate for 30 minutes at room temperature, away from direct or intense light. This was followed by addition of 100 µl stop solution into all wells. The absorbance of each well was measured using an Anthos HT3 Microtiter Plate Reader at 450 nm, using 650 nm as the reference wavelength within 15 minutes of adding the stop solution. The presence or absence of anti-HBe antibodies in each sample was determined by comparing the absorbance value of unknown samples to that of the cut-off value (Diasorin ETI-AB-EBK PLUS kit insert). The cut-off value was calculated using the manufacturer's provided formula which is determined by multiplying the mean absorbance of the calibrator values by 0.500 after subtraction of the substrate blank absorbance value (Diasorin ETI-AB-EBK PLUS kit insert).

#### **3.3.4. Anti-HBc (total) testing**

Following testing for HBsAg, a random sample of 161 HIV-positive and 154 HIV-negative samples were chosen from those that had tested HBsAg-negative on the AxSYM in order to test for total antibodies to HBV core antigen. This was done to determine the prevalence of exposure to HBV in HIV-infected and HIV-uninfected pregnant women. Testing for anti-HBc was performed using the Murex Anti-HBc (total) immunoassay kit (Murex Biotech, Kent, England), according to the instructions outlined by the manufacturer.

The Murex Anti-HBc (total) test is a competitive enzyme immunoassay for the detection of total antibodies against HBc antigen (Murex Anti-HBc (total) kit insert). The microwells of the kit are coated with recombinant HBcAg. Serum or plasma from patients and controls are incubated in the microwells and any anti-HBc present in the sample or control binds to the HBcAg immobilized on the well surface. Excess antibodies in the sample are removed by a wash step using wash buffer on a microplate washer. A second incubation is carried out during which the conjugate binds to any HBcAg on the well surface not blocked by anti-HBc from the test sample. The conjugate is composed of monoclonal anti-HBc combined with horseradish peroxidase. After washing to remove any unbound conjugate, a substrate solution containing 3,3',5,5'-TMB and hydrogen peroxide is added to the wells. Wells which do not contain anti-HBc and therefore bind to conjugate will develop a blue/green colour which is converted to orange when the enzyme reaction is stopped with the addition of sulphuric acid (Murex Anti-HBc (total) kit insert). The intensity of colour is determined spectrophotometrically. The optical density is greatest in the absence of anti-HBc and falls with increasing concentrations of anti-HBc in the sample.

The microwell plate and the reagents used were allowed to equilibrate to room temperature for 30 minutes. Sample diluent of 50  $\mu$ l was added to each well for every sample tested. This was followed by addition of 50  $\mu$ l of patient sample or control to the corresponding well containing sample diluent. The microplate was then covered with a lid and incubated for 30 minutes in a 37°C incubator. After the incubation period, the microplate was washed using 500  $\mu$ l of wash fluid for five cycles using a Bio-Rad microplate washer. Thereafter, 50  $\mu$ l of conjugate was added to each well and the plate was allowed to incubate for 30 minutes at 37°C in an incubator. The wash step was again repeated, followed by addition of 100  $\mu$ l of substrate solution to each well. The plates were covered using a lid and incubated again 37°C in an incubator for 30 minutes. Stop solution of 50  $\mu$ l of 1M sulphuric acid was then added and the colour intensity of each well was measured using an Anthos HT3 microwell plate reader at 450 nm using 690 nm as the reference wavelength. The result for each sample was determined by comparing its optical density to the cut-off value. The value of the cut-off was calculated according to the formula provided by the manufacturer: the mean absorbance of the positive control was added to the mean absorbance of the negative control and the sum was divided by two (Murex Anti-HBc (total) kit insert).



### 3.3.5. Anti-HDV testing

Samples were tested for HDV using the ETI-AB-DELTAK-2 (DiaSorin S.p.A, Salugia, Italy), which is an ELISA kit for qualitative determination of total antibodies to hepatitis Delta antigen (total anti-HD) in serum or plasma samples. Similar to the test for antibodies to HBeAg, the ETI-AB-DELTAK-2 test kit is a competitive assay based on the use of polystyrene microwells coated with biotinylated anti-HD IgG (human) and recombinant HDAg (ETI-AB-DELTAK-2 kit insert). Manufacturer's protocol and recommendations were followed during testing for total antibodies to HDV.

Briefly, 50 µl of sample/controls was added to the appropriate well. This was followed by addition of 100 µl of enzyme tracer into all wells except the blank well. The wells were sealed using cardboard seals supplied within the kit. The enzyme tracer contains amongst other components, human anti-HD Fab fragments conjugated to HRP which compete against anti-HD contained in the sample/control for binding to the HDV antigen adsorbed onto the surface of the microwell plate (ETI-AB-DELTAK-2 kit insert). The plate was then incubated for three hours at 37°C. Thereafter, the plate was washed on a Bio-Rad microplate washer for five cycles using a wash fluid volume of 400 µl and allowing a soak period of 30 seconds between each cycle. The plate was blotted onto paper towel after the final wash cycle to remove excess fluid. A 100 µl volume of chromogen/substrate solution was then added to the plate which was then sealed again and left to incubate at room temperature (25°C) in the dark for 30 minutes. The substrate/chromogen solution contains hydrogen peroxide and a tetramethylbenzidine derivative which is oxidised to a blue colour.

After the 30 minutes incubation, 100 µl of blocking reagent was then added to the plates. The blocking reagent contains 1N sulphuric acid that stops further colour changes and changes the colour of the reaction from a blue colour to a stable yellow. The optical densities were then read at 450/630 nm using an Anthos HT3 automated plate reader. Cut-off value was calculated by using the formula-

Cut-off value =  $0.5 \text{ NC}\bar{x} + 0.5 \text{ PC}\bar{x}$  , where  $\text{NC}\bar{x}$  is the mean absorbance for the negative control and  $\text{PC}\bar{x}$  is the mean absorbance for the positive control (ETI-AB-DELTAK-2 kit insert).

Samples with optical densities  $\leq$  cut-off value were considered positive for anti-HD while samples with optical density values  $\geq$  cut-off value were considered negative for anti-HD.



### **3.3.6. Quality control for serology assays**

Quality of results was ensured during testing by strictly following the manufacturers' protocols and instructions. Cut-off values were calculated and interpretation made following each individual kit protocol. Manufacturer kit controls and calibrators were used in every run. In addition, well characterized positive and negative in-house controls were incorporated in each run. On occasions where a result was in the "grey zone" such a sample was repeated in duplicate to get a definitive result.

## **3.4. Molecular tests**

The molecular tests that were used included real-time PCR for quantitative detection of HBV DNA and nested PCR followed by direct sequencing using Sanger's chain termination method to determine HBV genotype as well as to detect the presence of drug-resistance or vaccine-escape mutations. Quantitative detection of HBV DNA was performed according to the protocol developed by Garson et al. with minor modifications (Garson et al, 2005). Genotyping and sequence analysis was done according to the protocols designed and in use at the Blood-Borne Virus Unit at the HPA Colindale (London, UK).

### **3.4.1. HBV viral load testing**

#### **3.4.1.1. DNA Extraction from serum**

HBV DNA was extracted, according to the manufacturer's protocol, from the serum samples using the QIAamp MinElute Virus Spin kit (QIAGEN, Hilden, Germany), a method that purifies nucleic acid and removes inhibitors of PCR amplification.

Before extraction, five microlitres of mCMV internal control at a concentration of 400 copies/ $\mu$ l was added to each 200  $\mu$ l of lysis buffer (Buffer AL). A 200  $\mu$ l aliquot of serum was added to 25  $\mu$ l QIAGEN protease solution in a sterile 1.5 ml microcentrifuge tube. Two-hundred  $\mu$ l of the mCMV and buffer AL mixture was then added to the tube and mixed by pulse-vortexing for 15 seconds. The sample was then incubated at 56°C for 15 minutes in order to release a maximum yield of DNA from the degraded virions. The tube was then briefly centrifuged to remove liquid droplets from the cap. A volume of 250  $\mu$ l of 99.99% ethanol (Sigma-Aldrich, St Louis, MO) was then added to the tube and the mixture was mixed by pulse-vortexing. The mixture was incubated for five minutes at room temperature.

The lysate was applied to a QIAamp MinElute column in a 2 ml collection tube and centrifuged at 8 000 rpm for one minute. This enabled the adsorption of the DNA onto the silica gel membrane of the spin column while allowing the rest of the liquid to pass through the column. The tube containing the filtrate was discarded and replaced with a clean 2 ml collection tube, and the QIAamp MinElute column was then washed with 500 µl Buffer AW1 and centrifuged at 8 000 rpm for one minute. The wash step was repeated using 500 µl Buffer AW2 and centrifugation at 8 000 rpm for one minute in order to remove any residual contaminants. Again, the collection tube with the flow-through was discarded and replaced with a new collection tube and the column was subsequently washed using 500 µl of 99.99% ethanol. Since residual ethanol in the eluate may inhibit downstream applications, the spin column was centrifuged again at full speed (14 000 rpm) for three minutes in a clean 2 ml collection tube. To ensure complete removal of any residual ethanol, the columns were placed onto clean collection tubes and incubated on a dry heat block at 56°C for three minutes to evaporate the ethanol completely. The MinElute columns were then transferred to sterile, labelled 1.5 ml microcentrifuge tubes followed by application of 60 µl elution buffer (buffer AVE). The columns were allowed to incubate at room temperature for five minutes followed by centrifugation at 14 000 rpm for one minute to elute the DNA from the columns. The eluted HBV DNA was stored at -20°C. Known HBV positive samples and normal human plasma that was negative for HBV markers were used as positive and negative controls for the extraction procedure.

#### **3.4.1.2. Quantitative detection of HBV DNA using quantitative PCR**

Quantitative detection of HBV DNA was performed using real-time PCR on the Rotor Gene 6000 real-time PCR machine (Corbett Sciences, Australia) according to the protocol developed by Garson et al. (Garson et al., 2005). Serial dilutions of a HBV standard ( $1 \times 10^8$  IU/ml) were made in HBV DNA negative human plasma to give a range from  $1 \times 10^2$  –  $1 \times 10^7$  IU/ml. The HBV standard was calibrated against the WHO HBV DNA standard (NIBSC, UK) with a viral load of  $1 \times 10^6$  IU/ml before use. Table 3.1 shows the primer and probe sequences that were used to amplify a highly conserved segment of the surface antigen gene as well the primer and probe sequences that were employed to amplify and detect the mCMV internal control. All primers and TaqMan probes were used at a concentration of 100 µM supplied by Applied Biosystems. A reaction volume of 25 µl was used. The PCR master mix reagents and their volumes are shown in the Table 3.2.

**Table 3.1 Oligonucleotide primers used for quantitative detection of HBV DNA**

<b>Primer/Probe</b>	<b>Sequence</b>
HBV Primer 1	5'- GTGTCTGCGGCGTTTTATCA-3'
HBV Primer 2	5'- GACAAACGGGCAACATACCTT-3'
HBV Probe	5'FAM-CCTCTTCATCCTGCTGCTATGCCTCATC-TAMRA
mCMV Primer 1	5'-AACCCGGCAAGATTTCTAACG-3'
mCMV Primer 2	5'-ATTCTGTGGGTCTGCGACTCA -3'
mCMV Probe	5'-VIC-CTA GTC ATC GAC GGT GCA CAT CGG C-3'TAMRA

The 2X QuantiTect QPCR mastermix was sourced from QIAGEN (QIAGEN, GmbH, Hilden, Germany). Amplification and detection was carried out under the following cycling conditions: 1 cycle of 95°C for 15 minutes and 45 cycles of 95°C for 15 seconds and 60° for 60 seconds. After completion of each run, results were analyzed using the Rotor-Gene 6000 Series Software 1.7 (Corbett Life Sciences, Australia). For quality control of each run, negative and positive working controls were used. The positive control was supplied by NIBSC (NIBSC, UK). Nuclease-free water (QIAGEN, Hilden, Germany) was also used as a non-template control.

**Table 3.2 Reagent components of the quantitative HBV PCR reaction mix**

<b>Reagent</b>	<b>Concentration (pmol/μl)</b>	<b>Volume/sample (μl)</b>
HBV primer 1	100	0.1
HBV primer 2	100	0.1
HBV probe	100	0.05
mCMV primer 1	100	0.1
mCMV primer 2	100	0.1
mCMV probe	100	0.05
2X QuantiTect QPCR mastermix	N/A	12.5
Nuclease-free water	N/A	2
Template	Variable	5

### 3.4.1.3. Determination of Limit of Quantification

The lower limit of quantification was determined by performing serial dilutions of the NIBSC HBV DNA standard down to a level of 10 IU/ml. Five replicates of each serial dilution were then tested for HBV DNA in a single assay. The assay was performed twice to effectively give 10 replicates of each standard dilution. The lowest concentration of HBV DNA that could be detected in all 10 replicates was then considered as the limit of quantification.

### 3.4.2. Direct Nucleotide Sequencing of HBV Surface Antigen Region

All samples that had detectable HBV DNA during quantitative detection of HBV DNA were subjected to direct sequencing. The remnant extract from the quantitative detection assay was used. Pre-nested and nested PCR were performed using the primer sequences shown in the Table 3.3.

**Table 3.3 Oligonucleotide primers used for pre-nested PCR amplification of the HBV polymerase gene**

<b>Primer</b>	<b>Sequence</b>	<b>Nucleotide position on HBV genome</b>
HBV Z	5' AGCCCTCAGGCTCAGGGCATA 3'	1179 - 1199
HBV 3	5' CGTTGCCCKDGCAACSGGGTAAAGG 3'	2478 - 2455
HBV M	5' GACACA CTTTCCAATCAATNGG 3'	2306 - 2287
HBV P	5' TCATCCTCAGGCCATGCAGT 3'	1292 - 1311

Nucleotide positions of primers are numbered according to Pugh et al. (1986).

#### 3.4.2.1. First round PCR

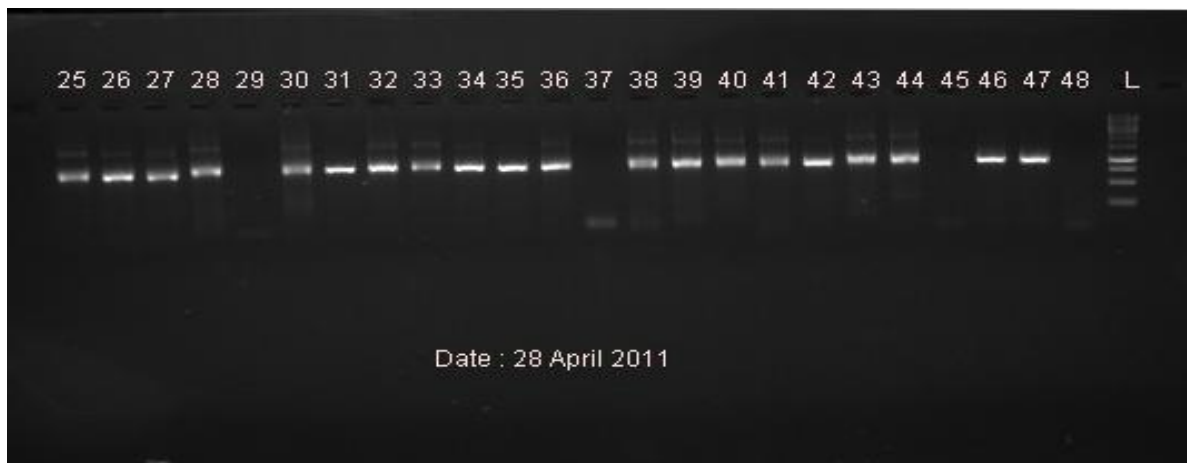
The PCR reaction master mix for each single pre-nested reaction consisted of: 0.5 µl of 10 mM deoxynucleotide triphosphate (dNTP) mix (Bioline, London), 0.5 µl of the primer HBV 3 at a concentration of 20 pmol/µl, 0.5 µl of primer HBV Z at concentration 20 pmol/µl, 2.5 µl of 10XPCR buffer (Invitrogen, California), 0.75 µl of 50 mM magnesium chloride (MgCl<sub>2</sub>) (Invitrogen, California), 0.1 µl *Thermus aquaticus* (Taq) Polymerase (Invitrogen, California), 5 µl of DNA template and 15.15 µl of nuclease-free water to make a total volume 25 µl. The following cycling conditions were used for pre-nested amplification: 95°C for 5 minutes, 34 cycles of 94°C for 30 seconds, 55°C for 30 seconds 72°C for 60 seconds followed by a final extension cycle at 72°C for two minutes.

### 3.4.2.2. Second round PCR

The PCR reaction master mix for each single nested reaction was composed of: 1  $\mu$ l of 10 mM dNTP mix (Bioline, London), 1  $\mu$ l of primer HBV P at a concentration of 20 pmol/ $\mu$ l, 1  $\mu$ l of primer HBV M at concentration 20 pmol/ $\mu$ l, 5  $\mu$ l of 10XPCR buffer (Invitrogen, California), 1.5  $\mu$ l of 50 mM MgCl<sub>2</sub> (Invitrogen, California), 0.2  $\mu$ l Taq Polymerase (Invitrogen, California), 1  $\mu$ l of DNA template and 39.3  $\mu$ l of nuclease-free water to make a total volume 50  $\mu$ l. The cycling conditions for the second round PCR were as follows: denaturation at 95°C for 5 minutes, 34 cycles of 94°C for 30 Seconds, 50°C for 30 Seconds, 72°C for 60 seconds followed by a final extension at 72°C for 2 minutes.

### 3.4.2.3. Nested PCR Product Visualization

Following the second round PCR, product formation was visualized by gel electrophoresis. A 2% gel was prepared by adding two grams of Promega Agarose to 100 ml of 1X tris-acetate-ethylene diamine tetra acetic acid (TAE) buffer. 1X TAE buffer was prepared from a 50X stock solution that was prepared by mixing 57.1 ml of glacial acetic acid (Merck Chemicals, Germany), 242 g Tris base (Boehringer Mannheim, USA), 100 ml of 0.05M ethylene diamine tetra acetic acid (EDTA) and distilled water. The suspension was heated in a microwave oven on high power for five minutes to completely dissolve the agarose. To the gel, 10  $\mu$ l of 5 mg/ml of ethidium bromide was then added and mixed by swirling. The gel was cooled under running water before being poured into the electrophoretic tank and allowed to set for at least an hour at room temperature. Samples were wet-loaded using 6X Orange DNA loading dye (Fermentas, Lithuania). O'GeneRuler 1 kilobase (kb) DNA ladder (Fermentas, Lithuania) was used as the molecular marker. Electrophoresis was then performed using a voltage of 90V for 25 minutes. The gels were then viewed on the UVITEC Prochemi (Cambridge, UK) image acquisition system. PCR products were seen by exposing the gel to ultraviolet light at 245 nm and collecting the data using the UVIPROCHEMI image acquisition system. Afterwards, images were enhanced and edited using UVIBAND software (Cambridge, UK). A 1 kb fragment was expected on samples positive for PCR and an example of the pictures obtained is shown in Figure 3.1



**Figure 3.1** Example of an electrophoresis migration pattern for positive samples on nested PCR of the HBV polymerase gene. The picture shows the expected 1kb bands that were seen in samples that could be amplified.

#### 3.4.2.4. PCR product clean-up

Samples on which the expected band could be seen from the electrophoresis gel were then cleaned up using the Illustra GFX PCR DNA & Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK). The kit allows for purification and concentration of DNA from PCR mixtures using four essential steps of sample capture, sample binding, washing and elution. Sample capture is achieved by use of a sample capture buffer type 3 that denatures proteins from the PCR mixture. Binding of the DNA occurs when the sample is applied onto the Illustra GFX MicroSpin columns that have a silica membrane. Washing then follows using wash buffer type 1 which permits removal of salts and other contaminants from the membrane bound DNA. PCR primers also pass with the filtrate at this step and are not retained. This is followed by elution using the elution buffers provided within the kit or nuclease-free water.

To purify and concentrate the amplified HBV DNA, GFX columns were placed onto labeled collection tubes for each sample. Five hundred  $\mu$ l of capture buffer type 3 was added directly to each column. An aliquot of 30  $\mu$ l of PCR product was added to each column in its corresponding labeled tube followed by pipetting up and down four times. The tubes were then centrifuged for one minute at 13000 rpm. The filtrate was discarded and the GFX MicroSpin column was placed back inside the same collection tube. A 500  $\mu$ l volume of wash buffer type 1 was pipetted to all tubes and these were centrifuged at 13000 rpm for one minute. The filtrate and collection tubes were discarded. Columns were placed onto new labeled microcentrifuge tubes. To release the purified DNA, 40  $\mu$ l of nuclease free water was added to each column which was then left to incubate for one minute at room temperature. The tubes were then centrifuged at 13000 rpm for one minute to elute the sample DNA. The

GFX MicroSpin columns were then discarded after centrifugation while the eluate was retained in the microcentrifuge tube.

#### **3.4.2.5. Measurement of Purified PCR product**

To ensure that HBV DNA had not been lost during purification, its presence in the purified product was determined spectrophotometrically using the NanoDrop 1000 spectrophotometer (ThermoFisher Scientific, USA). It utilises a patented sample retention technology that uses surface tension alone to hold the sample in place thereby eliminating the need for cuvettes and other sample containment devices and allows for rapid clean up. A 1 µl volume of the purified product was loaded onto the instrument and the concentration of DNA in each sample was determined, wiping with paper towel to clean the pedestals between samples. The results from the machine were printed out.

#### **3.4.2.6. Nucleotide Sequencing of HBV DNA from purified DNA**

All purified samples were sequenced for the surface antigen gene using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA). The kit performs fluorescence-based cycle sequencing reactions on single-stranded or double-stranded DNA templates and on PCR fragments. The technology of the BigDye Terminator v3.1 Cycle Sequencing Kit is based on Sanger dideoxynucleotide chain termination DNA sequencing method, which uses PCR to incorporate fluorescent dye-labelled dideoxynucleotides into a population of oligonucleotides synthesised from a single strand of the target DNA. The kit contains all necessary reagents for the sequencing reaction in a reaction ready-to-use format and all that is needed is to provide the template and the template-specific primers. The primers used to sequence the polymerase genes and their sequences are shown in Table 3.4.

**Table 3.4 Primers used for sequencing of the polymerase region**

<b>Primer</b>	<b>Sequence</b>	<b>Nucleotide positions on HBV genome</b>
HBV P	5' TCA TCC TCA GGC CAT GCA GT	1292 - 1311
HBV M	5' GAC ACA CTT TCC AAT CAA TNG G	2306 - 2287
HBV H	5' TAT CAA GGA ATT CTG CCC GTT TGT CCT	1767 - 1793
HBV N	5' ACTGAGCCAGGAGAAACGGACTGAGGC	1991 - 1965

For a sample to be sequenced, it had to contain a DNA concentration >10 ng/μl on the NanoDrop 1000. For samples that had a DNA concentration greater than 30 ng/μl, dilutions were made in an appropriate volume of nuclease-free water were performed to obtain a concentration between 15–20 ng/μl before sequencing. The sequencing PCR master mix for each reaction was made as shown in Table 3.5.

**Table 3.5 PCR Master Mix for HBV Sequencing Reaction**

<b>Reagents</b>	<b>Concentration</b>	<b>Quantity</b>
Terminator ready reaction mix	-	1 μl
ABI sequencing buffer	-	3 μl
Template	Between 15-25ng	1 μl
Primer	2.0 pmol/ul	1 μl
Water	-	4 μl
Total volume	-	10 μl

Four reactions were set up according to the Table 3.5 for each sample corresponding to each primer that was used. Nine microlitres of the sequencing PCR master mix was pipetted onto well on a MicroAmp PCR plate. The DNA template was then pipetted onto each predetermined well on the MicroAmp PCR plate before amplification on the ABI 9700 thermocycler (Applied Biosystems, California). The following cycling conditions were used



for the sequencing PCR: 30 cycles of 96°C for 20 seconds, 50°C for 20 seconds and 60°C for four minutes, followed by an infinite hold at 4°C. The hold allowed for the MicroAmp PCR plate to be left on the machine overnight in instances where the next step could not be started immediately.

#### **3.4.2.7. Cleaning of sequencing PCR reactions**

Cleaning up of the sequencing PCR reactions was performed before the sequences were read on the genetic analyzer. This was done in order to remove unincorporated dye terminators prior to injection of samples on the genetic analyzer. The BigDye XTerminator® Purification Kit (Applied Biosystems, California) was used to clean up the sequencing reactions. The kit is based on a DNA sequencing reaction purification method that removes unused BigDye® terminators and salts that would affect injection of sample and base calling if not removed.

The BigDye® XTerminator purification kit consists of SAM solution and XTerminator solutions. To purify a single reaction well, the cleaning up solution was made by combining 49.5 µl of SAM Solution with 11 µl of Xterminator suspension. The mixture was then vigorously vortexed for 10 seconds. A volume of 55 µl of the premixed Xterminator reagent was added to each reaction on the MicroAmp Optical 96-well reaction plate. The MicroAmp Optical plate was sealed using a MicroAmp Optical sheet (Applied Biosystems, California) and placed on a Multi-microplate Genie microplate shaker (Scientific Industries, New York) where it was vortexed for 30 minutes at 2000 rpm. Afterwards, the MicroAmp plate was centrifuged for two minutes at 1000 x g to remove any droplets from the optical cover. The sealing optical sheet was removed and a septa mat was placed on the plate. The plate was then placed for sequence analysis on the ABI3130xl Genetic Analyzer (Applied Biosystems, California).

#### **3.4.3. Quality control for molecular assays**

Caution was observed to avoid contamination which leads to false positive results in PCR tests. Gloves were worn when performing all procedures and not transferred between the five dedicated rooms of the laboratory where different steps were performed. The rooms were dedicated for extraction, reagent preparation (clean area), gel electrophoresis and combined amplification and detection. Dedicated pipettes and filtered tips were also used and these were not moved between sections. Nuclease free water was used as a non-template control in all

assays. A working control from NIBSC was used for real-time PCR assays as mentioned previously. Working spaces were kept clean by thorough cleaning using 10% Virkon (Antec International, Suffolk, UK) followed by 70% ethanol.

### **3.5. Sequencing data and phylogenetic analysis**

Sequence analysis and assembly was performed using Lasergene software, version 8 (DNASTAR Inc, WI, USA). Lasergene is a suite of sequence handling software and databases. The suite contains different programmes that allow for sequence editing and map creation, contiguous assembly and multiple and pair wise alignments amongst other functions. Files were exported from the ABI3130xl Genetic Analyzer and imported into Lasergene software, v8. Consensus sequences were assembled from the four primer sequences and edited using SeqMan. The consensus sequences were exported from SeqMan. Genotypes and sub genotypes were obtained using online genotyping databases of Stanford University (<http://hivdb.stanford.edu/HBV/HBVseq/development/HBVseq.html>), International Public Health Repository for Hepatitis B ([www.hepseq.org/Public/Tool/annotator\\_tool.php](http://www.hepseq.org/Public/Tool/annotator_tool.php)) and the Max Planck Institute (<http://www.geno2pheno.org>).

For detection of amino acid mutations in the polymerase and surface antigen regions, sequences were aligned with wild type sequences using ClustalV. The presence of an amino acid that is not known to be a natural polymorphism for that position was recorded as a mutation. The frequency of mutations on a particular codon was recorded. Phylogenetic trees were constructed using MegAlign.

### **3.6. Statistical analysis**

Data was analyzed using Statistica software, version 10 (StatSoft, Oklahoma, USA). Fisher's exact test was used to analyze categorical (prevalence) data where there were small sample numbers. The Pearson chi-square test was used when sample numbers were sufficiently large. HBV DNA load values were  $\log_{10}$  transformed. The Mann-Whitney test was used to compare median HBV viral loads between the HIV-negative and the HIV-positive groups. Medians were chosen to compare the viral load data as the values did not follow a normal distribution. Descriptive analyses are presented using median and 95% confidence intervals. Statistical significance was defined as two-tailed  $p < 0.05$ .

## CHAPTER FOUR

### 4. Results

#### 4.1. Demographic data

A total of 3089 samples were selected for the study. The demographic characteristics of the women from whom the samples were drawn are shown in Table 4.1.

**Table 4.1 Demographic data of patients sampled**

Characteristic	HIV-uninfected	HIV-infected
<b>Number of women selected</b>	1546	1543
<b>Age</b>		
Median	26 years	26 years
Interquartile range	23 - 31	23 - 31
Range	12 - 44	12 - 44
<b>Gravidity</b>		
Median	2	2
Interquartile range	1 - 3	1 - 3
Range	0-10	0-10
<b>Parity</b>		
Median	1	1
Interquartile range	0 - 2	0 - 2
Range	0-7	0-7
<b>Race</b>		
African	1297 (83.9%)	1323 (85.7%)
Mixed-race	203 (13.2%)	179 (11.6%)
Unknown	46 (3.0%)	41 (2.7%)
<b>Education: ≤ Grade 10</b>	*747/1517 (49.2%)	*901/1531 (58.9%)

\* denotes that some samples are not included in the analysis due to the data not being recorded in the original database.

## 4.2. Serology results

Of the 3089 samples that were screened for HBsAg, the following results were obtained. Of the 1546 HIV-uninfected mothers, 50 samples were initially repeatedly reactive for HBsAg on the AxSYM giving an HBsAg prevalence of 3.2%. A neutralization test performed on the HIV-uninfected samples that had tested repeatedly positive for HBsAg confirmed true surface antigen carriage in only 44/50 samples resulting in an effective prevalence of 2.85%.

Sixty out of a total of 1543 HIV-infected women tested positive on the initial HBsAg screen test performed on the Abbott AxSYM resulting in a prevalence of 3.9%. Of the 60 initially reactive samples, 53 had a positive neutralization test giving a final HBsAg prevalence of 3.4%. These results are shown Table 4.2.

**Table 4.2 HBsAg testing results**

<b>Serology Marker</b>	<b>HIV-uninfected</b>	<b>HIV-infected</b>	<b>p-value</b>
Initial screen test on AxSYM	50/1546 (3.2%)	60/1543 (3.9%)	
HBsAg Neutralization	44/50 (88%)	53/60 (88.3%)	
Final HBsAg rate	44/1546 (2.8%)	53/1543 (3.4%)	0.35

Among the 203 HIV-uninfected women of mixed race, 5 (2.46%) had confirmed HBsAg carriage. Of the 179 HIV-infected women of mixed race, 5 (2.79%) had a positive HBsAg result confirmed by neutralization.

Of the 1297 HIV-uninfected African women, 37 (2.85%) were confirmed positive by neutralization for HBsAg carriage while 48 of 1323 (3.63%) of HIV-infected African women were also positive for HBsAg.

All the samples that were positive for HBsAg with enough remnant sample volume were tested for HBeAg and anti-HBe. Four of the forty-four confirmed HBsAg positive samples from the HIV-uninfected group could not be tested for HBeAg and anti-HBe due to an insufficient sample volume. The serology results following HBeAg and anti-HBe testing are shown in Table 4.3.

**Table 4.3 Results of HBeAg and anti-HBe testing of confirmed HBsAg positive samples**

<b>HBV marker</b>	<b>HIV-uninfected</b>	<b>HIV-infected</b>	<b>p-value</b>
<b>HBeAg (%)</b>	7/40 (17.5)	10/53 (18.9)	<i>1.00</i>
<b>Anti-HBe (%)</b>	33/40 (82.5)	43/53 (81.1)	<i>1.00</i>

Two of the HIV negative samples had optical density values that were just above the cut-off value while all ten HBeAg positive, HIV positive samples had optical densities that were beyond the upper limit of the plate reader on the HBeAg assay. One HIV negative sample was positive for both HBeAg and anti-HBe. However, the optical densities for both tests were low compared to all other samples. Also, one HIV negative sample was negative for both HBeAg and anti-HBe.

From the HBsAg negative samples, 155 HIV negative and 161 HIV positive samples were randomly selected for total anti-HBc testing. One of the HIV negative samples had an insufficient sample volume for testing and is excluded from the results analysis. One of the HIV positive samples had insufficient volume for testing while one other HIV positive sample had an equivocal result and is excluded from the results analysis. The results obtained are depicted in tabular form in Table 4.4.

**Table 4.4 Total anti-HBc testing on HBsAg negative samples.**

<b>Marker</b>	<b>HIV-uninfected</b>	<b>HIV-infected</b>	<b>p value</b>
Number of samples tested for anti-HBc	153	159	
Samples positive for anti-HBc, (%)	42 (27.5)	68 (42.8)	<i>0.006</i>

Total anti-HD was tested in 39 HBsAg positive, HIV negative samples. All the 39 samples were negative for anti-HD. Five of the HBsAg positive samples in the HIV-uninfected group were not tested for anti-HD because of an insufficient sample volume.

Of 53 HBsAg positive and HIV positive samples, only 50 were tested for total anti-HD. The remaining three samples could not be tested due to an insufficient sample volume. All the 50 samples that were tested for anti-HD were negative.

### 4.3. HBV DNA detection and quantification

The lower limit of quantification for the assay on the Rotor Gene 6000 was determined to be 20 IU/ml through repeat testing of standard serial dilutions. HBV DNA detection and quantification was attempted on all samples with confirmed HBsAg carriage as well as on samples that had tested positive for anti-HBc (total). A total of 44 HIV negative samples tested positive for HBsAg. Among the HBsAg-positive HIV-negative, 3/44 samples had insufficient volume for HBV DNA detection and quantification. As a result, only 41 of the HBsAg positive, HIV negative samples could be tested for HBV DNA using quantitative PCR (qPCR). All 53 HIV positive, HBsAg positive samples had enough volume for HBV DNA quantification.

Of the HBsAg positive, HIV negative samples 32/41 had detectable DNA while the remaining 9/41 had undetectable HBV DNA. Of the 32 samples with detectable DNA, 6 samples had HBV DNA below 20 IU/ml while 26 samples had levels  $\geq 20$  IU/ml. Of the HIV positive samples, 44/53 had detectable HBV DNA. Of the 44, there were 2 samples that had HBV DNA less than 20 IU/ml and the remaining 42 samples had HBV viral loads  $\geq 20$  IU/ml. The results obtained from the qPCR assays are shown in Table 4.5.

Of all the HIV-positive, HBsAg positive samples, 17/53 (32.1%) had HBV viral loads  $> 10^4$  IU/ml while 6/42 (13.4%) of all HIV-negative, HBsAg positive samples had viral loads  $> 10^4$  IU/ml (Odds ratio = 2.83; 95% confidence interval: 1.00 -8.01;  $\chi^2$ : P = 0.04).

HBV DNA was detected in all HBeAg positive samples from both groups. Of seven HIV-uninfected women with positive HBeAg serology, five samples contained HBV DNA load greater than  $4 \log_{10}$  IU/ml with two of these having greater than  $8 \log_{10}$  IU/ml. Of the 10 HIV-infected women with positive HBeAg serology, 90% had HBV DNA greater than  $4 \log_{10}$  IU/ml with 50% of these having greater than  $8 \log_{10}$  IU/ml.

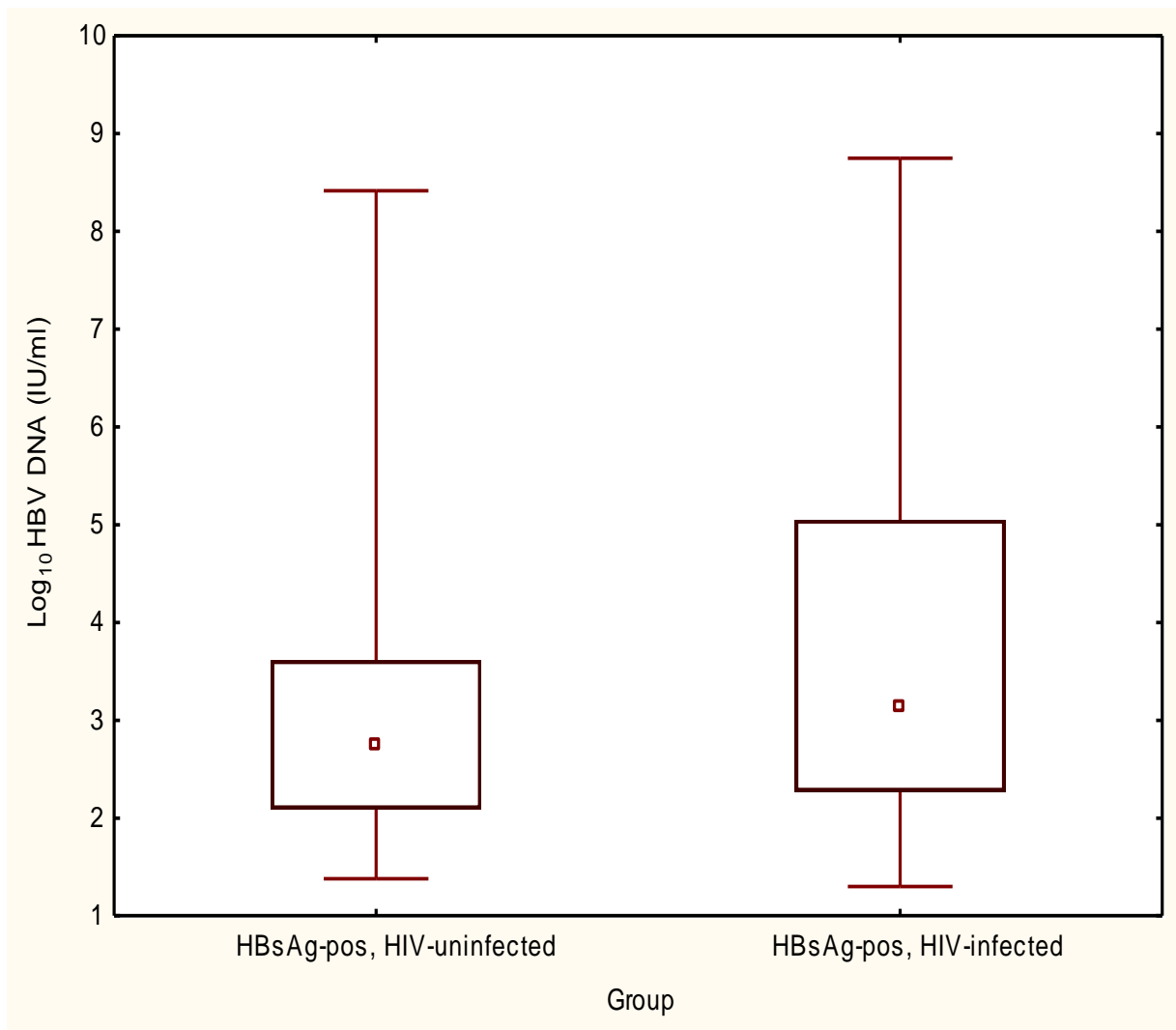
Twenty-six of thirty-three (78.8%) of the samples from HIV-uninfected women with a positive anti-HBe result had detectable DNA. Only one sample had HBV DNA greater than  $10^4$  IU/ml with no sample having more than  $10^8$  IU/ml.

Thirty-four of forty-three (79.0%) of the HIV-infected women with positive anti-HBe had detectable HBV DNA. Eight of the thirty-four samples (23.5%) contained HBV DNA  $\geq 10^4$  IU/ml. However, none of the eight samples had HBV DNA levels greater than  $10^8$  IU/ml although three samples had  $\geq 10^5$  IU/ml with one of these three having  $\geq 10^6$  IU/ml of HBV DNA.

**Table 4.5 HBV DNA detection and quantification in HBsAg positive samples**

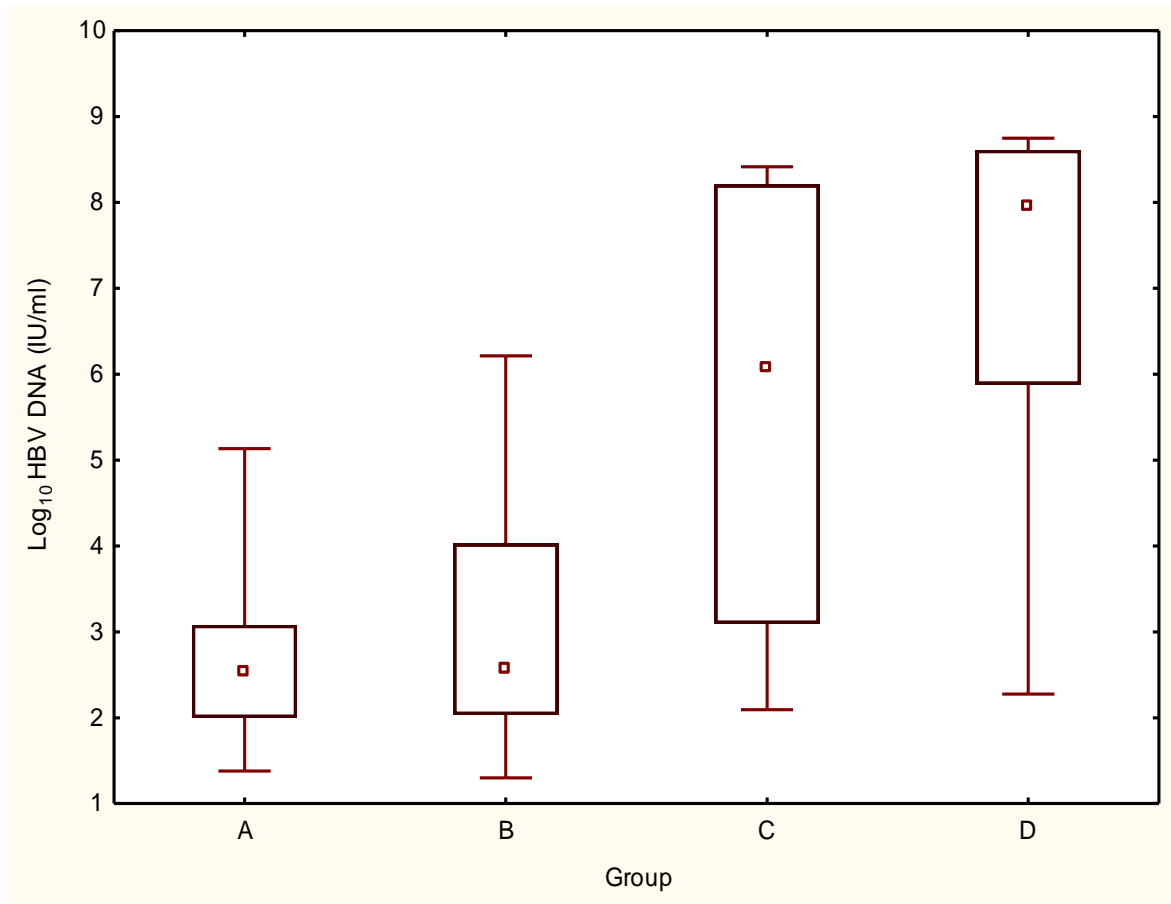
<b>Parameter</b>	<b>HIV-uninfected</b>	<b>HIV-infected</b>	<b>p value</b>
<i>HBsAg positive samples with detectable HBV DNA</i>	32/41 (78%)	44/53 (83%)	0.60
<i>Log<sub>10</sub> HBV DNA of all HBsAg positive</i>			
Median	2.73	3.11	0.32
Interquartile range	2.08 - 3.85	2.27 - 5.05	
<i>Log<sub>10</sub> HBV DNA HBeAg positive only</i>			
Median	6.07	7.93	0.23
Interquartile range	3.10 - 8.21	5.44 - 8.62	
<i>Log<sub>10</sub> HBV DNA anti-HBe positive only</i>			
Median	2.54	2.56	
Interquartile range	2.00 - 3.10	2.00 - 4.20	0.47

The results of the log<sub>10</sub> HBV DNA of all HBsAg positive samples according to HIV status are depicted in Figure 4.1 while the log<sub>10</sub> HBV DNA viral load values for HBeAg positive samples only and anti-HBe positive samples for the two groups classified according to HIV status are shown in a combined graph in Figure 4.2.



**Figure 4.1 Log<sub>10</sub> HBV viral loads according to HIV status.** The graph shows the medians (centre small box), 25%-75% percentiles (outer box) and the minimum and maximum values (whiskers).





**Figure 4.2 Log<sub>10</sub> HBV Viral loads for HBeAg and anti-HBe positive samples.** The graph shows the medians (centre small box), 25%-75% percentiles (outer box) and the minimum and maximum values (whiskers). A: Anti-HBe positive, HIV-uninfected, B: Anti-HBe positive, HIV-infected, C: HBeAg positive, HIV-uninfected, D: HBeAg positive, HIV-infected

#### 4.4. Occult HBV testing

The serologic results of HBsAg negative samples that were randomly selected for anti-HBc testing are shown in Table 4.7. The same table also has the results that were obtained on testing for HBV DNA using quantitative PCR. HBV DNA detection and quantification was performed only on samples that tested positive for anti-HBc. Seven of the forty-two HIV negative, anti-HBc positive samples had HBV DNA greater than 20 IU/ml while two had less than the quantification limit. Only five of the sixty-eight HIV positive, anti-HBc positive samples had HBV DNA greater than 20 IU/ml. The five remaining HIV positive samples with detectable DNA had HBV viral loads that were less than 20 IU/ml which was the quantification limit as stated previously. Statistical analyses are shown in table 4.7.

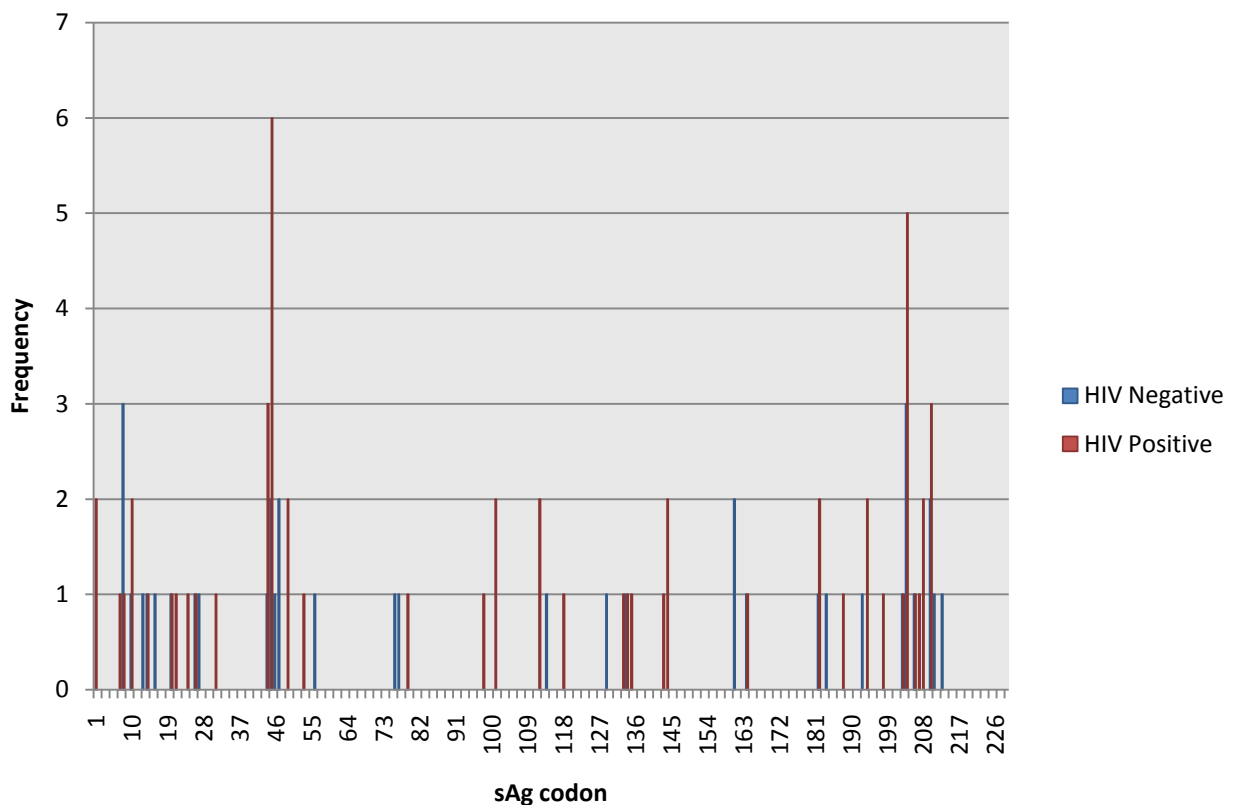
**Table 4.7 Serology and HBV DNA for occult infections**

<b>Parameter</b>	<b>HIV-uninfected</b>	<b>HIV-infected</b>
Samples positive for total anti-HBc (%)	42/153 (27.5)	68/159 (42.8)
Number of samples with detectable HBV DNA among anti-HBc positive samples	8/42 (19.0%)	10/68 (14.7%)
HBV DNA of anti-HBc positive (IU/ml) Median (IQR)	51(40-84)	59 (37-151)

#### 4.5. Sequencing results

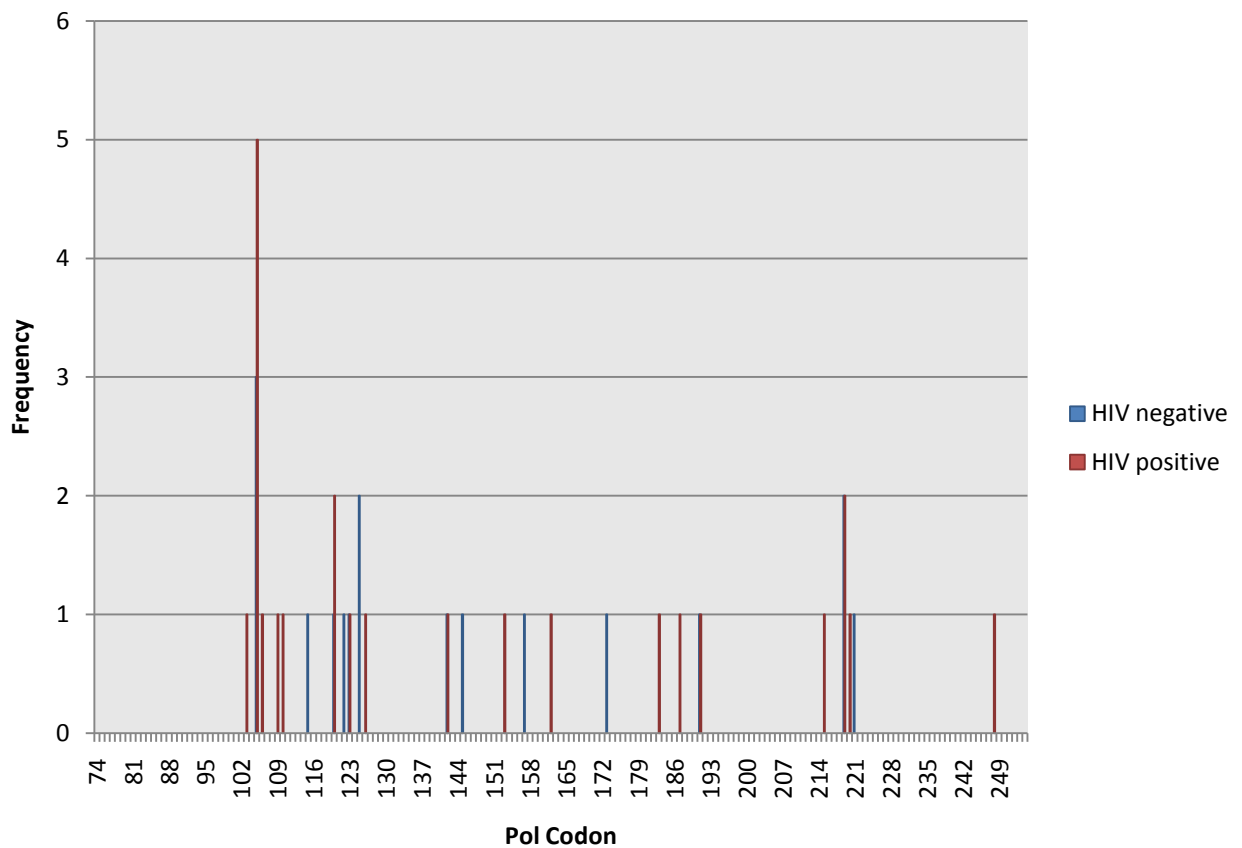
All samples with detectable DNA were subjected to a nested PCR on the polymerase gene region that also acts as the surface antigen coding region in a frame shifted manner. A nested PCR was performed on 54 HIV positive samples and 41 HIV negative samples. Of the 54 HIV positive samples, a band was seen on 43 and these were subsequently sequenced while 25/41 HIV negative samples had the 1 kb band on gel electrophoresis after the nested PCR. Forty-two of the HIV positive samples carried HBV genotype A1 while only one woman carried HBV genotype D, while twenty-one patients in the HIV-uninfected group had genotype A1 and the remaining four participants were infected with genotype D.

No drug-resistance associated or vaccine-escape mutations were detected in the HIV-infected group. The V173L mutation that is associated with resistance to LAM was detected in one patient among the HIV-uninfected group. The patient carried HBV genotype D. No vaccine-escape mutations were detected in the HIV-uninfected patients. The amino acid sequences obtained were aligned with reference wild-type sequences to determine presence of other mutations that are as yet undefined on the surface antigen and polymerase regions. There was no particular clustering of mutations within the analyzed regions although some codons had frequencies that were higher than other positions. Most of the sequences obtained contained the S207N polymorphism which is characteristic of African HBV isolates. The frequency of the mutations that were obtained on different codons of the surface antigen is shown in Figure 4.3. The frequency of mutations observed within codons 74 to 254 of the polymerase region are depicted in Figure 4.4. The highest number of mutations was observed on codons 105 and 209 of the polymerase region in both HIV-uninfected and HIV-infected women.



**Figure 4.3** Frequency and location of amino acid substitutions within HBsAg, comparing HIV-infected and HIV-uninfected women.

The results shown in figure 4.3 illustrate that no classic vaccine escape mutations such as the G145R were detected in this cohort. However, there were some mutations found within the  $\alpha$  determinant (amino acid positions 124-147) of the major hydrophilic region (amino acid positions 99-169) to which vaccine-elicited antibodies are directed (Wu et al., 2010). Also there were no particular codons where mutations are restricted although there were more mutations within the HIV-infected group occurring at codons 45 and 204 of the surface antigen region.



**Figure 4.4** Frequency and location of mutations within the different codons of the polymerase region according to HIV status.

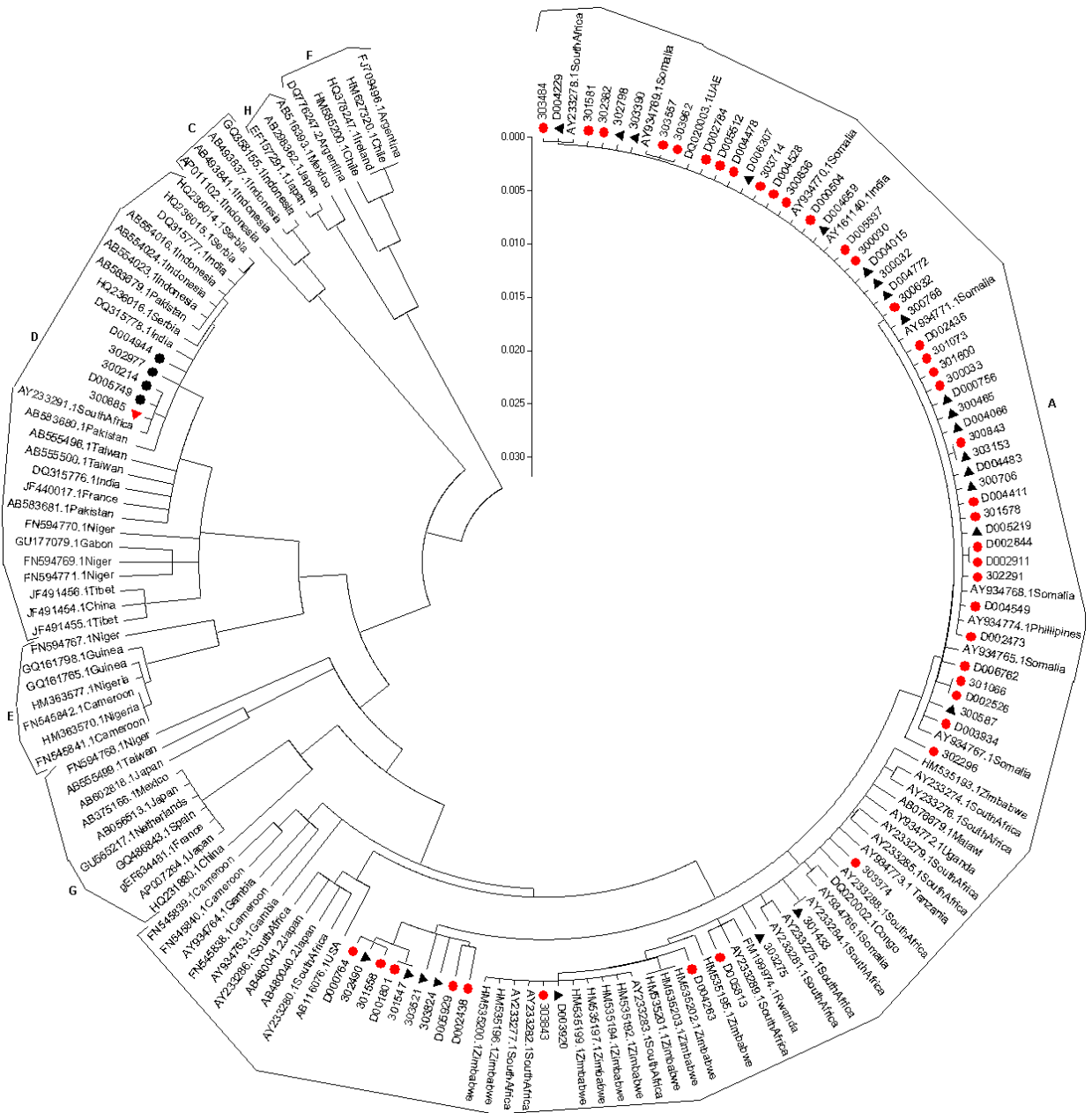
#### 4.6. Characteristics of HBV genotypes

Table 4.6 Characteristics of the HBV genotypes in isolated sequences

Characteristic	Genotype A	Genotype D
HBeAg positivity, (%)	14/63, (22)	3/5, (60)
<i>HBV DNA</i> , $\log_{10}$ Median, (IQR)	2.8, (2.2-4.5)	5.9, (2.6-6.1)

#### 4.7. Phylogenetic analysis

The sequences were aligned with other reference sequences from other geographic regions including South Africa. The sequences clustered well with other African-derived sequences. There was close clustering with other previously characterized South African sequences. The phylogenetic tree obtained from alignment of the sequences from this study with other HBV sequences from Africa and elsewhere is shown in Figure 4.5.



**Figure 4.5 Phylogenetic analyses of HBV genomes.** 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 Maximum Composite Likelihood method (Tamura, Nei, and Kumar, 2004). Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). Sequences shown with a red triangle (genotype D) or red circle (genotype A) are from HIV-infected women from this study. Sequences shown with a black triangle (genotype A) or black circle (genotype D) are from HIV-uninfected women.

## CHAPTER FIVE

### 5. Discussion

#### 5.1. Serology results

##### 5.1.1. HBsAg prevalence rate

An overall HBsAg carriage prevalence of 3.2% was obtained for the cohort (HIV-uninfected and HIV-infected women) in this study. When grouped according to HIV status, the prevalence of HBsAg was 2.8% in HIV-uninfected and 3.4% in HIV-infected women. There was no statistically significant difference in HBsAg carriage according to HIV status in this study although there was a slightly higher prevalence in the HIV-infected than in the HIV-uninfected group.

The overall prevalence obtained in this study is in agreement with results of previous studies conducted on pregnant women in South Africa. The prevalence rate of HBsAg in pregnant South African women, without regard to HIV status, has previously been estimated to range from 1.2% to 8.3% (Burnett et al., 2007; Guidozi et al., 1993). Tsebe et al. also reported HBsAg prevalence of 3.2% in mothers of vaccinees (Tsebe et al., 2001). The HBsAg prevalence values of this study are also similar to more recent figures reported in Zimbabwe where the overall HBV prevalence rates were between 3.3% and 3.7% (Mavenyengwa et al., 2010). However, the overall prevalence reported in this study is lower than has been previously found in other countries within SADC. An earlier study by Madzime et al. in Zimbabwe reported an HBsAg prevalence of 25% (Madzime et al., 1999) while Ahmed et al. reported 13% in Malawi (Ahmed et al., 1998). The reason for the difference in prevalence may be due to the fact that there may be pockets of higher prevalence areas within South Africa and other African countries (Sinha and Kumar, 2010; Kew, 1996). Another reason for the difference of results between this study and others may be due to use of different methods and testing strategy. In this study, positive HBsAg results were confirmed by neutralization.

The similarity in prevalence of HBV among HIV-uninfected and HIV-infected women is consistent with other studies on African women where no statistically significant differences in HBsAg carriage have been observed when results are grouped according to HIV status (Rouet et al., 2004; Oshitani et al., 1996). The prevalence of HBsAg in HIV-uninfected and HIV-infected pregnant women was previously reported to be between 4.8-6.2% and 5.8-9.5% respectively in earlier studies that were previously conducted in other provinces of South Africa (Burnett et al., 2007; Klugman et al., 1991). In a study on Zambian pregnant women,

Oshitani *et al.* observed HBsAg prevalence of 7.1% and 5.4% in HIV-infected and HIV-uninfected women, respectively (Oshitani *et al.*, 1996). In Malawi, the prevalence of HBsAg was 12% in HIV-uninfected women and 16% in HIV-infected women (Ahmed *et al.*, 1998). The studies by Oshitani *et al.*, 1996 and Burnett *et al.*, 2007 were done on both rural and urban women and this may have contributed to the higher rates they reported. A recent South African study conducted on rural HIV-infected women from the Eastern Cape reported an HBsAg prevalence of 5.5% (Boyles and Cohen, 2011). However, it is not clear whether the prevalence of HBV differs between rural and urban areas. Earlier studies revealed significant differences in the HBsAg prevalence between rural and urban children (Abdool Karim *et al.*, 1988).

Most studies on African populations do not seem to find different prevalence rates of HBsAg between HIV-infected and HIV-uninfected people and this has been attributed to the fact that most of the people who go on to contract HIV may have already been exposed to HBV in childhood because of the horizontal route of transmission that is prevalent in sub-Saharan Africa (Puoti *et al.*, 2008; Burnett *et al.*, 2005). This is in contrast to the developed countries where carriage of HBsAg carriage in HIV-infected cohorts may reach up to 10 times than is observed in rest of the population who are HIV-uninfected (Puoti *et al.*, 2008; Kellerman *et al.*, 2003). This is mainly attributed to the fact that transmission of HBV in the West peaks with onset of sexual activity and there HIV and HBV are contracted almost simultaneously (Puoti *et al.*, 2008).

### **5.1.2. HBeAg and anti-HBe prevalence**

The prevalence rate of HBeAg carriage in HBsAg positive pregnant women in this study was found to be 17.5% and 18.9% among HIV-uninfected and HIV-infected women, respectively. Prevalence rates that have been previously reported in sub-Saharan Africa vary widely and lie between 2-24% (Sinha and Kumar, 2010). HBeAg carriage in South Africa has previously been reported to be at 4.6% among HBsAg-positive pregnant women (Guidozzi *et al.*, 1993). The study by Guidozzi *et al.* also reported a much lower prevalence of HBsAg compared to other South African studies. A limited number of studies have been conducted in South Africa to investigate the prevalence of HBeAg among HBsAg-positive pregnant women. However, in Zambia the prevalence of HBeAg was found to be 12.3% in HIV-uninfected women and 25% in HIV-infected women who were positive for HBsAg (Oshitani *et al.*, 1996). The HBeAg prevalence obtained in this study is however higher than the value of 3.3% that was



reported in neighboring Zimbabwe (Madzime et al., 1999). The only other study to report a high prevalence of HBeAg among HBsAg-positive patients in South Africa was performed on samples drawn from HIV-infected patients who were about to start therapy where 9/19 patients were found to be HBeAg-positive (Firnhaber et al., 2008). It is apparent that the patients studied by Firnhaber et al. were immunocompromised due as evidenced by CD4 counts <200 cells/ $\mu$ l.

The HBeAg carriage rates reported from studies conducted on Southern African populations are similar to what has been reported in other parts of Africa. In Ivory Coast, the HBeAg carriage rates in patients positive for HBsAg have been reported as 22.2% among HIV-infected women compared to 9.4% in HIV-uninfected pregnant mothers (Rouet et al., 2004). There is slightly higher but non-significant higher carriage of HBeAg in HBsAg positive HIV-infected pregnant women than in their HIV-uninfected counterparts. This may be attributed to the immunosuppression caused by co-infection with HIV (Oshitani et al., 1996). In this study, two of the seven women in the HIV-uninfected group who were positive for HBeAg had very low optical densities that suggested a rather weak positive result. One of the samples with a low HBeAg optical density was also positive for anti-HBe. The woman from whom the sample was drawn could potentially have been in the process of seroconverting from HBeAg to anti-HBe and this could have caused a double positive result because viral antigen and antibody titers are both low (Wang et al., 2011). The same explanation may also hold true for the one patient who had a double negative result (Wang et al., 2011).

### **5.1.3. Total anti-HBc prevalence**

Total anti-HBc is a marker for previous exposure to HBV. The prevalence of anti-HBc in HIV-infected pregnant women was 42.8% which was statistically significantly higher than the 27.0% observed in the HIV-uninfected group ( $p < 0.05$ ). These results suggest that a greater proportion of HIV-infected women are exposed to HBV compared to HIV-uninfected women. These findings are similar to what has been reported in other studies conducted in sub-Saharan Africa. In South Africa, anti-HBc positivity in HBsAg-negative HIV-infected patients has previously been reported at 61.7% compared to 27.3% in HIV-uninfected women (Mphahlele et al., 2006). Another study conducted by Burnett et al. in South Africa reported anti-HBc carriage of 37.3% in HIV-infected pregnant women and 23.6% in the HIV-uninfected group (Burnett et al., 2007). Barth et al. also reported an anti-HBc prevalence of 28.1% among ART-naïve HIV-infected patients who were negative for HBsAg (Barth et al., 2011).

It is difficult to comment on the clinical implications of anti-HBc results without information on the anti-HBs status of the women. Testing for anti-HBs would have provided more information on the HBV immune status of the women in the study. Anti-HBs are detected when an individual has recovered from HBV infection or when a person has successfully responded to HBV vaccine (Ganem and Prince, 2004). Presence of anti-HBs would also have provided better understanding on whether the women who tested positive for anti-HBc were true positives. Anti-HBs would also explain the great number of samples with no detectable HBV DNA since only 14.7% samples among the HIV-infected group and 21.4% in the HIV-uninfected group had detectable DNA in their plasma. Alternatively, it would also have been verified if the women with positive anti-HBc results are part of the group of HIV-infected patients that have been widely described to have isolated anti-HBc positive serology (Mphahlele et al., 2006; Piroth et al., 2002; Owiredu et al., 2001; Grob et al., 2000;).

#### **5.1.4. Anti-HDV prevalence**

Infection with HDV only occurs in individuals who are carriers of HBV and whose serum has detectable HBsAg (Wedemeyer, 2010; Ojo et al., 1998). HDV leads to increased severity of liver disease in HBV carriers (Ojo et al., 1998). None of the samples tested positive for anti-HDV in this study. This is similar to findings of an early study in the pre-HIV era on South African patients in which no delta antigen or antibodies to delta antigen were detected (Kew et al., 1984). More recently, no HDV carriage was found among a cohort of HIV-infected patients who were HBsAg-positive and about to start therapy (Firnhaber et al., 2008). A limited number of studies have been conducted to investigate the prevalence of HDV on African populations. Total anti-HDV antibodies were detected in 17.6% of chronic HBV carriers in Cameroon (Foupouapouognigni et al., 2011), 6.8% of asymptomatic carriers in Tunisia had positive HDV serology (Djebbi et al., 2009) and 6.5% of patients with chronic active liver disease in Nigeria (Ojo et al., 1998). No HDV was found amongst blood donors and university students in the Nigerian study (Ojo et al., 1998).

Although no evidence of HDV was found in this study, it does not necessarily dismiss the presence of the delta antigen in South African patients. An investigation on patients with active chronic HBV liver disease may show different results. In Somalia, 50% of patients with histologically proven chronic liver disease who had HBsAg were found to have anti-HDV (Aceti et al., 1991). It also appears that HDV is more likely to be found in patients with chronic active HBV liver disease than in those that are healthy or asymptomatic.

## **5.2. Molecular results**

### **5.2.1. HBV DNA in HBsAg positive samples**

The results showed a trend towards higher replication in HIV/HBV co-infected pregnant women compared to HBV mono-infected women. However, the difference in median HBV viral loads in the two groups was not statistically significant. The more significant finding was the difference in the proportion of samples that contained HBV DNA greater than  $10^4$  IU/ml within the two groups. Among the HIV-positive, HBsAg positive samples, 32% had HBV viral loads  $>10^4$  IU/ml compared to only 14% among the HIV-negative, HBsAg positive samples ( $p < 0.05$ ). A cut-off value of  $10^4$  IU/ml was considered as it has been shown to carry a relative risk of vertical transmission of 2.41 compared with lower viral load (Candotti et al., 2007). These results clearly indicate that there is a higher proportion of HIV-infected pregnant women than HIV-uninfected pregnant women that have increased rates of HBV replication. Previous studies have shown that HIV/HBV co-infected patients have higher HBV replication rates than HBV mono-infected patients although none have investigated the viral loads in pregnant women particularly using a cut-off value that has a known risk of perinatal infection.

### **5.2.2. HBV DNA in HBeAg positive only**

A trend toward higher median HBV DNA load values in the HIV-infected group than in the HIV-uninfected group was observed. When considering only samples that were positive for HBeAg, the HIV-infected group had an HBV DNA load median value of 7.93  $\log_{10}$  compared to a median value of 6.07  $\log_{10}$  obtained in the HIV-uninfected women. The differences observed between the medians were not statistically significant ( $p > 0.05$ ). However, the trend is in agreement with literature that states that there is higher replication of HBV in HIV/HBV co-infected patients than in HBV mono-infected patients (Hoffman and Thio 2007). It may also be argued that since the comparison is on HBeAg positive samples, the difference may not be statistically significant because highly replicating virus was being compared across both groups. The small numbers that were involved may also explain the reason for the findings being not statistically significant. Nevertheless, there were 5/10 (50%) samples from HBeAg positive HIV-infected women compared to 2/7 (28.6%) samples from HBeAg positive HIV-uninfected women that had HBV DNA  $\geq 10^8$  IU/ml. HBeAg positivity together with HBV DNA greater than  $10^8$  copies/ml ( $\sim 10^7$  IU/ml) has been associated with perinatal transmission of HBV (Wiseman et al., 2009).

### **5.2.3. HBV DNA in anti-HBe positive only**

Thirty two samples from pregnant women infected only with HBV contained detectable anti-HBe with a median HBV DNA load of 2.54 log<sub>10</sub> IU/ml while forty three samples from women infected with HIV and HBV contained detectable anti-HBe with a median HBV DNA load of 2.56 log<sub>10</sub> IU/ml.

As was observed among the samples with HBeAg, the HBV DNA load values in the HIV-infected group were higher than in the HIV-uninfected pregnant women even though the difference did not reach statistical significance. More significant was the higher proportion of women infected with HIV who had HBV DNA values  $\geq 10^4$  IU/ml compared to the mono-infected group. The high HBV DNA levels observed in some of the HBeAg negative, anti-HBe positive samples suggest a need for vigilance to identify pregnant women who may potentially transmit HBV to their infants despite presence of anti-HBe which is associated with less infectivity.

Earlier studies from Italy and Taiwan showed that the presence of anti-HBe was protective against vertical transmission of HBV (Gussetti et al., 1983; Stevens et al., 1979). However, presence of anti-HBe alone was later disproved to be protective against HBV transmission (Candotti, Danso, and Allain, 2007; Kazim et al., 2002). The stance that anti-HBe was protective against transmission probably arose from the fact that the technology to measure HBV DNA was not yet available. It is now known that the level of viraemia is the most important risk factor in determining risk of vertical transmission rather than the mere presence of anti-HBe (Candotti et al., 2007). Anti-HBe may be found together with high HBV viral load values in the serum of patients who have mutations in the basal core promoter gene of HBV where there is no synthesis and secretion of HBeAg. In Ghana where genotype E is predominant, it was shown that an HBV DNA load of  $>10^4$  IU/ml was associated with a relative risk of vertical transmission of 2.41 compared with lower viral load (Candotti et al., 2007).

### **5.2.4. HBV DNA in anti-HBc positive samples and occult infections**

Among the HIV negative HBsAg negative samples, 41/153 tested positive for anti-HBc (total) and 19% had detectable HBV DNA. This is in contrast to 68/159 samples from HIV-infected, HBsAg negative women that tested positive for total anti-HBc of whom only 14.7% had detectable HBV DNA.

The rates of HBV viraemia amongst anti-HBc positive patients obtained in this study is different from what has been previously reported in South African patients. A previous study conducted on hospitalized patients at Dr. George Mukhari Hospital, revealed that 33.3% of HIV-infected patients with anti-HBc alone as the sole positive serology marker had HBV DNA in the absence of HBsAg while none of the HIV-uninfected patients with a similar serology pattern had detectable HBV DNA (Mphahlele et al., 2006). Also, another study performed at the same hospital revealed an occult HBV prevalence rate of 23% amongst HIV-infected patients (Lukhwareni et al., 2009). The rates of occult HBV infection in HIV-infected patients reported in these studies are much higher than was obtained in this study. However, the study populations that were considered at Dr George Mukhari Hospital are different from the cohort investigated in this study. The patients studied at Dr George Mukhari Hospital were either admitted in hospital or about to start antiretroviral therapy and may therefore have been more immunosuppressed than the HIV-infected women investigated in this study.

It is assumed that pregnant HIV-infected women are likely to be more immunocompetent than symptomatic HIV-infected individuals though unpublished data from Tygerberg Hospital suggests that their CD4 counts are just as low as some AIDS patients in the general population. The changes in epidemiology of HBV in the setting of HIV co-infection are more apparent as HIV infection progresses to AIDS.

#### **5.2.5. HBV genotyping**

There are currently eight genotypes widely described for HBV ranging from genotype A to H (Kramvis et al., 2005). Only genotype A1 and genotype D3 isolates were obtained from the samples that were analyzed in this study. Genotypes were determined for 25 samples from the HIV-uninfected pregnant women and for 43 women from the HIV/HBV co-infected group. Of the 25 samples in the HBV mono-infected group, 21 samples belonged to genotype A1 while the remaining four belonged to HBV genotype D3. Forty two samples among the HIV/HBV co-infected group belonged to HBV genotype A1 while only one sample belonged to HBV genotype D3. These findings are consistent with literature on the genotypes found within South Africa. Genotype A1 has been isolated and described as being dominant in South Africa (Kramvis and Kew, 2007a; Kramvis and Kew, 2007b; Kimbi et al., 2004). Genotype D has also been isolated from infected HBV carriers in South Africa previously (Kramvis and Kew, 2007b). The genotype D samples isolated in this study were of sub-genotype D3 consistent with the prior descriptions from South Africa.

Samples with genotype D had higher HBV DNA levels when compared to those with genotype A. The median HBV viral load for the genotype D isolates was 5.9 log<sub>10</sub> IU/ml compared to 2.8 log<sub>10</sub> IU/ml for samples with genotype A. The characteristics of the genotypes are in agreement with other published studies which showed that HBV carriers with genotype D have higher HBV viral loads when compared to patients with genotype A (Kramvis and Kew, 2005; Tanaka et al., 2004). Furthermore, 60% of samples belonging to genotype D were positive for HBeAg while 22% of the samples with genotype A1 isolates were HBeAg positive. This is also in agreement with previous studies that have shown that HBV carriers with genotype D are likely to be positive for HBeAg compared to those infected with genotype A1 (Tanaka et al., 2004). The observation that a higher proportion of genotype D samples were positive for HBeAg compared to genotype A samples also helps to explain the higher HBV DNA values observed.

The consensus sequences from this study were aligned with other sequences from South Africa deposited in GenBank and phylogenetic trees drawn from the alignment. There was phylogenetic clustering of the sequences from this study with other sequences that have been previously isolated from South African and other sub-Saharan African patients. This suggests that there may be a high degree of similarity in the surface antigen region of HBV genotypes A and D strains that are circulating within South Africa and sub-Saharan Africa at large. However, whole genome sequencing would allow validation of this hypothesis.

#### **5.2.6. Mutation analysis**

Amino acid alignments against wild type consensus genotype specific sequences were created for the HBsAg regions. These alignments were used to identify mutations within the antigen to which antibodies against the vaccine are directed. No vaccine-escape mutations were found in this study in either the HBV mono-infected group or the HIV/HBV co-infected group. This is encouraging data for the vaccination program as it suggests that wild type HBV strains are in circulation and thus the vaccine is likely to continue to be effective. The presence of vaccine-escape mutants would potentially render the current vaccine ineffective thereby exposing infants to infection despite immunisation.

However, some amino acid changes were identified within the major antigenic region between codons 120 and 150 of the surface antigen in both the HIV-uninfected and HIV-infected group. The frequency of these mutations was higher in the HIV-infected group than

in the HIV-uninfected group but it is difficult to ascertain whether this is due to HIV infection or because of the smaller number of HIV negative samples that could be analyzed.

One sample from an HIV-uninfected woman with genotype D carried the valine to leucine change at codon 173 which forms part of the antiviral resistance profile for LAM (Benhamou et al., 1999). It cannot be established if the woman had exposure to LAM or if this was a spontaneous mutation. LAM resistance mutations in therapy naïve HBV-infected patients have previously been reported in South African and were significantly higher in HIV/HBV co-infected individuals (Selabe et al., 2007).

### **5.3. Strengths and limitations of study**

The strengths of this cross-sectional study were that it had a simple study design, there was access to a large sample number and also that this was a collaborative research allowing use of techniques that have not been used in previous studies on pregnant women in Africa. The study also generates data on HBV viral loads in pregnant women which has not been reported before.

The limitations of the study are as follows. No antiretroviral therapy or previous treatment information was provided for HIV-infected patients. Since this data is not collected during antenatal surveys, it could be possible that some of the HIV-infected patients may have had exposure to antiretroviral therapy which also controls HBV replication. This may have potentially contributed to lowering HBV DNA levels in the HIV-infected patients. Some samples with low HBsAg levels may have been missed by dilution although this is unlikely because dilutions were made on positive samples which were diluted by a factor of up to 10 000 and HBsAg could still be detected. It therefore unlikely that true HBsAg positive samples will have been missed. Also, there were numerous freeze-thaws on samples which could lead to inaccurate results particularly in the serology tests, some of which are affected by the presence of fibrin. However, to reduce the effect of fibrin the samples were centrifuged prior to any testing. In addition, no data on immune status (CD4 counts) was available. Although data from different studies is conflicting on the effect of CD4 count on HBV disease progression, the study would have benefitted by knowing the immune status of the cohort that was investigated. Another limitation is that the study did not test for HBV DNA in all screened patients due to the cost. This may cause an underestimation of the actual prevalence of HBV within the population and it is possible that some samples that were positive for HBV DNA may have been missed due to the absence of screening serological

markers that were used. HBV DNA has been reported to occur in the absence of any serological markers (Lukhwareni et al., 2009; Hino et al., 2001).



## CHAPTER SIX

### 6. Conclusion

This study has shown that there is a trend towards higher HBV replication in HIV-infected than in HIV-uninfected pregnant women. There was no difference in the proportion of women infected with HBV in both the HIV-uninfected and HIV-infected women. Of all HBsAg positive women, 24% had HBV DNA load greater than  $10^4$  IU/ml and were at increased risk of transmitting HBV to their infants. More importantly, the study has shown that a higher proportion of HIV/HBV co-infected pregnant women have increased replication rates of HBV when compared to HBV mono-infected pregnant women. This study contributes to data that supports administering a birth dose of HB vaccine. Data from South Africa has shown that neonates born to HIV-infected mothers are significantly less likely to have protective levels of maternally-derived anti-HBs leaving them susceptible to horizontally transmitted infection (Jones et al., 2011). However, they do have robust immune responses to vaccine and it will therefore be safe to administer a birth dose of vaccine to these infants so that they can produce their own protective levels of anti-HBs. However, the efficacy of administering the birth dose to HIV-infected neonates will need to be evaluated because a separate study from South Africa reported that there was reduced detection and decreased levels of protective anti-HBs to HBV vaccine (which leaves them susceptible to horizontal infection) in HIV-infected compared to HIV-infected children under the age of 2 years who were recruited at a paediatric outpatient facility (Simani et al., 2009). Administration of HBV vaccine at birth may be feasible for children born at health facilities but maybe be costly to administer in some places such as rural areas where there is a significant number of home-births (Kramvis and Clements, 2010). There will be a need to devise ways in which the vaccine can be administered to neonates born away from the health facilities and this may prove to be expensive, particularly when funds are scarcely available (Kramvis and Clements, 2010).

The finding that 24% of all HBsAg positive women in this study had HBV viral loads greater than  $10^4$  IU/ml also suggests a need for closer observation of both HIV-infected and HIV-uninfected pregnant women who are HBV infected in order to provide the necessary care and intervention that will decrease their likelihood to vertically transmitting HBV to their infants. Necessary interventions may only be possible when there is a policy to screen pregnant women for HBV within the public health system under which most of these women will be

served. Currently, the HBsAg positive women in this cohort are unlikely to have been tested for HBV and would be at risk of transmitting the infection to their children.

The results also point to the fact that there may be low levels of perinatal transmission currently occurring in the Western Cape when considering the number of women who had high HBV DNA levels and were HBeAg positive. Provision of high titre hepatitis B immunoglobulin would cater for children who will be born to infected mothers with high HBV DNA levels but the costs are prohibitive for developing countries like South Africa (Robson and Kirsch, 1991). Instead, LAM or TDF administration in the third trimester to women who are infected with HBV and at risk of transmitting the infection to their children presents a more feasible option and has been shown to be safe to use in pregnancy (Bzowej, 2011; Kose et al., 2011; van Zonneveld et al., 2003). Currently, HIV-infected women with CD4 <350 cells/ml are receiving TDF and LAM as part of first line therapy. However, LAM or TDF are not widely available for HBV mono-infected patients in the public health sector under which most people are served.

Further studies are needed to evaluate the extent of perinatal transmission especially considering the number of women that were found to contain HBeAg in this study.

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# Addendum A

## Ethical Approval – Health Ethics Research Committee



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23 June 2010

**MAILED**

Dr M Andersson  
Department of Medical Virology  
8th Floor, Clinical building  
Stellenbosch University  
Tygerberg campus  
7505

Dear Dr Andersson

"Cross sectional analysis of the prevalence and character of chronic hepatitis B infection in HIV infected and HIV uninfected women."

**ETHICS REFERENCE NO: N09/11/319**

**RE: APPROVED**

It is a pleasure to inform you that a review panel of the Health Research Ethics Committee has approved the above-mentioned project on 22 June 2010, including the ethical aspects involved, 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. You may start with the project. Notwithstanding this approval, the Committee can request that work on this project be halted temporarily in anticipation of more information that they might deem necessary.

Please note a template of the progress report is obtainable on [www.sun.ac.za/ids](http://www.sun.ac.za/ids) and 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 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@ogwc.gov.za](mailto:healthres@ogwc.gov.za) Tel: +27 21 483 3907) and Dr Hélène Visser at City Health ([Helene.Visser@capetown.gov.za](mailto: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.

23 June 2010 10:26

Page 1 of 2



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Approval Date: 22 June 2010

Expiry Date: 22 June 2011

Yours faithfully

**MRS. MERTRUDE DAVIDS**  
RESEARCH DEVELOPMENT AND SUPPORT  
Tel: 021 938 9207 / E-mail: [mertrude@sun.ac.za](mailto:mertrude@sun.ac.za)  
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23 June 2010 10:28

Page 2 of 2



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## Addendum B

### Ethical approval – Department of Health



DIRECTOR GENERAL  
HEALTH  
REPUBLIC OF SOUTH AFRICA

PRETORIA  
Private Bag X928, PRETORIA, 0001, 12th Floor, House of Trade and Industry 236 (HTI) Building, Conyns and P. Heffer Street, PRETORIA, 0002 Tel (012) 512 9196 Fax (012) 525 0093  
CAPE TOWN  
P.O. Box 3875, CAPE TOWN, 8000, Room 404, 120 Plain Street, CAPE TOWN, 8000 Tel (021) 461 2140 Fax (021) 461 6864

Dr M Andersson  
Department of Medical Virology  
8<sup>th</sup> Floor, Clinical Building  
Stellenbosch University  
Tygerburg campus  
7505

Dear Dr Andersson

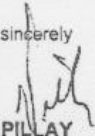
**Request for use of blood specimen from the Antenatal Sentinel HIV and Syphilis Survey for Cross Sectional analysis of the prevalence and character of chronic hepatitis B infection in HIV infected and uninfected women.**

Reference to your request, I give the University of Stellenbosch the permission to utilize the 2008 National Antenatal Sentinel HIV Syphilis Prevalence survey specimens for the purpose of preparing for a cross sectional analysis of the prevalence and character of chronic hepatitis B infection in HIV infected and uninfected women research project, in terms of the Human Tissue Act 65 of 1983.

These specimens should be used only for the purpose applied for if not, it will be a contravention of your side. It is my belief that results from this secondary analysis will provide us with new knowledge of Hepatitis B.

I hope that the results of this exercise will be shared with both National and Western Cape Departments.

Yours sincerely

  
DR Y PILLAY  
ACTING DIRECTOR-GENERAL: HEALTH

DATE: 24/10