ASSESSING THE IMPACT OF HEPATITIS B IMMUNIZATION IN OVER 5 YEAR OLDS FROM SELECTED PROVINCES OF SOUTH AFRICA

BY

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ASSESSING THE IMPACT OF HEPATITIS B IMMUNIZATION IN OVER 5 YEAR OLDS FROM SELECTED PROVINCES OF SOUTH AFRICA

By

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MASTER OF MEDICAL SCIENCE (MEDICAL VIROLOGY)

In the Department of Virology

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DECLARATION

I, **EDINA AMPONSAH-DACOSTA**, hereby declare that the work presented in this dissertation is original and has not been previously submitted for the purpose of a degree at this or any other institution.

This dissertation is being submitted in fulfilment of the requirements for the degree of Master of Medical Science (Medical Virology), in the Department of Virology, School of Pathology and Pre-clinical Sciences, Faculty of Health Sciences, at the University of Limpopo, Medunsa Campus. Works of other investigators, where cited, have been duly acknowledged.

..... **.....**

Signature **Date**

DEDICATION

I dedicate this work to my brothers, Maxwell and David Amponsah-Dacosta, for their unwavering support which has spurred me on throughout the duration of my studies. I owe both of you so much more.

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ABSTRACT

Introduction: The hepatitis B virus (HBV) causes a serious type of liver disease referred to as hepatitis B, which is associated with various fatal sequelae following the onset of chronic infection. As a result of the global burden of chronic HBV infection, HBV-related mortality is currently estimated at 620 000 annual deaths worldwide (Hwang and Cheung, 2011). The World Health Assembly (WHA) recommended in 1992 that the hepatitis B vaccine be incorporated into national immunization programmes universally, especially in the hyperendemic regions of the world, in order to curb the global burden of hepatitis B (WHO, 1992). Accordingly, South Africa introduced the hepatitis B vaccine into the national Expanded Programme on Immunization (EPI-SA) in April 1995. Almost 17 years later, South Africa has not conducted any nationwide serosurveys to monitor the population impact of the hepatitis B vaccine. Instead, a number of field and laboratory studies have been conducted only in vaccinated children within the first 5 years of life and as such reports on the short-term impact made by the hepatitis B vaccine in the country have largely relied on these studies (Tsebe *et al*., 2001; Schoub *et al*., 2002; Simani *et al*., 2008). The aim of the current study therefore was to assess the population impact made by the hepatitis B vaccine post its introduction into EPI-SA using an age stratified, cross-sectional study. The objectives were to compare the prevalence of HBV exposure between the post- and pre-vaccination populations of South Africa, determine the influence of HIV infection on the prevalence of HBV exposure between the post- and pre-vaccination populations of South Africa by performing a subset analyses, and lastly, to perform molecular characterization of hepatitis B surface and polymerase genes in HBV DNA positive individuals.

Materials and Methods: This was an explorative and descriptive retrospective, cross-sectional study based on recently tested and stored blood samples from the NHLS Diagnostic Laboratory in the Department of Virology. For the purpose of this study, these samples were obtained after Ethics approval, and two target populations identified based on the year (i.e. 1995) the hepatitis B vaccine was introduced into EPI-SA; a post-vaccination population consisting of 605 blood samples from individuals aged 1-15 years and a pre-vaccination population consisting of 601 blood samples from individuals aged 16-25 years. The post-vaccination population was further stratified by age as follows; 1-5, 6-10 and 11-15 years, in order to assess immunity and chronic carriage of HBV across the different age groups. All samples were tested for the following primary serological markers; HBsAg, anti-HBc and anti-HBs, to determine the prevalence of HBV chronic carriage, past HBV exposure and immunity to HBV infection respectively. Samples were further assessed for the

incidence of acute HBV infection by testing for IgM anti-HBc. All serological testing was performed using the Elecsys® 2010 Immunoassay System. Samples with serological evidence of infection or exposure to HBV were selected and screened for HBV DNA using a real time PCR assay to determine the prevalence of active HBV infection within this group. Study subjects with records of their HIV status, either positive or negative, were also pooled for subset analyses in order to determine the influence of HIV infection on immunity and chronic carriage of HBV. Finally, samples positive for HBV DNA were subjected to molecular characterization of the hepatitis B surface (S) and polymerase (*pol*) genes.

Results: Following serological screening, immunity to HBV infection was found to be significantly (p<0.001) higher (56.7%) in the post-vaccination population than in the pre-vaccination population (15.5%). Within the post-vaccination population alone, immunity was found to wane with increasing age from 76.1% in those 1-5 years of age to 50.0% in those 6-10 years and 44.2% in those 11-15 years of age. Chronic carriage on the other hand was significantly (p=0.008) reduced in the post-vaccination population with 1.5% HBsAg prevalence as compared to 4.0% in the pre-vaccination population. Within the different age strata, chronic carriage increased with increasing age (0.5% in 1-5 years; 1.3% in 6-10 years; 2.5% in 11-15 years). Overall, no acute HBV infection was detected within the post-vaccination population, while a 14.6% prevalence rate of acute HBV infection was found for the pre-vaccination population. From the subset analyses, immunity was found to be significantly (p<0.001) higher in the HIV uninfected population as compared to the HIV infected population; 82.5% versus 22.0% in the post-vaccination population and 26.7% versus 0% in the pre-vaccination population, while chronic carriage was found to be higher in the HIV infected population than in the HIV uninfected population. Following molecular characterization of the HBV S gene, it was revealed that the majority of the viral isolates were genotype A, with only 1 genotype D isolate found. A number of notable amino acid variations were also detected within the antigenic region of the HBsAg of viral isolates, including the K122R, N131T, T143S, and E164D mutations.

Conclusion: Introduction of the hepatitis B vaccine into EPI-SA has shown remarkable success in children under the age of 5 years. Overall, immunity and chronic carriage of HBV within the post-vaccination population has been greatly impacted by hepatitis B immunization. Within the HIV infected population, susceptibility to HBV infection remains a cause for concern. Finally, although amino acid variations within the viral HBsAg are present, vaccine escape-related mutants appear to be rare or even absent within the South African population.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
µl	Microlitre
3TC	Lamivudine
aa	Amino acid
ADV	Adefovir
ALT	Alanine aminostransferase
Anti-HBc	Antibody to hepatitis B core antigen
Anti-HBe	Antibody to hepatitis B e antigen
Anti-HBs	Antibody to hepatitis B surface antigen
BCP	Basal core promoter
bp	Base pairs
cccDNA	Covalently closed circular DNA
CTL	Cytotoxic T lympocyctes
DNA	Deoxyribonucleic acid
DR	Direct repeats
dsDNA	Double stranded DNA
Enh	Enhancer
EPI	Expanded Programme on Immunization
ER	Endoplasmic reticulum
ETV	Entecavir
FTC	Emtricitabine
HAV	Hepatitis A virus
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HBx	Hepatitis B x protein
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus

HDV	Hepatitis D virus
HEV	Hepatitis E virus
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
IG	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
kb	Kilobases
kDa	Kilodalton
Ldt	Telbivudine
LHBs	Large hepatitis B surface protein
MHBs	Medium hepatitis B surface protein
MHC	Major histocompatibility complex
MHR	Major hydrophilic region
mL	Millilitre
mRNA	Messenger RNA
MTCT	Mother to child transmission
NRTI	Nucleos(t)ide reverse transcriptase inhibitors
nm	Nanometre
ORF	Open reading frame
PCR	Polymerase chain reaction
pgRNA	Pregenomic RNA
Pre-C	Pre-core
Pre-S	Pre-surface
Pol	Polymerase
qPCR	Quantitative PCR
rcDNA	Relaxed circular DNA
RNA	Ribonucleic acid
RT	Reverse transcriptase
ssDNA	Single stranded DNA

TDF	Tenofovir disoproxil fulmarate
ULN	Upper limit of normal
WHO	World Health Organization

LIST OF NUCLEOTIDES

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

LIST OF AMINO ACIDS

A	Alanine
R	Arginine
N	Asparagine
D	Aspartic acid
C	Cysteine
E	Glutamic acid
H	Histidine
I	Isoleucine
L	Leucine
K	Lysine
M	Methionine
F	Phenylalanine
P	Proline
S	Serine
T	Threonine
W	Tryptophan
Y	Tyrosine
V	Valine

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CHAPTER 1

1. BACKGROUND AND EXPERIMENTAL PROPOSAL

1.1 Background

Hepatitis, which is essentially inflammation of the liver, has both an infectious and non-infectious aetiology. Non-infectious causes include autoimmune liver disease (autoimmune hepatitis, AIH), metabolic liver disease, traumatic injury to the liver and long-term exposure to hepatotoxic agents such as alcohol and drugs (Mauss *et al*., 2009). Infectious causes of hepatitis are bacteria, fungi, parasites and viruses. Specialized hepatitis-causing viruses have been identified however, and have been exclusively classified as hepatitis viruses, designated A-E (i.e. hepatitis A virus; HAV, hepatitis B virus; HBV, hepatitis C virus; HCV, hepatitis D virus; HDV, hepatitis E virus; HEV). Of these, HBV and HCV are the major causes of viral hepatitis worldwide (Harrison *et al*., 2009). Secondary causes of viral hepatitis also exist; these include members of the *Herpesviridae* (Cytomegalovirus, Herpes Simplex virus, Varicella-Zoster virus and Epstein-Barr virus), the Adenovirus, Rubella virus and some hemorrhagic fever viruses (Promrat and Wands, 2005).

1.2 HBV

Hepatitis B is a major cause of mortality worldwide, associated with various complications including hepatic failure and hepatocellular carcinoma (HCC). The causative agent; HBV, invades the liver and causes acute hepatitis which may resolve without complication but in some cases may lead to a chronic form of the disease. The likelihood of developing chronic hepatitis B is heightened in infections acquired during infancy and carries some degree of fatality (Ganem and Prince, 2004; Wasmuth, 2009). This chronic disease can however be managed with various antiviral interventions as well as immunomodulators. Suitably, hepatitis B can be prevented altogether, especially earlier on in life through vaccination (Wedemeyer, 2009). Incorporation of the hepatitis B vaccine into routine immunization programmes has helped alleviate the disease burden in various regions of the world including South Africa. This pivotal role played by the vaccine in preventing hepatitis B makes it imperative that assessments be in place to ensure that its efficacy is maintained.

1.3 Study problem

HBV accounts for up to 620 000 hepatitis B-related deaths each year worldwide (Hwang and Cheung, 2011). Compounding the situation is the increased risk for HIV/HBV co-infection especially in regions with an equally high prevalence of HIV as is the case in South Africa. Fortunately, a prophylactic vaccine against hepatitis B is available. The World Health Assembly (WHA) recommended in 1992 that the hepatitis B vaccine be incorporated into national immunization programmes universally, especially in the hyperendemic regions of the world (WHO, 1992). South Africa introduced the hepatitis B vaccine into the national Expanded Programme on Immunization (EPI-SA) in April 1995 (Burnett *et al*., 2012). Follow-up studies to its introduction revealed that the hepatitis B vaccine was effective at reducing early childhood HBV infections, which are responsible for the high prevalence of chronic carriage (Tsebe *et al*., 2001; Schoub *et al*., 2002; Simani *et al*., 2008). Despite its reported success, reduced vaccine efficacy has been noted, reported to be associated with the presence of underlying HIV infection in vaccinees (Simani *et al*., 2008). In addition, there are other challenges that threaten the success of hepatitis B vaccination in preventing and potentially eliminating HBV infections globally. These include imperfect hepatitis B vaccine coverage which could be a result of the inaccessibility of the hepatitis B vaccine to infants in impoverished regions, waning of protective anti-HBs titres over time following primary hepatitis B vaccination at infancy in different geographic regions, immune suppression in previously vaccinated individuals, vaccine non-response in a limited percentage of vaccinated individuals and the rare occurrence of breakthrough infections where vaccinated individuals still acquire the HBV infection.

There is a need therefore, for evaluation of the impact made by the nationwide hepatitis B immunization programme (WHO, 2011). Limitations to previous studies carried out in this regard included the lack of sero-analysis in over 5 year-olds as well as a parallel cross-sectional comparison of hepatitis B prevalence in a backdrop of individuals who were born before and after inclusion of hepatitis B vaccine into the country's EPI (Tsebe *et al*., 2001; Schoub *et al*., 2002; Simani *et al*., 2008). This study aimed therefore at addressing these limitations by carrying out an age stratified, cross-sectional study to assess the impact made by the hepatitis B immunization programme in individuals between the ages of 1-15 years, who would have received the vaccine as of its introduction in 1995, in comparison to the prevalence of HBV infection in an older population born prior to implementation of the immunization programme.

1.4 Aim of the study

To assess the population impact made by the hepatitis B vaccine post its introduction into EPI-SA using an age stratified, cross-sectional study.

1.5 Rationale

In order to assess population immunity and chronic carriage of HBV almost 17 years after the introduction of the hepatitis B vaccine into EPI-SA, two representative populations were targeted; a post-vaccination population comprising blood samples from individuals aged 1-15 years and a pre-vaccination population comprising blood samples from individuals aged 16-25 years. The post-vaccination population was further stratified by age as follows; 1-5, 6-10 and 11-15 years, in order to assess immunity and chronic carriage of HBV across the different age groups. Study subjects with records of their HIV status were also pooled for subset analyses so as to investigate the influence of HIV infection on immunity and chronic carriage of HBV.

Serological screening for specific markers such as HBsAg, anti-HBc and anti-HBs is a commonplace task for the diagnosis of HBV infection as well as the determination of immunity to HBV infection. Molecular methods such as real time and conventional nested PCR assays directly detect and quantify viral DNA which assists in confirmation of an active HBV infection, and provide a channel for analysing HBV gene sequences for possible mutations. These diagnostic methods have revolutionized the study of HBV as well as the management of HBV infections making them very useful to this study.

1.6 Objectives

1. To compare the prevalence of HBV exposure between the post- and pre-vaccination populations of South Africa by:

    a. Defining the markers of current and past HBV exposure as well as recovery or immunity to infection in different age strata: 1-5, 6-10 and 11-15 years (post-vaccination population) and 16-25 year olds (pre-vaccination population).

b. Assessing the prevalence of acute HBV infection in different age strata: 1-5, 6-10, 11-15 and those 16-25 years of age.

2. To determine the influence of HIV infection on the prevalence of HBV exposure between the post- and pre-vaccination populations of South Africa by performing a subset analyses.

3. To perform molecular characterization of hepatitis B surface and polymerase genes in HBV DNA positive individuals.

1.7 Expected significance of the study

In the absence of routine nationwide serosurveys that would assess the impact of the hepatitis B immunization programme in the country, South Africa has relied on a number of laboratory and field studies, the majority of which have assessed the efficacy of the hepatitis B vaccine (short-term impact) in vaccinated children under the age of 5 years (Tsebe *et al*., 2001; Schoub *et al*., 2002; Simani *et al*., 2008). None of these studies have evaluated immunity and chronic carriage of HBV in a South African population without knowledge of vaccination status. It is thus expected that results obtained from this study will provide insight into the impact made by hepatitis B immunization in individuals 1-15 years of age who should have received the vaccine during infancy. In addition, such a study could possibly influence the decision by the South African National Department of Health to carry out a nationwide serosurvey to assess the long-term impact of the hepatitis B vaccination programme almost two decades after introducing the hepatitis B vaccine into EPI-SA.

CHAPTER 2

2. LITERATURE REVIEW

2.1. History of HBV

Knowledge on hepatitis B and its related sequelae has improved radically since 1967 when HBV was first described to be the causative agent. This discovery was made following the detection of a viral antigen (named the 'Australia antigen') in Australian individuals of aboriginal race who had been diagnosed with what was referred to then, as serum hepatitis (Blumberg *et al*., 1967). By observing the serum of the infected patients using electron microscopy, viral particles were identified, some composed entirely of the antigen (Huang *et al*., 1972; Kidd-Ljunggren *et al*., 2002). The purified form of this viral antigen was found to be transmissible to susceptible individuals who developed typical presentations of acute or chronic liver disease. Further analysis revealed that the 'Australia antigen' is the surface protein of the virus and as such was named the hepatitis B surface antigen (HBsAg). The main antigenic determinant 'α' was also identified and this together with variations in the surface antigen allowed for serological subtypes of HBV to be differentiated (Kidd-Ljunggren *et al*., 2002). Since then, the advent of molecular techniques including the polymerase chain reaction (PCR) and genotyping, has allowed for further characterization of the virus based on nucleotide polymorphisms into genetic variants. These discoveries gave way to further research, allowing for an increased understanding of various aspects of HBV infection, including its epidemiology, pathogenesis, treatment and prevention.

2.2 Epidemiology of HBV

2.2.1 Transmission of HBV

Hepatitis B is contracted by exposure to HBV via direct or indirect contact with infected bodily fluids. HBV is commonly present in genital secretions and blood which are the main source of spread (Lavanchy, 2004). The virus is however not transmitted through the gastrointestinal or respiratory tracts and as such the disease cannot be acquired through casual contact such as shaking hands, hugging and talking with an infected individual or through ingestion of food or water thought to be contaminated with the virus (WHO, 2008). The virus is reported to be nearly 100 times more infectious than the human immunodeficiency virus (HIV) and 10 times more infectious than HCV, making it highly transmissible (Lavanchy, 2004; Gupta *et al*., 2008).

Three major modes of transmission are recognized, namely; the horizontal, sexual and vertical routes of transmission (Lavanchy, 2004; Wasmuth, 2009). Horizontal transmission of hepatitis B from an infected source to an uninfected individual may occur through direct contact with infected blood or blood products. Current stringent screening of blood and blood products and prevention of recognized risk groups from being donors has effectively reduced spread of HBV infection as well as other blood-borne diseases through transfusions and transplantations (Jackson *et al*., 2003; Lavanchy, 2004). Thus horizontal transmission is largely driven by percutaneous inoculation through sharing of needles among injection drug users, use of contaminated instruments during acupuncture, tattooing and body piercings and occupational exposures such as accidental needles stick and sharps injuries (Lavanchy, 2005; Wasmuth, 2009). Iatrogenic exposure to HBV is also possible in the event that inadequately sterilized instruments are used during medical procedures. Prolonged familial contact with chronically infected individuals has also been implicated as a source of spread within households, particularly through direct contact via non-intact skin as well as sharing of razors and syringes (Lavanchy, 2005; Gupta *et al*., 2008; Wasmuth, 2009).

High risk sexual behaviour such as having multiple sexual partners and continuous unprotected sexual intercourse with HBsAg positive partners is the main cause of HBV infection in the adolescent and adult-age populations. About 5% of these adult-age-acquired infections develop into chronic hepatitis B. Chronically infected individuals do not immediately present with symptoms and as such are unaware of the HBV infection. They are thus one of the primary sources of HBV spread especially when multiple sexual partners are involved (Atkins and Nolan, 2005; Lavanchy, 2005). Other common risk factors associated with sexual transmission include high number of years of sexual activity and a history of other sexually transmitted infections. In the case of men who have sex with men (MSM), receptive anal intercourse is a major determinant of acquisition of HBV infection (Khan, 2002; Atkins and Nolan, 2005).

Vertical transmission (mother-to-child transmission; MTCT) of HBV commonly occurs in the event that a mother has an active infection during pregnancy or at the time of birth. The risk of transmission is directly proportional to the viral replication rate in the mother. Pregnant women with no detectable HBV DNA are thus less likely to pass the infection on to their babies while the risk in mothers with high levels of viremia may be up to 90% (Tran, 2009; Wasmuth, 2009). HBV infection acquired in infancy or childhood carries a great deal of

importance as up to 90% of such cases become chronic carriers. Transmission may occur prenatally (*in utero*), perinatally (at delivery) or postnatally (after delivery) (Xu *et al*., 2002; Chowdhury and Eapen, 2009; Tran, 2009). *In utero* transmission is relatively rare as the virus typically does not cross the placenta (Zhang *et al*., 2004). When it does occur, mechanisms for *in utero* transmission may include transplacental leakage of infected maternal blood to the foetus due to placental tears or HBV infection of villous capillary endothelial cells of the placenta during acute infection, particularly within the third trimester, resulting in infection of the foetus (Xu *et al*., 2002; Zhang *et al*., 2004; Zhu *et al*., 2010). Perinatal transmission is the principal means of infant HBV infection, passed on from the infected mother during labour, at which point the infant comes into direct contact with maternal blood or cervical secretions (Chowdhury and Eapen, 2009; Tran, 2009). Postnatal transmission of the infection is mainly by direct contact with infected bodily fluids of the mother through cuts in the skin. Postnatal transmission of HBV can be prevented through timely administration (within 24 hours after birth) of immunoprophylaxis (hepatitis B immunoglobulin and/or hepatitis B vaccine) to the infant (Petrova and Kamburov, 2010; Shi *et al*., 2011).

2.2.2 Global distribution of HBV

As one of the most notable infectious diseases worldwide, hepatitis B is globally distributed, with an estimated one-third of the world's population having been exposed to HBV infection. This accounts for over 380 million chronic HBV infections to date and up to 620 000 deaths each year worldwide (WHO, 2009a; Hwang and Cheung, 2011). The global prevalence of HBV infection (based on the prevalence of HBsAg carrier rates in the general population) is extremely variable, ranging from <0.1% in the low endemic zones, which comprise mainly of adult-age infected individuals, to ~20% in the designated hyperendemic areas where high cases of childhood-acquired HBV infections are reported (Hwang and Cheung, 2011) (Figure 2.1).

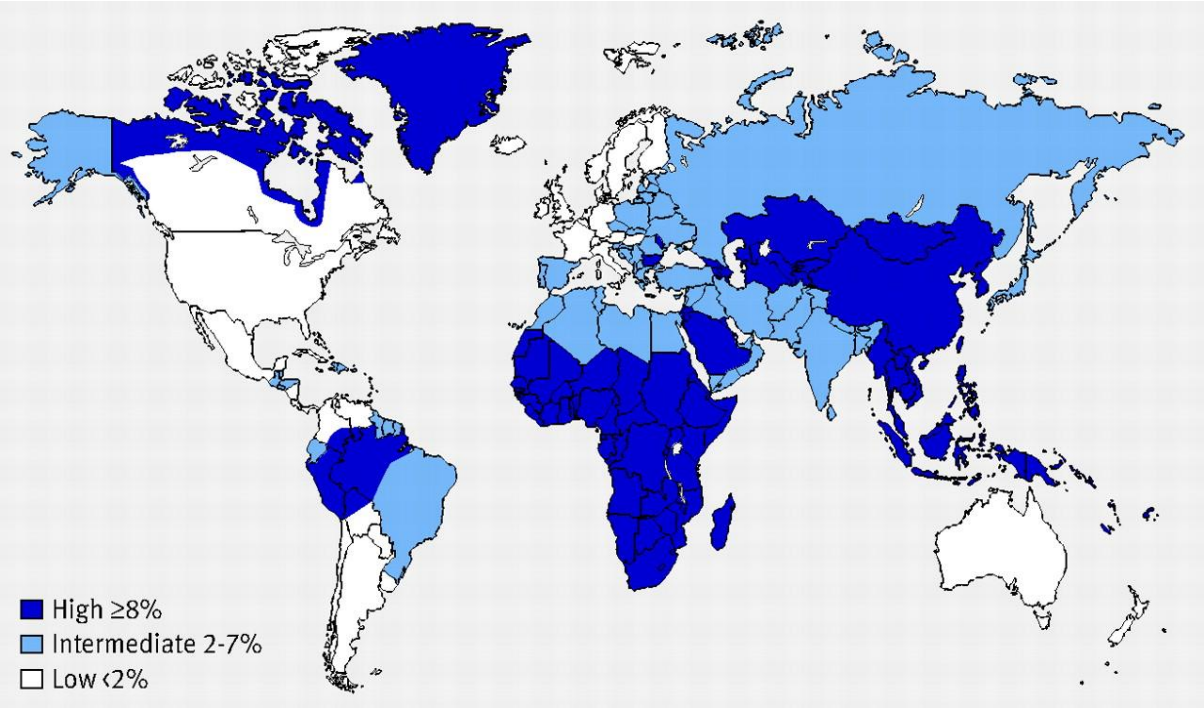


Figure 2.1: Diagrammatic representation of the global distribution of hepatitis B (WHO, 2002)

The extent of the global prevalence of HBV infection is best articulated in terms of each of the six WHO regions of the world; The Americas, Europe, Eastern Mediterranean, The Western Pacific, South East Asia and Sub-Saharan Africa (Figure 2.1). The prevalence of HBV infection in The Americas varies from low to intermediate with the highest prevalence rates found to be in the Amazon basin (≥10%) and the lowest in the Southern part of South America (<2%) (Echevarria and León, 2003; Ropero *et al*., 2005). Northern and Western Europe present with low prevalence rates of HBV infection (0.1-2%) while Eastern and Southern Europe have a more intermediate endemicity (1-7%) (Rantala and van de Laar, 2008). In the Eastern Mediterranean, the state of the epidemic ranges from <1% in countries such as Egypt, Oman and Saudi Arabia while Somalia and Southern Sudan bear the brunt of the disease burden in the region with prevalence rates as high as 8% (WHO, 2009b). With the exception of Australia, Japan and New Zealand where the HBV prevalence rate is <2%, the rest of the Western Pacific region have an estimated chronic carriage rate of >8% (Rani *et al*., 2009). The overall HBsAg prevalence rate for South East Asia on the other hand is estimated at >10%. However, the actual prevalence in the member states varies greatly with countries such as India, Thailand and Sri Lanka having prevalence rates between 1-5%, Bangladesh and Indonesia with 5-10% and the highly endemic countries being Democratic

People's Republic of Korea and Myanmar with >10% HBsAg prevalence rates (Khin, 2006; WHO, 2010).

Of the six WHO regions, Sub-Saharan Africa is second to South East Asia in terms of hepatitis B hyper-endemicity, harbouring over 50 million (~18%) of the world's total hepatitis B chronic population (Burnett *et al*., 2005; Kramvis and Kew, 2007). While the high prevalence rate in South East Asia is driven by perinatally acquired infections due to the high prevalence of active HBV infection in females of childbearing age in the region, the HBV burden in Sub-Saharan Africa is mainly due to infections acquired in early childhood via horizontal routes of transmission (Kramvis and Kew, 2007; Ott *et al*., 2012) The average chronic carriage rate of HBV infection in the African region is estimated at 10%. However, prevalence rates range from 10-12.5% in East and West Africa, 12.2% in Central Africa, to ≥8% in Southern Africa (André, 2000; Mphahlele *et al*., 2002; Parkin, 2006). The impact of the infection remains varied among the representative countries in the sub-continent with Gambia, Kenya, Senegal, Tanzania, Zambia and Zimbabwe described as being some of the main endemic zones (Mphahlele *et al*., 2002).

South Africa is one such endemic zone within Sub-Saharan Africa, accounting for over 3 million chronically infected individuals (Kew, 2008). It is reported that at least more than 70% of the country's population has had previous exposure to HBV, accounting for the majority of the liver-related diseases and deaths in the country (Tsebe *et al*., 2001). The HBsAg prevalence rate in the general population is reported to be between 7-10% with the HBV infection found to be more prevalent in the rural areas as compared to the urban regions (Kew, 2008).

2.3 Taxonomy of HBV

HBV is regarded as the prototype for a family of viruses referred to as the *Hepadnaviridae*. This comprises a group of hepatotropic, double stranded DNA viruses, which encompass an RNA intermediate in the course of their life cycle (Lüsbrink *et al*., 2009). They are the only known family of animal viruses classified under class VII of the Baltimore Classification System and possess one of the smallest genomes ranging from 3-3.5 kb in size (Lüsbrink *et al*., 2009). The genome organization of the *Hepadnaviridae* is a characteristic highly

condensed, circular, overlapping structure which favours their unique replication strategy (Lüsbrink *et al*., 2009).

Two genera of the *Hepadnaviridae* exist, differentiated on the basis of their host tropism; those that infect mammals (orthohepadnaviruses) and those that infect birds (avihepadnaviruses) (Scaefer, 2007). HBV belongs to the orthohepadnaviruses, a genus it shares with the Chimpanzee hepatitis B virus, Gorilla hepatitis B virus, Orangutan hepatitis B virus and the Woodchuck hepatitis B virus (Seeger and Mason, 2000; Scaefer, 2007). Studies have shown that primate orthohepadnaviruses are exclusive to their hosts. Likewise, humans are known to be the natural definitive hosts of human HBV, in whom it causes acute or chronic liver infection which may lead to various fatal complications.

2.4 Morphology and structure of HBV

Electron microscopic studies reveal that HBV is a pleomorphic virus, existing in three distinct morphological forms (Figure 2.2); the 20 nm quasi-spherical particle, the tubular or filamentous form of varying sizes and the 40 nm complete spherical particle referred to as the Dane particle (Bruss, 2007). The quasi-spherical and filamentous forms are regarded as sub-viral particles as they are devoid of viral DNA, consisting exclusively of the viral surface protein embedded in host cell lipids. The presence of HBV DNA-negative Dane particles in infected serum has also been described, composed entirely of viral surface and core proteins (Kimura *et al*., 2005; Harrison *et al*., 2009). These DNA-negative, sub-viral particles are predominant in serum, synthesized in excess over the Dane particles and reaching serum concentrations over 10 000 folds. They are suggested to act as decoys for the host immune system but due to their lack of genomic material, sub-viral particles are non-infectious (Kann and Gerlich, 2005).

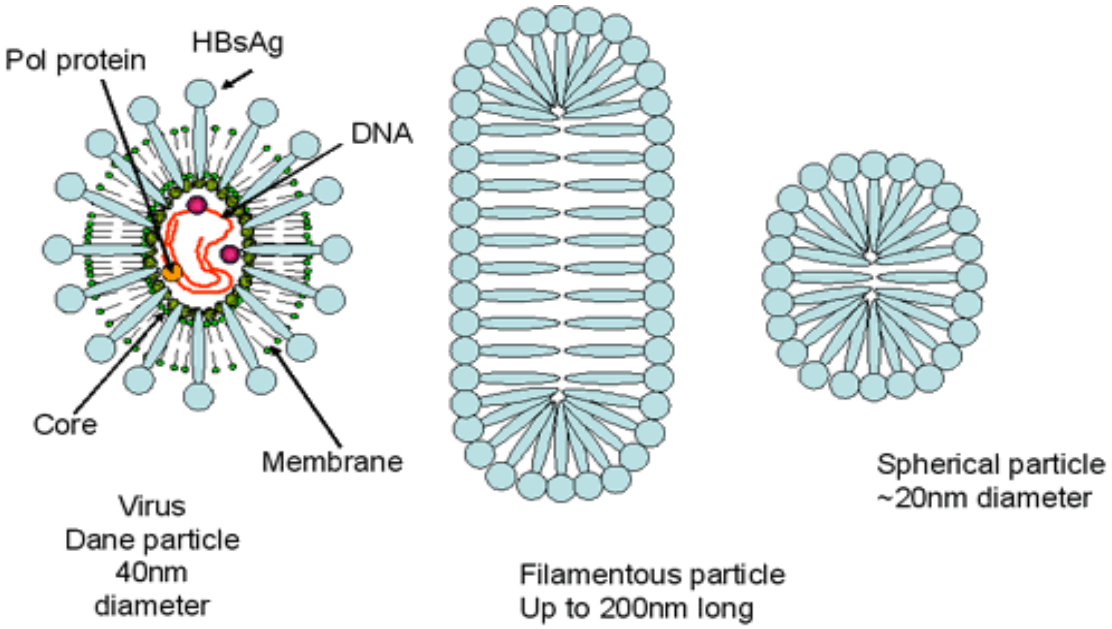


Figure 2.2: Diagrammatic representation of the three HBV morphologies (http://pathmicro.med.sc.edu/virol/hep-bstruct.gif, accessed on 15th July, 2011)

The contrary is true for the Dane particles which are the intrinsic HBV infectious virions that direct the course of liver disease and its associated complications. Electron microscopy reveals a double-shelled structure of similar form to the quasi-spheres but with a difference in terms of size (≥40 nm in diameter) (Kann and Gerlich, 2005). The inner shell of the HBV virion comprises a 27 nm electron dense core protein which together with the viral DNA, form the icosahedral viral nucleocapsid (Kann and Gerlich, 2005). This nucleocapsid, which also houses the viral polymerase protein, is surrounded by the outer shell; a membrane composed of the viral surface proteins together with host cell lipids which are acquired when the virion is transported through the endoplasmic reticulum (ER) and Golgi apparatus or while it buds through and exits the host cell it infects (Lüsbrink *et al*., 2009). This outer membrane is essentially the lipid envelope, complete with glycoprotein spikes which are encoded by the viral genome (Kaito *et al*., 2006; Lüsbrink *et al*., 2009).

2.5 Organisation of the HBV genome

The HBV genome is a relaxed circular (rc), partially double-stranded (ds) DNA molecule formed in a highly condensed structure. The plus strand of the dsDNA is the incomplete (2/3 the length of the entire genome) non-coding strand while the minus strand is the full-length strand, encoding all the relevant viral proteins. At the 5' end of the (+) strand DNA is a

residual pre-genomic RNA (pgRNA) oligomer derived from the viral replication stage. The (-) strand DNA on the other hand is found in a covalent bond with the polymerase protein and flanking each end of this strand are short redundant sequences; DR1 and DR2, shown in Figure 2.3, which are essential to the viral replication process (Lüsbrink *et al*, 2009).

Averaging about 3.2 kb in size, the viral genome comprises four conserved open reading frames (ORFs), namely; the S, X, C and P ORFs which encode the pre-surface/surface, X, pre-core/core and polymerase proteins respectively (Figure 2.3). The entire S ORF consists of three domains; the Pre-S1, Pre-S2 and the S domains, and partially overlaps with the P ORF. Similarly the C ORF, consisting of the Pre-C and C domains, has a partial overlap with the P ORF. The X ORF on the other hand partially overlaps with both the P and C ORFs.

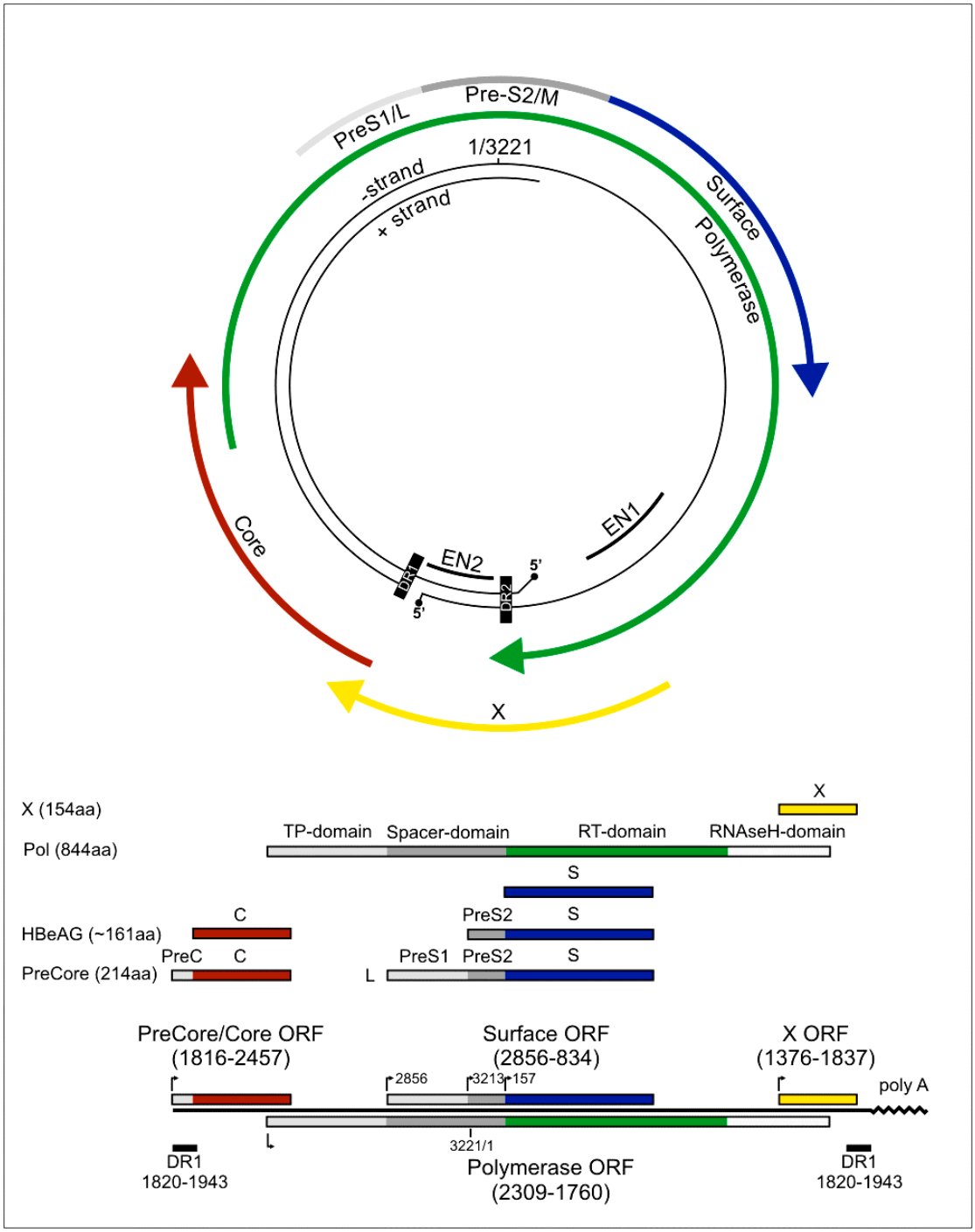


Figure 2.3: Diagrammatic representation of the genome organization and transcripts of HBV (Lüsbrink *et al*, 2009)

The S ORF is expressed to produce three related surface proteins (large; LHBs, medium; MHBs and small surface proteins; SHBs), all of which are components of the viral envelope. The S domain of the S ORF encodes the HBsAg which is one of the most useful markers for detecting the presence of an HBV infection. The X ORF encodes a 154 amino acid long

trans-activating factor, the X-protein or HBx, which among other undefined functions activates transcription of various viral as well as host cellular genes (Bouchard and Schneider, 2004). The viral e-antigen (HBeAg) and core protein (HBcAg), encoded by the pre-C and C domains respectively, elicit host immune responses during the course of infection. The core protein also has a structural function and makes up the viral nucleocapsid. The polymerase protein, encoded by the P ORF, is composed of three functional domains; the terminal protein (TP), the reverse transcriptase (RT) and the ribonuclease H enzyme (RNase H), all involved in viral replication (Bruss, 2007; Lüsbrink *et al*, 2009).

2.6 The HBV life cycle

The HBV life cycle incorporates a unique replication strategy involving reverse transcription, which is related to the replication strategy employed by members of the *Retroviridae* such as HIV, Human T Lymphotropic Virus types I and II (HTLV I and II), Simian Immunodeficiency virus (SIV) and the Rous Sarcoma Virus (RSV). This sets HBV apart from all other human DNA viruses as it incorporates an RNA intermediate into its life cycle. The viral life cycle begins when HBV gains entry into a susceptible host cell in which it usurps normal cell functioning and proliferates to produce multiple copies of progeny virions. These virions are then released from the infected cell and can go on to infect other neighbouring cells, starting the cycle all over again. The HBV life cycle comprises three basic steps, namely; attachment and entry of the virus, viral genome replication, and finally virion assembly and release (Seeger and Mason, 2000; Schädler and Hildt, 2009).

2.6.1 Viral attachment and entry

Viral recognition and binding of susceptible host cells constitute the initial steps of HBV entry (Cooper *et al*., 2003). HBV has a tropism for the cells of the liver which serve as the primary replication sites of the virus due to the presence of an HBV-specific cell receptor, recently discovered to be sodium taurocholate cotransporting polypeptide (NTCP) (Yan *et al*., 2012). Receptor mediated attachment to these liver cells is suggested to occur through a multi-binding mechanism, involving an initial reversible non-cell type specific binding of the LHBs protein of the viral envelope to a host cell “attachment receptor” known as heparan sulphate proteoglycans (Glebe, 2007; Schulze *et al*., 2007; Urban *et al*., 2010). The virus is then located to the HBV-specific cell receptor, NTCP, to which it binds irreversibly using the major

cell attachment epitope; a QLDPAF motif between amino acids 21-47 of the pre-S1 domain of the LHBs protein (Neurath *et al*., 1986; Paran *et* al., 2001; Cooper *et al*., 2003; Schädler and Hildt, 2009). This high affinity binding step is followed by fusion of the viral envelope protein and the host cell membrane, facilitated by a putative “fusion motif” on the S domain of the SHBs protein (Lu and Block, 2004; Xie *et al*., 2010). This fusion process results in a conformational change in the host cell membrane and subsequently, entry of the virus into the host cell cytoplasm by an apparent pH-dependent process.

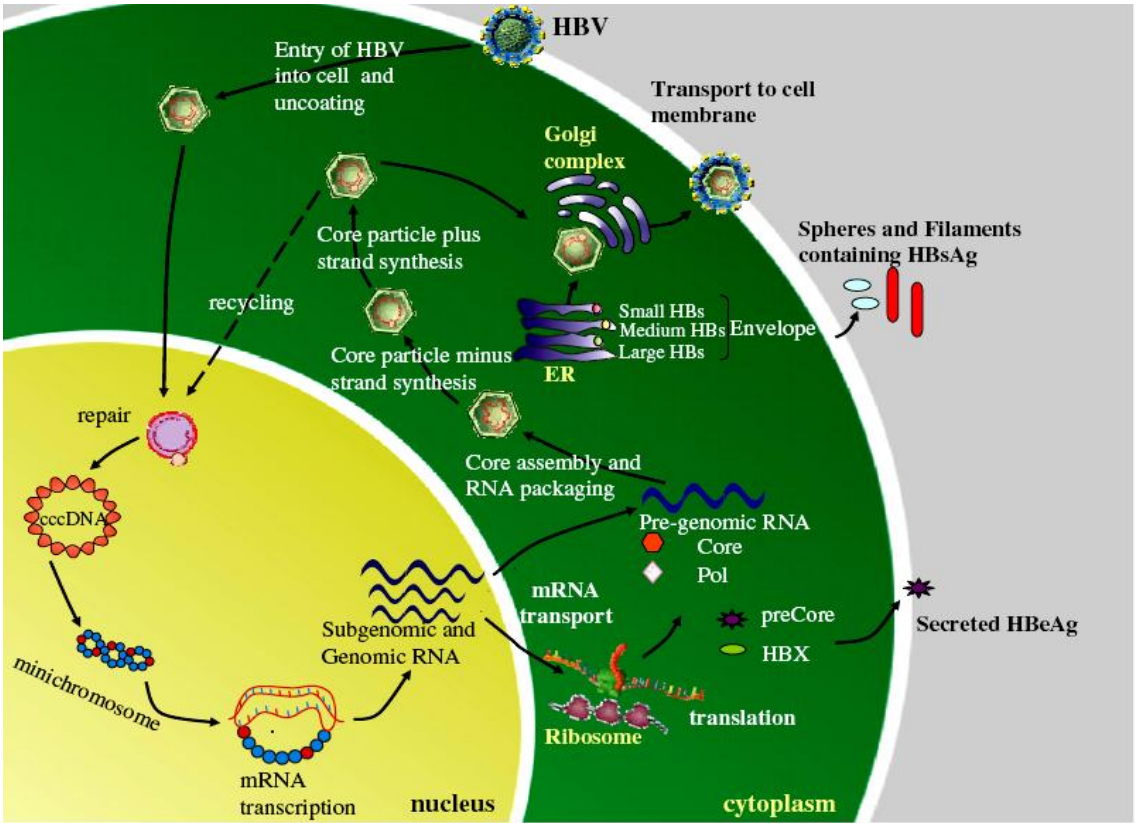


Figure 2.4: Schematic representation of the HBV life cycle (Raimondo *et al*., 2007)

2.6.2 Viral genome replication

Infection with HBV is only certain when the virus replicates and produces multiple copies of infective viral particles. To make the viral genome available for replication, it has to be transported to the host cell nucleus where the necessary replication factors are found. This is made possible by uncoating of the viral envelope to release the nucleocapsid enclosing the HBV genome and the bound polymerase protein. This nucleocapsid is then actively transported through the cell cytoplasm to the nuclear pore where the genome is released

into the nucleoplasm, leaving the nucleocapsid at the nuclear envelope (Kann *et al*., 2007). The relaxed circular DNA is then repaired to form covalently closed circular DNA (cccDNA), using the viral polymerase to extend the incomplete (+) DNA strand followed by covalent ligation of the 5' and 3' ends of both DNA stands (Sohn *et al*., 2008). The cccDNA is then bound by host histone proteins and organized into a chromatin-like structure referred to as a minichromosome (Figure 2.4) (Urban *et al*., 2010). This minichromosome serves as a template for the synthesis of multiple copies of sub-genomic as well as genomic RNA transcripts, processes catalyzed by host RNA polymerase II (Beck and Nassal, 2007). These RNA transcripts are then transported from the nucleus to the cytoplasm where the sub-genomic RNA transcripts are translated into viral surface proteins (LHBs, MHBs and SHBs). Following their synthesis at the host cell ER, these surface proteins may bud out of the cell in large quantities as subviral particles as shown in Figure 2.4. The polymerase and core proteins on the other hand, are translated from a genomic RNA (pre-genomic RNA) transcript. The core proteins essentially form progeny nucleocapsids while the polymerase proteins bind to a stem and loop structure (ε) on the 5' end of pre-genomic RNA (pgRNA) in the cell cytoplasm. This polymerase binding process facilitates selective packaging of pre-genomic RNA, together with the bound polymerase protein, into newly forming nucleocapsids (Beck and Nassal, 2007; Urban *et al*., 2010).

Within the nucleocapsid, the process of reverse-transcription of the pg-RNA into a (-) strand DNA begins. This process is initiated by protein-priming at the 5' ε region, catalyzed by the reverse transcriptase (RT) domain of the bound polymerase protein. This is followed by translocation of the synthesized primer from the 5' ε region of the pg-RNA to its 3' DR1 region. The RT enzyme then catalyzes the extension of the primer to synthesize a (-) strand DNA. Concurrent with (-) strand DNA synthesis is the degradation of the pg-RNA template (leaving behind the pg-RNA terminal region with the DR1 sequence) by the RNase H domain of the polymerase protein (Rieger and Nassal, 1996; Feng and Hu, 2009). After completion of the (-) strand DNA, the initiation primer is translocated a second time to the 3' DR2 region where synthesis of complimentary (+) strand DNA is initiated. During synthesis of the (+) strand DNA, a third translocation step occurs, switching the end of the growing (+) strand DNA from the 5' to the 3' end of the (-) strand DNA template which causes a relaxed circularization (rc) of the growing molecule. Extension of the (+) strand DNA is therefore incomplete with the final product being a partial double stranded rcDNA molecule within each progeny nucleocapsid (Beck and Nassal, 2007; Nassal, 2008; Feng and Hu, 2009; Lentz and Loeb, 2011). These progeny nucleocapsids may recycle the viral relaxed circular

DNA back into the nucleus for cccDNA synthesis and replication. This is evident in that each infected cell may contain up to 50 copies of viral cccDNA, the source of persistent chronic infection.

2.6.3 Virion assembly and release

An alternative pathway for the progeny nucleocapsids involves interaction with newly synthesized envelope proteins at the host cell ER. By budding through the ER, the progeny nucleocapsids are encased within the enveloped proteins which are lodged within the ER lumen (Bruss, 2004). Only replication competent or mature nucleocapsids (those containing relaxed circular DNA) are enveloped and assembled into progeny virions. These virions are then transported through the cell cytoplasm to the cell membrane where they are released as mature viral particles capable of infecting neighbouring cells (Bruss, 2004).

2.7 Genetic variability of HBV

High genetic variability is a characteristic feature of HBV, a result of the error-prone nature of the viral reverse transcriptase (RT), which lacks a proofreading function and thus is the cause of development of polymorphisms within the viral genome. However, the extreme overlapping of the HBV genome ensures that fixation of these polymorphisms is curbed to a minimum (Datta, 2008). These conflicting factors result in HBV having an intermediate rate of substitutions as compared to other DNA and RNA viruses. The mutations that do persist are naturally selected and account for the various HBV variants (HBV quasispecies) which can be classified into serological subtypes, genotypes and sub-genotypes. Inter- and intra-genotypic recombinant strains have also been described, arising from genome recombination of co-infecting genotypes or members of the same genotype respectively. Other mutant variants also exist, possessing mutations in particular genes and expressing different properties from the wild type virus, altering the natural course of disease (François *et al*., 2001; Norder *et al*., 2004; Simmonds and Midgley, 2005).

2.7.1 HBV genotypes and sub-genotypes

Genetic variants of HBV were first recognized by analyzing the viral surface protein to detect polymorphisms involving single amino acid substitutions at specified positions. This protein

possesses serotype determinants, '*d*', *'r*', '*w*' and '*y*', which together with the main antigenic determinant 'α' (amino acids 99-170 of the S protein), give rise to the nine serological subtypes of HBV (*ayw1-ayw4, ayr, adw2, adw4, adrq-, adrq+*) (Kidd-Ljunggren *et al*., 2002; Weber, 2005). These serological subtypes have a differential geographic distribution and are associated with the viral genotypes; each being encoded by more than just one genotype (Norder *et al*., 2004; Kay and Zoulim, 2007).

Determination of HBV genotypes is based on genome sequence diversity instead of antigenic variations in the surface protein. There exists within these genotypes still a great deal of diversity, resulting in further characterization of some of the genotypes into sub-genotypes. Thus based on full genome analysis, an HBV genotype is defined as a sequence with ≥8% divergence (Norder *et al*., 2004; Kramvis *et al*., 2005). To date, 8 genotypes of HBV are widely recognized, labelled A-H, however based on the evolutionary ability of the virus, the possible emergence of new genotypes overtime cannot be excluded. Accordingly, a putative genotype, I, has been proposed by Yu *et al* (2010) to be the ninth genotype following their isolation of a new strain (*W29*) in the northwest of China which showed substantial divergence from the other 8 genotypes. This proposal is however not yet widely accepted as the exact parental strains of this group remain undefined. Globally, the majority of the 8 HBV genotypes are geographically distinct while some, like genotypes A and D are ubiquitous as shown in Figure 2.5. The pattern of HBV genotype distribution is not absolute as human population migration and increased risk behaviours, among other factors, can alter the prevalence of genotypes in a region. At present, genotypes B and C are restricted to Asia and Oceania while G has been reported in the USA and France. Genotypes F and H on the other hand are commonly found in the Americas (Kay and Zoulim, 2007; Datta, 2008).

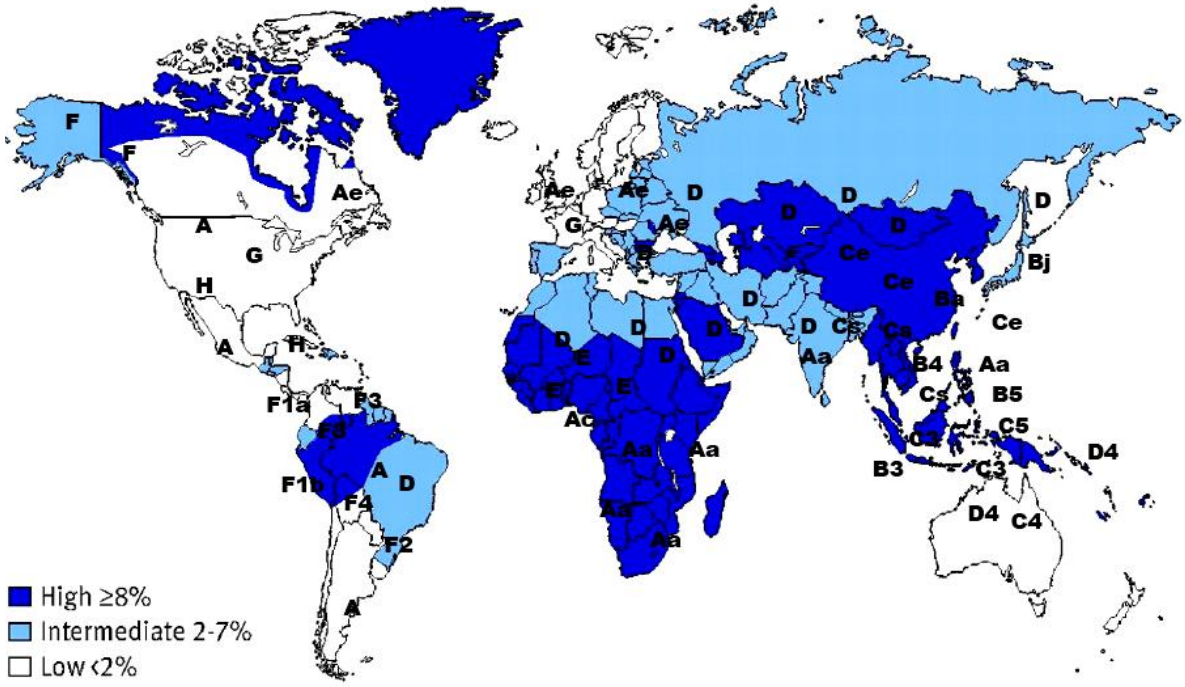


Figure 2.5: The global distribution of HBV genotypes and sub-genotypes. Aa/A1 ('a' for Africa/Asia), Ae/A2 ('e' for Europe), Ba/B2 ('a' for Asia), Bj/B1 ('j' for Japan), Ce/C1 ('e' for east) and Cs/C2 ('s' for south) (adapted from Datta, 2008)

The distribution of genotypes in Sub-Saharan Africa is equally variable; genotype E is the most prevalent genotype distributed across western Africa, from Senegal to Namibia whereas genotype D is more pronounced in the northern regions of the sub-continent. Genotype A on the other hand is ubiquitous to the African region. Likewise, within South Africa, the prevalence of HBV is chiefly driven by genotype A, followed by genotypes D and B (Kramvis *et al*., 2005; Kramvis and Kew, 2007).

The HBV genotypes are also further characterized into sub-genotypes which are defined by ≥4% (but <7.5%) nucleotide divergence (Kramvis *et al*., 2008). Sub-genotypes have been described for genotypes A (A1/Aa, A2/Ae and A3-A5), B (B1/Bj, B2/Ba and B3-B4), C (C1/Cs, C2/Ce and C3-C5), D (D1-D5) and F (F1a, F1b and F2-F4) (Kay and Zoulim, 2007; Scaefer, 2007). The HBV genotypes and their respective sub-genotypes show some extent of heterogeneity in their geographic distribution (Figure 2.5).

Genome recombination among the different HBV genotypes takes place through the exchange of genes during mixed infections which occur as a result of simultaneous

transmission of, or sequential infection with, the different genotypes. This genetic exchange occurs more often at specific gene sites, referred to as recombination hotspots, than at other regions within the viral genome. The gene regions prone to frequent recombination events include the Pre-S1, Pre-S2/S, C and P genes (Yang *et al*., 2007; Fallot *et al*., 2011). Recombinant variants are identified by the presence of a mosaic genome representing gene fragments from the parental genotypes. This results in generation of novel variants of the virus, a source of further genetic variation. HBV recombinants have been identified involving genotypes A/C, A/D, A/E, A/G, B/C, B/D and D/C. Intragenotypic recombination has also been described to occur within genotypes A, D and F/H (Simmonds and Midgley, 2005; Günther, 2006). Interspecies recombination events between non-human primate and human HBV variants have also been documented (Yang *et al*., 2007). The infections caused by the different HBV variants have been shown to differ in terms of disease severity and progression as well as response to therapy (Kramvis *et al*., 2005; Mahtab *et al*., 2008). Thus the endemicity of hepatitis B in a particular region may be driven by the variant prevalent in that population.

2.7.2 HBV gene specific mutants

Development of mutations is not restricted to any one particular gene but instead occurs in any of the four viral ORFs as well as the regulatory elements. Thus HBV mutants may result from mutations in the pre-S/S, X, pre-C/C or P genes. Due to the extreme overlapping of the viral genome, a mutation in one gene may inadvertently select for a mutation in another that is in direct overlap with it. Mutant HBV strains develop as a result of errors during gene replication and may remain stable over time, eventually predominating over the wild type variant as a consequence of specific selective pressures such as the host immune response or prophylactic and therapeutic interventions against infection. Similar to their wild type compatriots, some HBV mutants are transmissible and as such have relevant medical and public health implications. Other mutations are however deleterious; rendering the virus defective and affecting viral infectivity (François *et al*., 2001; Echevarría and Avellón, 2006).

2.7.2.1 Pre-core (pre-C) and core (C) gene mutants

The C ORF is divided into the pre-core (pre-C) and core (C) domains by two in-frame initiation codons (ATG). The pre-C mRNA encodes the protein precursor of HBeAg while the C domain encodes the core protein which is the viral nucleocapsid (Lüsbrink *et al*, 2009).

Both HBeAg and the core protein have diagnostic relevance; the presence of HBeAg in serum is indicative of ongoing viral replication and thus an active HBV infection while antibody to the core protein (anti-HBc) implies exposure to HBV (Chevaliez and Pawlotsky, 2008).

Two mutant variants have been described; the pre-C mutants and the core promoter mutants. Expression of HBeAg is inhibited in pre-C mutants during viral translation where a double nucleotide mutation (G1896A/C1858T) results in the insertion of a premature stop codon (TAG). These pre-C mutants are also reported to naturally occur in chronic carriers of HBV infection following seroconversion of HBeAg by specific antibodies (anti-HBe). The lack of HBeAg synthesis by the pre-C mutants allows for viral immune escape, particularly from antibodies against HBeAg (anti-HBeAg) (Tong *et al*., 2005). Core promoter mutants usually have nucleotide substitutions in the basal core promoter (BCP) region with the mutational hotspots being A→T at position 1762 and G→A at position 1764. Mutations in the BCP region, in contrast to pre-C mutants, reduce the expression of HBeAg at the viral transcription level (Parekh *et al*., 2003; Yokosuka and Arai, 2006). The A1762T/G1764A BCP mutation and the G1896A pre-C nonsense mutation, which is often accompanied by a G1899A mutation, are associated with the re-activation phase of chronic hepatitis B (Cao, 2009).

The core promoter region is regulated by elements referred to as enhancers (Enh I and Enh II) and these are also prone to mutations. A combination of mutations in the Enh II/BCP/pre-C regions such as C1766T/T1768A, A1762T/G1764A/C1766T, T1753C/A1762T/G1764A, and T1753C/A1762T/G1764A/C1766T mutations have been associated with increased HBV DNA replication efficiency and cumulative down-regulation of HBeAg expression. These mutations are medically relevant as a link between increased viral replication and development of fulminant hepatitis is suggested. Mutations in the core promoter region are also reported to be a risk factor for development of severe liver disease and HCC (Yokosuka and Arai, 2006; Jammeh *et al*., 2008; Cao, 2009).

2.7.2.2 X gene mutants

The HBV X protein (HBx), comprising of 154aa residues, is the smallest of the HBV proteins. It is encoded within the X ORF which contains regions of high conservation. A multifunctional protein; HBx is composed of an N-terminal negative regulatory/anti-apoptotic domain and a C-terminal transactivation/pro-apoptotic domain. These domains act to regulate the activation of transcription at various viral and cellular promoters (including that of proto-oncogenes), as well as host cell proliferation during the course of infection. The X protein also binds to and inhibits a host tumor suppressor protein (p53), and is capable of activating various cell signal pathways (Murakami, 2001; Bouchard and Schneider, 2004; Yokosuka and Arai, 2006). In view of these functions, mutations within the X gene are associated with a loss of the growth-suppressive property of the X protein and induction of anti-apoptotic events, leading to uncontrolled cell proliferation and consequently hepatocarcinogenesis (Léon *et al*., 2005).

The coding sequence of the X gene is partially overlapped by the core promoter region and for this reason mutations in the core promoter, particularly the double mutation; A1765T and G1767A, induce amino acid substitutions M130K and V131I in the X protein. The M130K and V131I X gene mutations have been linked with the development of severe liver damage as well as HCC (Léon *et al*., 2005). Further alterations in the X gene may arise as a result of deletions within the BCP region which have been reported to induce frame-shift mutations within the X protein which then results in the synthesis of truncated X proteins. These truncated X proteins lack the p53-dependent transcriptional repression binding site and have been shown to enhance the transforming ability of cellular oncogenes. It is suggested therefore that the truncated X proteins may be associated with HCC development (Yokosuka and Arai, 2006). An X gene mutant with impaired viral fitness may also result, bearing 8 nucleotide deletions in the X gene from nucleotide positions 1770 to 1777. This deletion truncates 20 amino acids from the C-terminal domain of the X protein which is responsible for suppressed viral replication and reduced expression of HBV DNA (Léon *et al*., 2005; Yokosuka and Arai, 2006).

2.7.2.3 Polymerase (P) gene mutants

The P ORF takes up almost 80% of the entire HBV genome with up to 845aa residues, and comprises five sub-domains (A-E) and four domains (the N-terminal domain, the spacer

domain, the polymerase domain and the C-terminal domain). The C sub-domain contains the tyrosine-methionine-aspartate-aspartate (YMDD) catalytic motif of the viral reverse transcriptase enzyme. The N-terminal domain encodes the terminal protein (TP) which serves as a primer for viral genome replication while the C-terminal domain encodes the RNase H enzyme responsible for cleaving viral pg-RNA. The polymerase domain on the other hand encodes the viral reverse transcriptase enzyme which is indispensible to the viral replication process (Yokosuka and Arai, 2006).

Accordingly, antiviral drugs developed for treatment of chronic hepatitis B include nucleos(t)ide reverse transcriptase inhibitors (NRTIs) which act to suppress viral replication by preventing viral genome amplification. However, the success of NRTIs in managing chronic hepatitis B has been hampered by the emergence of therapy escape (drug resistant) mutants which possess mutations within the polymerase domain including the YMDD motif in the C sub-domain (Figure 2.6). Prolonged monotherapy with an NRTI is said to be responsible for selection of these drug resistant variants. The drug resistant mutations that develop are specific to individual NRTIs but still have the potential to induce some degree of cross resistance to other NRTIs. For example, the rtM204I and rtM204V mutations that confer resistance to Lamivudine also encode resistance to Telbivudine and may reduce sensitivity to Entecavir (Shaw *et al*., 2006; Zoulim and Locarnini, 2009). Figure 2.6 shows the relevant drug resistance mutations to some of the NRTIs currently available for treatment of chronic hepatitis B. These drug resistance mutations complicate patient management as reduced susceptibility to therapy may result in treatment failure and advanced liver disease. Due to the transmissibility of drug resistant mutants, mutations in the P gene also pose a public health concern (Shaw *et al*., 2006; Cao, 2009).

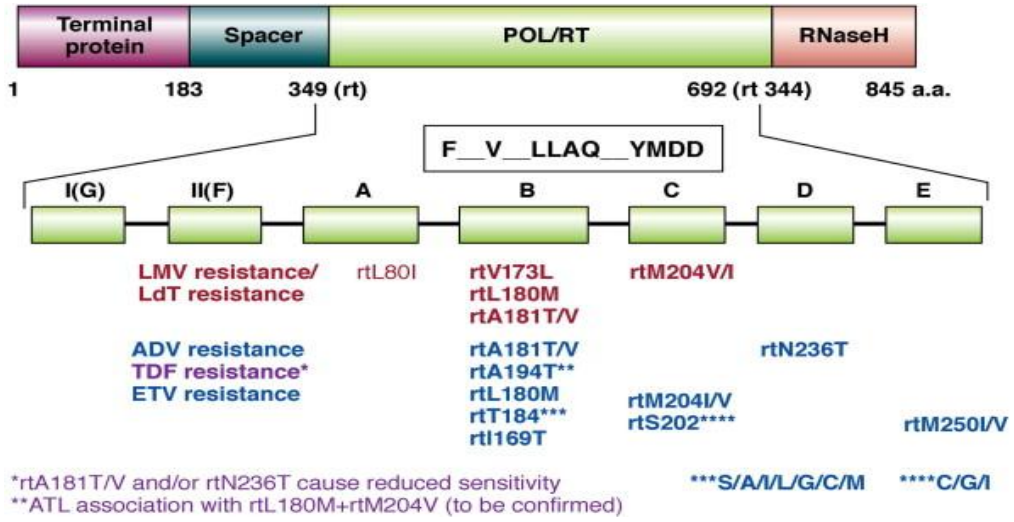


Figure 2.6: The location of primary antiviral drug resistance mutations to nucleos(t)ide reverse transcriptase inhibitors; Lamivudine (LMV), Telbivudine (LdT), Adefovir dipivoxil (ADV), Tenofovir (TDF) and Entecavir (ETV), in the HBV polymerase protein (Zoulim and Locarnini, 2009)

2.7.2.4 Pre-surface (pre-S) and surface (S) gene mutants

The pre-S domain is located upstream of the S domain and is divided into pre-S1 and pre-S2 regions (Lüsbrink *et al*., 2009). The pre-S1 domain bears the epitope for HBV-specific host cell receptor, NTCP, and as such is responsible for binding of the virus to host cells (Neurath *et al*., 1986; Yan *et al*., 2012). The pre-S2 together with the pre-S1 and S domains encode the large surface protein (LHBs). The LHBs is essential to virion assembly, and its intracellular levels are linked with the release of viral particles from the infected cell. The medium (MHBs) and small (SHBs) surface proteins on the other hand are encoded by the pre-S2/S and S domains respectively and appear to be uninvolved in virion assembly and release. Mutations in the pre-S2 start codon result in a lack of synthesis of the pre-S2 protein (Yokosuka and Arai, 2006). Deletions in the entire pre-S region or its binding site have also been associated with reduced synthesis of SHBs (typically the most abundant of the three surface proteins) while increasing LHBs levels proportionally. This results in accumulation of LHBs at the host cell ER where the viral envelope is synthesized, and leads to stressing of the ER and consequently the infected hepatocytes as well. This is commonly observed as the development of ‘ground glass’ hepatocytes in HBV-related HCC and chronically infected individuals (Wang *et al*., 2006; Yokosuka and Arai, 2006).

The viral envelope protein (encoded by pre-S1/pre-S2/S domains) comprises of 389 or 400 amino acid (aa) residues, depending on the HBV variant. A total of 226aa of this protein constitute the HBsAg. The main antigenic determinant, 'α', is a component of the HBsAg and serves to prime the host immune system to elicit neutralizing antibodies during an infection. These protective antibodies bind to the Major Hydrophilic Region (MHR), a conformational epitope of the HBsAg. This immune response makes neutralization of the virus possible and effects immune clearance of an HBV infection (Echevarría and Avellón, 2006).

Amino acid substitutions in the S domain are the main cause of development of HBV surface gene mutants. This particularly occurs within the 'α' antigenic determinant, resulting in a conformational change to the epitopes and thus altering the antigenic properties of the HBsAg. The host neutralizing antibodies may then be unable to bind to MHR due to the conformational changes and as such the mutations that arise may confer immune escape properties to the virus (Weber, 2005; Echevarría and Avellón, 2006). Immune escape mutants may be selected for by immune pressure from the host immune response to infection or immunomodulators, such as hepatitis B immunoglobulin (HBIG), which mainly contain antibodies against HBsAg. The virus thus persists and in the absence of effective host immune protection or immune therapy, causes a heightened infection (Zuckerman and Zuckerman, 2003).

Diagnostic escape is another property of HBV S gene mutants. Manufacturers of diagnostic assays that screen for HBsAg usually target different regions of the viral surface protein using conjugated antibodies. These monoclonal and polyclonal antibodies may be unable to detect mutant variants of HBV and thus mutations in the regions targeted by these assays may allow the virus to remain undetected, leading to under-diagnoses of infection (Echevarría and Avellón, 2006; Yokosuka and Arai, 2006; Alavian *et al*., 2012). This presents with a major public health problem as these assays are used in routine diagnosis as well as screening of blood donations, organ donors and pregnant women. Diagnostic escape mutants are also responsible for under-diagnosis of occult HBV infections. This is as a result of reduced immunoreactivity of the mutant surface protein with commercially available assays leading to undetectable HBsAg despite the presence of viral DNA (Alavian *et al*., 2012). Impaired production of HBsAg leading to diagnosis of occult HBV infection is also linked with mutations in the pre-S region. Occult HBV infections are a medical and public health concern as asymptomatic carriers usually remain undiagnosed and untreated,

and serve as a major source of spread of HBV infection (Torbenson and Thomas, 2002; Purdy, 2007).

Commercially available hepatitis B vaccines contain subunit preparations of HBsAg and so may select for HBV surface gene mutants with vaccine escape properties. Administration of HBIG therapy has also been implicated in the selection of HBV vaccine escape mutants (Carman, 1997; Yokosuka and Arai, 2006). The vaccine escape mutants are not neutralized by the antibodies induced by the current vaccines as they do not cover for HBV mutant strains. For this reason, vaccine escape mutants persist and cause HBV infection in fully vaccinated individuals, referred to as breakthrough HBV infections (Carman *et al*., 1990; Carman, 1997; Zuckerman and Zuckerman, 2003). Vaccine escape mutants are stable and thus may be transmitted to uninfected individuals. This creates a major concern as vaccination is currently the most effective preventive measure available (Weber, 2005). The most notable mutation responsible for HBV vaccine escape is a point mutation (G→A) at nucleotide position 587 of the S gene, consequently replacing glycine (G) with arginine (R) at amino acid residue 145 within the 'α' antigenic determinant (G145R). Other common mutations involved in HBV vaccine escape include D144A, P142S, Q129H, I/T126N/A and M133L (Carman *et al*., 1990; Carman, 1997; Torresi, 2002; Yokosuka and Arai, 2006).

In addition to the primary pre-S/S gene mutations, mutations in the P gene can further select for S gene mutants and vice versa due to the overlapping orientation of the two genes. A triple lamivudine resistance mutation; rtV173L/rtL180M/rtM204V for example, has been reported to induce amino acid substitutions within the surface protein (E164D/I195M) which consequently results in enhanced viral replication as well as reduced antigenicity of HBsAg (Torresi *et al*., 2002). Thus immune, vaccine as well as diagnostic escape mutants may be selected for by mutations in the overlapping P gene. Diagnostic escape mutants selected for in this manner cause patients who were previously treated with Lamivudine (and developed resistance to it) to appear to have cleared the infection as HBsAg is undetectable (Sheldon and Soriano, 2008). Mutations in the surface protein (viral envelope protein) which are induced by P gene mutations, such as insertion of stop codons, may also impair viral fitness. However compensatory mutations within the polymerase domain help to counteract these deleterious mutations and restore viral replication and viral fitness (Torresi, 2002; Torresi *et al*., 2002).

2.8 Pathogenesis of HBV infection

Exposure to HBV sets off a cascade of complex interactions between the virus and the non-immune host which results in a diverse spectrum of clinical manifestations raging from an asymptomatic state to an acute or fulminant hepatitis and finally to a chronic disease with progression to various sequelae. The pathogenesis of HBV infection is therefore a function of viral replication and host immune responses to the presence of the virus (Baumert *et al*., 2007; Dandri and Locarnini, 2012). Other factors such as host factors (immune status and genetics), viral factors (the size of the inoculum, the virulence of the HBV strain causing the infection and co-infection with other pathogens) as well as environmental factors (alcohol use and exposure to toxins) all influence the natural history of an HBV infection (Fattovich *et al*., 2008).

HBV is a hepatotropic virus and as such its major target organ is the liver which is composed of a diversity of cells including bile ductule epithelia, Kupffer cells and hepatocytes, all of which bear the bulk of the liver's functionality. The liver is vital for various reasons including its ability to process nutrients and store energy, remove toxins from the body, effect blood homeostasis and assist in immune response to microbial infection. For this reason injury or infection to this organ may interfere with any of its important roles, impairing the overall state of health. Of all the cells of the liver, HBV has a tropism for hepatocytes which constitute about 70% of the liver's cell mass (Seeger and Mason, 2000). The virus is also capable of infecting extrahepatic cells such as peripheral blood lymphocytes (PBL) and monocytes which may share the same HBV receptor with the hepatocytes. Following entry, HBV replicates within target cells and in so doing interferes with the normal functioning of the cell. This process does not induce cytopathic effects however, and as such the virus by itself is not the direct cause of cell and liver damage. It is reported that the host immune response to viral antigens presented on the surface of infected cells is the main contributing factor to liver injury (Ganem and Prince, 2004; Dandri and Locarnini, 2012).

2.8.1 Acute HBV infection

Following primary exposure to the virus, an asymptomatic incubation period ensues, typically lasting between one to four months. During this period no effective innate immune reaction to the presence of the virus is detected and it is this delayed response that accounts for the increased viral replication and consequential widespread infection of the hepatocyte

population (Seeger and Mason, 2000). Active viral replication is characterized by the presence of HBeAg (a tolerogen and indicator of infectivity) in serum, accompanied by a high titre viremia and HBsAg which may fluctuate over time but finally becomes negative after 2-4 months of the infection (Figure 2.7).

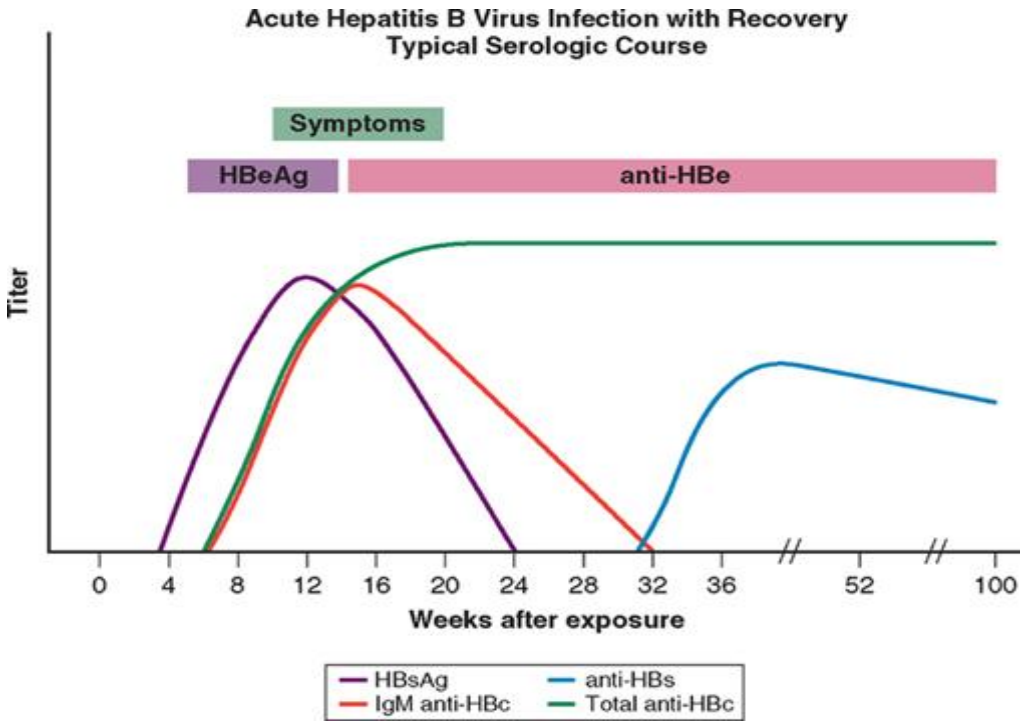


Figure 2.7: Graphical representation of the typical serologic course during an acute HBV infection (http://virology-online.com/viruses/HepatitisB.htm, accessed on 14th February, 2012)

In an attempt to contain the rapid spread of the virus, the host mounts an adaptive humoral immune response. High levels of antibodies against the viral core antigen (initially IgM anti-HBc and later total anti-HBc; IgM+IgG) and HBeAg (anti-HBe) are secreted but these do not provide any recognized immune protection (Huang *et al*., 2006). This is followed closely by production of neutralizing antibodies to HBsAg (anti-HBs) which bind to the large amounts of HBsAg and eliminate cell-free virions. During this process however, large amounts of immune complex (HBsAg/anti-HBs) deposits form, and these circulate in the blood stream to cause a type III hypersensitivity reaction characterized by skin rash, athralgia and in some cases nephritis (Bertoletti and Gehring, 2006; Huang *et al*., 2006).

A cell-mediated immune response to the infection is also recruited, typically involving virus-specific cytotoxic T lymphocytes (CTL; $CD8^+$ T cells) against viral antigens presented by major histocompatibility complex molecules (MHC class I) on infected cell surfaces. These $CD8^+$ T cells act to effect clearance of cell-bound virions by both cytolytic and non-cytolytic means, essentially reducing the viral load (Bertoletti and Gehring, 2006). Lysis of infected hepatocytes results in leakage of liver aminotranferases (ALT and AST) into the blood stream, indicating damage to the liver cells (Raimondo *et al*., 2003; Pungpapong *et al*., 2007). This damage results in onset of hepatitis associated with clinical symptoms such as right upper quadrant discomfort, nausea and jaundice among other non-specific symptoms. These symptoms may persist for up to three months after which they resolve with hepatocyte regeneration and undetectable viremia. Viral DNA (cccDNA) may not be completely cleared after recovery but may still be present within intact hepatocytes; a source of hepatitis B flares in the event of an immune-suppressed state. Complete recovery from acute hepatitis B is almost always certain but in rare cases (0.1-1%) death may result from liver failure due to onset of fulminant hepatitis. Acute HBV infection may also progress to a state of chronicity (Raimondo *et al*., 2003; Pungpapong *et al*., 2007).

2.8.2 Chronic HBV infection

Chronic hepatitis B is defined by persistent HBV infection with high titre serum HBsAg positivity for more than 6 months (Lok and McMahon, 2001). This follows failure of the host immune system to effectively clear an acute HBV infection after primary exposure or to protect against subsequent infection. Thus viral replication persists and is accompanied by HBeAg positivity for a long period of time with eventual seroconversion to anti-HBe after years of infection (Figure 2.8).

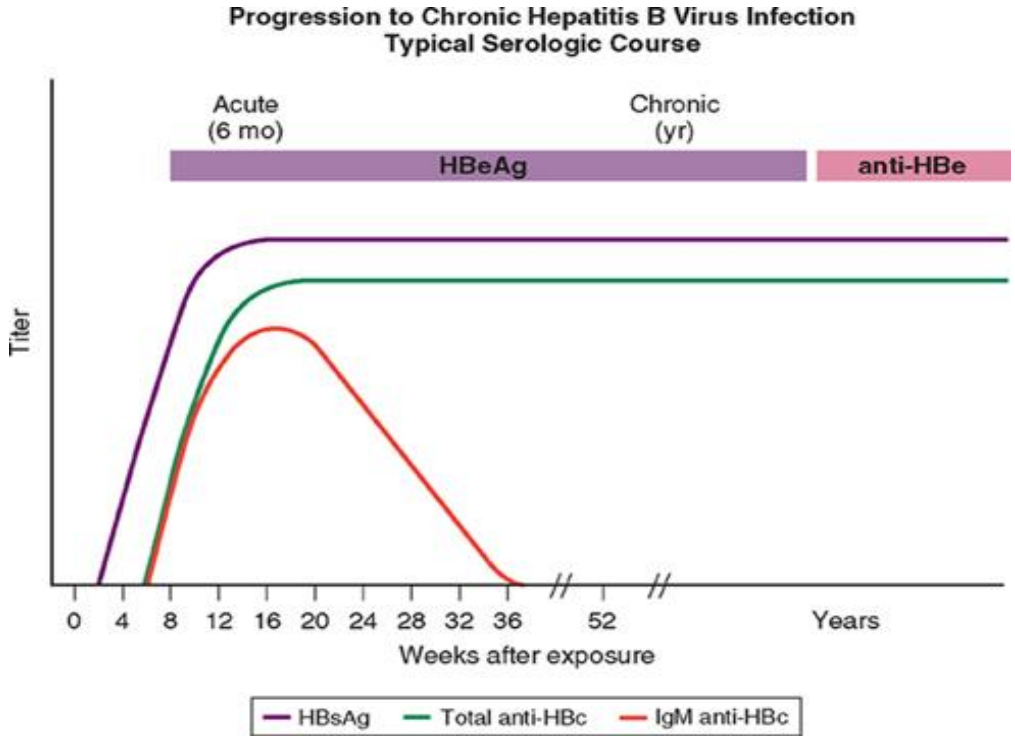


Figure 2.8: Graphical representation of the typical serologic course during the progression from acute HBV infection to chronic hepatitis B (http://virology-online.com/viruses/HepatitisB.htm, accessed on 14th February, 2012)

The eventual course of a chronic HBV infection is variable, typically involving four dynamic phases: the immune tolerance phase, the immune clearance phase, the inactive carrier phase and the reactivation phase. The ultimate outcome of chronic HBV infection is also varied, usually involving sequelae such as liver cirrhosis, rare cases of fulminant hepatitis and in some cases HCC, all of which can be fatal (McMahon, 2009; Shi and Shi, 2009).

2.8.2.1 Immune tolerance phase

The immune tolerance phase is clinically described as persistent HBeAg positivity with viral DNA levels ≥20 000 IU/mL and no effective immunological reaction to the presence of the virus (Tran, 2011). This is typically the first phase of chronic infection in perinatally- and childhood-acquired HBV infections but is rarely observed in those infected later on in life. Patients in this phase are highly infective due to the high level of viremia. However, despite extensive intrahepatic viral replication coupled with the absence of an efficient immune response, no liver injury is evident and thus infected hepatocytes stay intact and blood

alanine transaminase (ALT) levels remain persistently normal (Fattovich *et al*., 2008; Kao and Chen, 2008; Shi and Shi, 2009; Tran, 2011).

This state of immune inactivity is largely attributed to the tolerogenic property of viral HBeAg which has been reported to induce T helper (T_h) cell tolerance to HBeAg and to render the CTL response to HBcAg ineffective (Kao and Chen, 2008; Tran, 2011). The extent of viral replication and high levels of viral antigens secreted are also reported to exhaust HBV-specific $CD8^+$ T cell response. It is further proposed that regulatory T cells (T_{reg}) may also play a role in immune tolerance to infection by suppressing HBV-specific T cell response (Kao and Chen, 2008). In perinatally-acquired infections, it has been suggested that maternal HBeAg is transferred via the placenta to the foetus where the viral antigen induces clonal deletion or ignorance of foetal HBV-specific T cells (Bertoletti and Gehring, 2006; Kao and Chen, 2008; Tan *et al*., 2008). These mechanisms effect a quiescent disease state which may last up to several decades after which immune reactivity may be restored (Shi and Shi, 2009; Tran, 2011).

2.8.2.2 Immune clearance phase

As the host immune response is restored, an immune reaction is mounted in an effort to clear the infection during what is referred to as the immune reactive or clearance phase of chronic hepatitis B. The majority of adult-acquired HBV infections that go on to achieve a state of chronicity progress directly to this phase shortly after infection (Pungpapong *et al*., 2007; McMahon, 2009). The host immune response causes serum HBV DNA levels to decline, reaching up to 2000 IU/ml while blood ALT titre elevates as infected hepatocytes are destroyed (Fattovich *et al*., 2008; McMahon, 2009). Thus previously asymptomatic patients begin to experience bouts of clinical manifestations consistent with hepatic injury, referred to as acute exacerbation of chronic hepatitis B. The symptoms mimic acute HBV infection and are usually self limiting following liver inflammation which in some cases could be coupled with liver fibrosis. Cases of mortality during this phase are mainly the result of liver failure. In most immunocompetent patients however, remission of liver disease is the common outcome and is followed by spontaneous seroconvertion to anti-HBe with fluctuating levels of serum HBeAg (Lin and Kao, 2008; Shi and Shi, 2009).

2.8.2.3 Inactive carrier phase

The success of the immune clearance phase in effecting viral clearance is evident in the reduction of viral DNA levels to <2000 IU/ml. With continuous suppression of viral replication, a progression to the inactive carrier phase occurs, characterized by the presence of persistent HBsAg in serum and anti-HBe positivity in the absence of HBeAg following seroconversion (Hadziyannis, 2007; Fattovich *et al*., 2008). Host ALT levels also return to normal as the liver recovers from immune-mediated damage. Accordingly minimal or no hepatic inflammation is observed during this stage of chronic HBV infection. Over 300 million of the world's population are HBsAg inactive carriers, and these individuals remain asymptomatic for long periods of time but are still reservoirs of the infection. The prognosis during this phase is quite favourable with low risk of development of liver cirrhosis or HCC (Sharma *et al*., 2005; Hadziyannis, 2007). However, up to 30% of patients in this phase of the disease may spontaneously revert to the immune clearance phase with presentation of flares of acute hepatitis which may resort to liver damage or decompensated hepatitis. On the other hand, some patients may go on to develop anti-HBs and clear the viral HBsAg after sustained inactive infection, further improving the prognosis (Sharma *et al*., 2005; McMahon, 2009).

2.8.2.4 Reactivation phase

Reactivation of HBV infection following apparent resolution is as a result of resurgence of viral replication usually due to immune suppression. Reactivation which occurs in ≥30% of HBsAg inactive carriers can be discussed in three stages; an increase in HBV replication, the appearance of hepatic injury and finally recovery from infection (Hoofnagle, 2009). Although HBsAg inactive carriers develop anti-HBs which suppresses viral replication, low levels of viral DNA (cccDNA) still persist within infected hepatocytes and may begin to replicate if the immune system is compromised. This is characterized by an increase in viral DNA and a reverse seroconversion from anti-HBe to HBeAg (Fattovich, 2008). In the event of immune restoration, viral clearance ensues accompanied by a marked elevation in blood ALT level due to the immune-mediated destruction of infected hepatocytes. This leads to the appearance of hepatitis associated with jaundice and other clinical symptoms. These symptoms may be severe in some patients and can culminate in acute hepatic failure and death. The hepatitis stage may however persist and re-establish chronic HBV infection with acute exacerbations, which carry a risk of development of liver cirrhosis due to progressive hepatic injury. Recovery is possible in some cases and shows a decline in viral load and

blood ALT levels as the clinical symptoms of hepatitis abate. Patients may then return to the inactive phase of chronic HBV infection (Hoofnagle, 2009).

2.8.3 Occult HBV infection

Occult HBV infection is a form of chronic HBV infection characterized by the persistence of low levels of HBV DNA (<200 IU/ml). This state is mainly associated with suppressed viral replication and gene expression which accounts for the undetectable levels of HBsAg and in some cases negligible serum HBV DNA levels (Brèchot *et al*., 2001; Raimondo *et al*., 2007). An overlap exists between the time of recovery from an HBV infection and the development of occult HBV infection. This is due to the fact that following recovery low levels of HBV DNA still persist for long periods of time within infected cells in the form of episomes (cccDNA). The primed host immune response acts to suppress replication of these viral episomes which hinders synthesis of viral proteins and production of new viral particles. Occult HBV infection may also develop as a result of infection with mutant HBV variants characterized by low replication rates and impaired synthesis of viral proteins or having diagnostic escape properties (discussed under section 2.7.2.4). It is also suggested that co-infection with other pathogens may assist in down-regulation of viral replication, resulting in an occult HBV infection (Chemin and Trépo, 2005; Raimondo *et al*., 2007; de la Fuente *et* al., 2011).

The serological profile of occult HBV infection may include rarely detectable HBeAg with seronegativity or positivity for either anti-HBs or anti-HBc. It has also been reported that in 20% of occult hepatitis B cases, all serological markers remain entirely absent despite the presence of viral DNA (Brèchot *et al*., 2001; de la Fuente *et* al., 2011). Due to atypical serological markers of exposure, coupled with the absence of specific symptoms, occult HBV infection may go unnoticed for long periods of time. In the event of immune suppression however, occult HBV carriers may revert to an acute infection accompanied by the typical serological profile and clinical symptoms (de la Fuente *et* al., 2011). The significance of occult HBV infection lies in its ability to be transmitted from seemly healthy individuals to the uninfected population, one of the main sources of transfusion- and transplantation-related spread of HBV infection. The possibility of reactivation of infection also poses a great concern as occult HBV infection is reported to accelerate progression to chronic liver disease and liver cirrhosis and also carries a great risk for HCC development (Brèchot *et al*., 2001; Chemin and Trépo, 2005; Larrubia, 2011).

2.8.4 Long-term sequelae of chronic hepatitis B

Chronic hepatitis B may develop into various long-term complications, usually including liver failure, liver cirrhosis and HCC. These complications greatly impair the prognosis of the disease and increase the risk of mortality from HBV infection.

2.8.4.1 Liver cirrhosis

Cirrhosis or scarring of the liver is a direct result of chronic hepatic injury, characterized by the development of liver fibrosis and the appearance of scar tissue and regenerative nodules on the liver. The risk of development of cirrhosis in chronic hepatitis B is highest in the reactivation phase of the disease. Severe recurrent flares of infection to the liver coupled with continuous viral replication during this phase increase chances of development of liver scarring. Following the establishment of cirrhosis, the liver begins to decompensate, unable to replace damaged hepatocytes. The scar tissues that develop in-place-of the damaged hepatocytes alter the natural architecture of the liver and obstruct normal blood flow throughout the organ. A loss of liver function is the eventual outcome especially in advanced cirrhosis, which results in hepatic failure and death (Ganem and Prince, 2004; Shi and Shi, 2009).

2.8.4.2 Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) or hepatoma refers to a primary malignant tumour or cancer of the liver. It is the fifth most common cancer in men and the seventh in women, accounting for a total of 6% of all cancers occurring worldwide. HCC is a leading cause of cancer-related deaths globally, especially in regions endemic for hepatitis B. For this reason, chronic hepatitis B is reported to be the major risk factor for development of HCC with an infectious aetiology, accounting for more than half (~54%) of the world's total HCC cases (Parkin, 2006; Shi and Shi, 2009). The majority (70-80%) of these HCC cases develop in advanced cirrhotic patients, however HCC may also develop in the absence of liver cirrhosis. Two specific mechanisms have been implicated in HBV-related hepatocarcinogenesis, namely, the integration of viral DNA into the host genome during chronic infection and the expression of the viral X gene (Lupberger and Hildt, 2007; Chemin and Zoulim, 2009). Integration of HBV DNA is a random process but may occasionally target host gene families that regulate the survival of the host cell, its proliferation and immortalization. Thus mutations

(insertions) within these host genes may result in an alteration of their normal functioning such as a loss of function of the host tumour suppressor gene, uncontrolled cell proliferation and activation of proto-oncogenes, leading to malignant transformation of hepatocytes. Altered expression of the HBV DNA integrate is also reported, resulting in the synthesis of truncated pre-S (LHBs) and HBx proteins, both of which are involved in hepatocyte transformation (Lupberger and Hildt, 2007; Chemin and Zoulim, 2009; Neuveut *et* al., 2010). The viral HBx protein is duly termed an oncoprotein based on its ability to influence the cell cycle and interfere with cell signal transduction and expression of host genes involved in cell proliferation and oncogenesis. It is thus capable of altering the rate of hepatocyte proliferation and inducing liver tumourigenesis (Chemin and Zoulim, 2009; Neuveut *et* al., 2010). These mechanisms together with various risk factors such as the host immune response to infection and host genetic predisposition to HCC as well as extensive viral replication, occult HBV infection and infection with HBV mutant variants all contribute to the development of HCC following chronic disease. HCC generally presents with a poor prognosis and is associated with a high mortality rate as it is usually diagnosed in the advanced stages at which point medical interventions have limited impact (Shi and Shi, 2009).

2.8.5 HBV co-infections

Co-morbidities associated with HBV infection greatly influence the natural course of hepatitis B. Of note are those involving HBV and other hepatitis viruses, particularly HCV and HDV, as well as co-infections with HIV. The possibility of co-infection with these viruses is heightened due to similarities in their risk factors for infection and routes of transmission.

Co-infection of HBV with HCV is highly significant because of the frequency in its occurrence, especially in regions endemic for both viruses, and the poor prognosis associated with the resulting disease. An HBV/HCV co-infection is characterized by the presence of low levels of HBV DNA and viral proteins in serum. This is a result of the suppressive effect that the HCV “core” protein exerts on HBV transcription and gene expression, leading to the development of occult hepatitis B which is evident in the majority of HBV/HCV co-infections (Raimondo *et al*., 2007). In spite of the low HBV viral load in HBV/HCV co-infected individuals, HCV has been reported to accelerate progression to

hepatitis decompensation and liver cirrhosis and also increases the risk of HCC development in HBV/HCV infected individuals (Cheruvu *et al*., 2007).

The global estimate on the number of HBV/HIV co-infections is reported to be as high as 4 million, since ~10% of the global total of HIV infected individuals are HBV chronic carriers (Cheruvu *et al*., 2007; Burnett, 2008). In contrast to HBV/HCV co-infection, HBV/HIV co-infection has a lesser but remarkable association with HCC development. The relevance of HBV/HIV co-infections is mainly found in the influence that HIV has on the host immune system. Infection with HIV causes immune deficiency and for this reason the pathogenesis of hepatitis B in HBV/HIV co-infected individuals is characterized by a higher viral load and elevated levels of viral antigens with minimal immune-mediated hepatic injury (Puoti *et al*., 2002; Thio, 2003; Cheruvu *et al*., 2007). In the event of immune reconstitution, the possibility of hepatitis B reactivation and onset of multiple hepatic flares in HBsAg inactive carriers with HIV infection, creates a great cause for concern (Burnett, 2008). Those who remain immune-deficient are reported to be less likely to sustain protective anti-HBs levels and as such are also less likely to spontaneously clear the HBV infection, placing them at a higher risk of developing a chronic disease and increasing their risk for progressive liver morbidity and premature mortality (Thio, 2003; Puoti *et al*., 2006; Cheruvu *et al*., 2007).

2.9 Diagnosis of HBV infection

An infection with HBV can be diagnosed either during the acute or chronic stages of disease. Although presentation of certain clinical symptoms such as right upper quadrant discomfort, nausea and jaundice may be suggestive of an acute HBV infection, the bulk of acute HBV infections are subclinical and as such most diagnoses are made during the active stages of chronic disease. Definitive diagnosis of HBV infection is made with the aid of laboratory tests that assess serological, molecular, biochemical and histological markers of infection, which are analysed together with patient history (risk factors predisposing to an HBV infection) (Chevaliez and Pawlotsky, 2008).

Laboratory diagnosis of an HBV infection relies on definitively distinguishing the virus form all other possible aetiologies of hepatitis (Krajden *et al*., 2005). Due to the lack of reliable *in vitro* tissue culture systems for the cultivation of HBV, differential detection of the virus is

dependent on other laboratory tests. Serological tests, for example, are a commonplace diagnostic tool used in determining the presence of specific HBV antigens and their corresponding antibodies (Servoss *et al*., 2005). Other diagnostic tests include sensitive molecular methods which detect the presence of viral nucleic acids. An assessment of liver function with the use of biochemical tests also assists in differentiating viral hepatitis from non-viral causes of hepatic injury and helps monitor the prognosis of disease.

For laboratory diagnosis of an HBV infection, blood is the specimen of choice as it is a relatively non-invasive specimen and, since it circulates throughout the body, harbours an abundance of viral particles and secreted proteins during an infection (Krajden *et al*., 2005; Servoss *et al*., 2005). Other specimens may also be used in diagnosing HBV infections, including liver biopsies which are used in histopathology (grading and staging of liver disease) and immunohistochemical staining (detection of viral antigen in liver tissue). Since liver biopsies are invasive specimens, these methods are rarely used during clinical diagnosis of an HBV infection (Fang and Lau, 2004).

2.9.1. Serological testing

Screening for serological markers (viral antigens and host antibodies) helps establish a suspected HBV infection and distinguishes acute self limiting infections from the different phases of chronic disease. A number of automated systems exist for quantitative and qualitative determinations of serological markers within clinical specimens (serum or plasma), employing the enzyme immunoassay (EIA) principle together with colorimetric or chemiluminescence signal measurement of results (Servoss *et al*., 2005; Chevaliez and Pawlotsky, 2008). The diagnostic algorithm in Table 2.1 depicts the interpretation of results for the primary serological markers; HBsAg, anti-HBc, anti-HBs, and IgM anti-HBc. Secondary prognostic markers including HBeAg which gives an indication of persistent viral replication as well as infectivity and anti-HBe which denotes seroconversion and a less infectious state, may also be tested (Krajden *et al*., 2005).

Table 2.1: Diagnostic algorithm for hepatitis B serology (adapted from CDC, 2011).

TEST PROFILE	RESULTS	INTERPRETATION
HBsAg Anti-HBc Anti-HBs	Negative Negative Negative	Susceptible, never been infected
HBsAg Anti-HBc Anti-HBs	Negative Positive positive	Immune due to natural infection
HBsAg Anti-HBc Anti-HBs	Negative Negative Positive	Immune due to hepatitis B vaccination
HBsAg Anti-HBc IgM anti-HBc Anti-HBs	Positive Positive Positive Negative	Acutely infected
HBsAg Anti-HBc IgM anti-HBc Anti-HBs	Positive Positive Negative Negative	Chronically infected
HBsAg Anti-HBc Anti-HBs	Negative Positive Negative	Varied interpretation*

*1. Resolved infection (most common)
2. False-positive anti-HBc, thus susceptible
3.”low level” chronic infection
4. Resolving acute infection

2.9.2 Biochemical testing

In the event of an established HBV infection, routine liver biochemical tests aid in determining the presence of hepatic injury and give an indication of the prognosis of disease (Krajden *et al*., 2005). Varying measures of certain biochemical markers (aminotransferases, alkaline phosphatase, gamma-glutamyl transferase, bilirubin and albumin) assess liver viability and functioning. These biochemical markers may be released into circulation upon damage to liver cells or during an alteration in liver metabolism. They are however highly variable as they depend on several factors including age, gender, physical exercise, stress, time of day as well as food ingestion, and may also vary from one individual to the next. A commonly tested biochemical marker in hepatitis B diagnosis is serum ALT, an aminotransferase which is measured against cut-off values (upper limit of normal; ULN) to determine damage to the liver (Krajden *et al*., 2005). Sustained and elevated (above the ULN) intermittent levels of serum ALT are indicative of hepatic injury or inflammation and correlate with progressive liver disease. In some cases however, individuals with severe liver disease may not manifest elevated ALT levels at all, placing merit on other laboratory tests to arrive at a diagnosis (Krajden *et al*., 2005; Chevaliez and Pawlotsky, 2008).

2.9.3 Molecular testing

A number of nucleic acid based tests for the direct detection of viral DNA are widely available. These molecular methods make use of either signal (HBV Hybrid-Capture I, II) or target (conventional nested and real-time PCRs) amplification based assays specific for the qualitative and quantitative (sensitivities reaching up to 10^1-10^2 copies/mL for some PCR assays) detection of HBV DNA (Loeb *et al*., 2000; Pawlotsky, 2002; Servoss *et al*., 2005). Determining the presence of HBV DNA is an alternative to serological tests for HBeAg as it correlates with the presence of active HBV infection with high viral loads indicating infectivity. Thus molecular diagnostic methods provide supplementary information regarding viral replication during the course of an HBV infection, especially in cases where serological profiles are atypical.

2.10 Treatment of chronic HBV infection

The public health goal, second to preventing HBV infection, is reducing HBV-related morbidity and mortality. This is achieved through treatment with the aim of improving quality

of life by interrupting the development of potentially fatal sequelae (Papatheodoridis *et al*., 2012). For this reason, treatment of chronic HBV infection is intended for sustained suppression of viral replication thereby preventing hepatic flares and bringing about remission of liver disease well before the development of liver cirrhosis and HCC. An ideal response to therapy is defined by very low levels of serum HBV DNA (lower than detectable limit by real-time PCR assays) together with sustained loss of HBeAg or anti-HBe seroconversion, and improvement of liver disease with normalization of biochemical markers (de Franchis *et al*., 2003; Tillmann, 2007; Papatheodoridis *et al*., 2012).

Indication for treatment of chronic HBV infection is based on a combination of three basic criteria; serum HBV DNA level (>10^5 copies/mL or >3.7E+5 IU/mL), serum ALT level (above ULN) and in some cases liver histological grade and stage. Patient's age, severity of liver disease, likelihood of response to therapy, and the risk of adverse effects and complications also require consideration before making the decision to treat (de Franchis *et al*., 2003). Two different types of therapeutic agents are available for the treatment of chronic HBV infection namely, immunomodulators and antiviral drugs (Tillmann, 2007). Immunomodulators for the treatment of chronic HBV infection, such as interferon alpha (IFNα) and pegylated interferon α (peginterferon α; PEG-IFNα), effect an antiviral response by eliciting host cellular immune responses against cell-bound virions (Rang *et al*., 2002; Craxi and Cooksley, 2003). The advantages of PEG-IFNα therapy include finite duration of treatment, no resistance selection as well as durable response to the virus. As PEG-IFNα is also used in hepatitis C therapy, it is effective in the treatment of chronic HBV infection in HBV/HCV co-infected individuals (de Franchis *et al*., 2003). PEG-IFNα has a number of disadvantages however, such as its high cost, the need for frequent subcutaneous administration and risk of adverse effects (Craxi and Cooksley, 2003).

In contrast, nucleos(t)ide reverse transcriptase inhibitors (NRTIs; antiviral drugs) are easily administered orally and are generally free of significant adverse effects, making them the therapeutic agents of choice for chronic hepatitis B treatment (Zoulim and Perillo, 2008; Fung *et al*., 2011). The NRTIs available for treatment of chronic HBV infection include Lamivudine (3TC), Adefovir dipivoxil (ADV), Telbivudine (LdT), Entecavir (ETV), Emtricitabine (FTC) and Tenofovir disoproxil fumarate (TDF). In effect, these NRTIs act to inhibit the viral reverse transcriptase activity which then interrupts HBV DNA replication and consequently suppresses synthesis of infectious virions (Fung *et al*., 2011; Lampertico and

Liaw, 2012). The majority of these NRTIs also form part of the highly active antiretroviral therapy (HAART) regimen for treatment of HIV infection and as such ADV, LdT and 3TC+TDF/Truvada (Truvada is a combination of FTC and TDF) for example, are used in the treatment of chronic HBV infection in HBV/HIV co-infected individuals (Thio, 2003; Soriano *et al*., 2008). A major drawback to the success of antiviral treatment is the development of drug resistance following long-term exposure to some of the NRTIs as discussed under section 2.7.2.3 (Zoulim and Locarnini, 2009; Fung *et al*., 2011). First line use of the most potent NRTIs which have a high barrier to resistance such as ETV or TDF as well as combination therapy with 3TC+TDF or Truvada have been reported to be some of the best strategies for preventing resistance development to NRTIs (Zoulim and Locarnini, 2009; Pol and Lampertico, 2012).

To ensure successful treatment outcome, it is necessary to monitor response to therapy, which involves an evaluation of serum HBV DNA level, serological markers (HBsAg, HBeAg and anti-HBe) and serum ALT levels over the course of treatment. Optimal duration of therapy is not standardized but is dependent on sustained virologic (<2000 copies/mL) and serologic (loss of HBsAg and HBeAg/anti-HBe seroconversion) response to treatment with normalization of serum ALT levels post treatment cessation (Papatheodoridis *et al*., 2012).

2.11 Prevention of HBV infection

Three strategies are described for the prevention of HBV infection, namely; behaviour modification, passive immunoprophylaxis and active immunization (Hou *et al*., 2005). Behaviour modification aimed at reducing the risk of transmission of HBV infection involves the practice of risk-reduction measures such as safe sex and non-sharing of personal items (razors and syringes) as well as adherence to universal precautions in the health care setting in order to minimize nosocomial and occupational HBV exposures. Such measures are more effective in regions where the hepatitis B epidemic is chiefly driven by adult-acquired HBV infections as compared to regions such as Asia and Sub-Saharan Africa where high rates of infant- and childhood-acquired HBV infections are reported respectively (Hou *et al*., 2005; Franco *et al*., 2012). In regions like Asia and Sub-Saharan Africa, passive immunoprophylaxis offers a better means of reducing the incidence of primary HBV infection as compared to behaviour modification. Immunoprophylaxis such as hepatitis B immunoglobulin (HBIG) is administered as a pre-emptive measure in neonates of HBsAg

positive mothers and is also recommended for individuals following needle stick and sexual exposures or after liver transplantation (Hou *et al*., 2005). By and large, active immunization (vaccination) remains the single most effective preventive measure against primary HBV infection and consequently, chronic HBV infection and its fatal sequelae (WHO, 2009a). Safe and highly effective (>95%) hepatitis B vaccines are widely available, traditionally in the form of plasma-derived vaccines (containing highly purified viral surface protein from plasma of chronic HBsAg carriers) and currently in the form of recombinant DNA vaccines (containing the viral surface protein produced through recombinant DNA technology). The vaccine may be administered as a monovalent formulation or in a fixed combination with other vaccines (diphtheria-tetanus-pertussis, *Heamophilus influenzae* type b, hepatitis A and inactivated polio vaccines). In all cases, the hepatitis B vaccine is administered in 3 or 4 doses to elicit production of anti-HBs (optimum protective titre ≥10 mIU/mL) and to prime immune memory cells (B and T_h cells) against the HBsAg component which confers long-lasting (~20 years) immunity to HBV infection (Banatvala *et al*., 2000; WHO, 2009a; Zanetti *et al*., 2008). Timely administration of the hepatitis B vaccine in combination with HBIG as a post-exposure prophylaxis is also highly effective (>90%) in preventing neonatal HBV infections. Hepatitis B vaccination is also recommended for individuals at a high risk for HBV infection, such as health care workers, familial contacts of chronically infected individuals and persons with high-risk behaviours (Hou *et al*., 2005; Franco *et al*., 2012).

2.11.1 Hepatitis B vaccination

The World Health Assembly (WHA) recommended in 1992 that the hepatitis B vaccine be incorporated universally into national routine immunization programmes or national Expanded Programmes on Immunization (EPI) as a means to curb the global burden of hepatitis B (WHO, 1992). The most recent coverage report showed that the hepatitis B vaccine had so far been introduced into national EPIs in 179 of the 193 WHO member states. As a result of this, global hepatitis B vaccination coverage is currently estimated at 75%, reaching as high as 91% in the Western Pacific region while that in the South East Asian region is at 52% (WHO, 2012a). In Sub-Saharan Africa, vaccination coverage was reported at 67% as at the end of 2008 (Franco *et al*., 2012; WHO, 2012a).

2.11.2 Monitoring the impact of hepatitis B vaccination

Monitoring the impact of introducing universal hepatitis B immunization programmes may involve evaluating the prevalence of HBV exposure within a population by conducting serosurveys of current (HBsAg) and past infections (anti-HBc), and population infectivity (HBeAg/anti-HBe) as well as monitoring the incidence (IgM anti-HBc) of new infections, acute diseases (jaundice and acute hepatitis) and chronic sequelae (cirrhosis and HCC) (WHO, 2011). Such impact assessments may focus on either the short-term or long-term achievements of introducing the hepatitis B immunization programme.

2.11.2.1 Monitoring the short-term impact of hepatitis B vaccination

Monitoring the short-term impact of hepatitis B vaccination involves conducting serosurveys in targeted vaccinated cohorts after at least 5 years of implementing the immunization programme (Rani *et al*., 2009; WHO, 2011). The goal of monitoring the short-term impact of hepatitis B vaccination is to measure the prevalence of new or acute HBV infections particularly in children under the age of 5 years. This population represents the age group at which contracting HBV infection is highest, either through vertical or horizontal transmission routes, especially in hyperendemic regions (Shepard *et al*., 2006; Rani *et al*., 2009; WHO, 2011). The prevalence of HBsAg within this age group serves as an indication of future chronic carriage and related sequelae and as such can be used to monitor the immunization programme at least over the short term. Thus the short-term indicator of the success of the hepatitis B immunization programme will be a reduction in the prevalence of new HBV infections measured within the targeted cohort (Shepard *et al*., 2006; Rani *et al*., 2009).

Studies monitoring the short-term impact of implementing universal hepatitis B immunization have revealed a significant influence on the disease burden due to a reduction in the prevalence of new HBV infections on a global scale. This success is a collective of individual achievements by the various countries that heeded the WHO recommendation. Taiwan for example, was the first country to introduce the hepatitis B vaccine into their national EPI in July 1984. As a result, chronic carriage in vaccinated cohorts in Taiwan was found to have declined from 10% in 1984 to 0.6% in 2004 (Ni *et al*., 2007; Ni and Chen, 2010). Similar reductions have also been noted in vaccinated cohorts from other regions including Spain (0.8% to 0.3%), Columbia (8% to 1.1%), South Korea (8% to 3.7%), Alaska, USA (>6% to 0%) and Italy (2.5% to 0%) (Sanmarti *et al*., 2000; de la Hoz *et al*., 2008; Zanetti *et al*., 2008;

Park *et al*., 2010; McMahon *et al*., 2011; Coppola *et al*., 2012). The short-term impact of universal hepatitis B immunization has also been noted within the African region with countries such as The Gambia, the first in the sub-continent to include the hepatitis B vaccine into their EPI in 1986, observing a reduction in chronic carriage rate from 10% to 0.6% (Viviani *et al*., 1999). Similarly, Egypt recorded a 5% reduction (from <6% to ~1.5% in 2002) in chronic carriage rates after almost a decade of nationwide hepatitis B immunization (Sherbini *et al*., 2006).

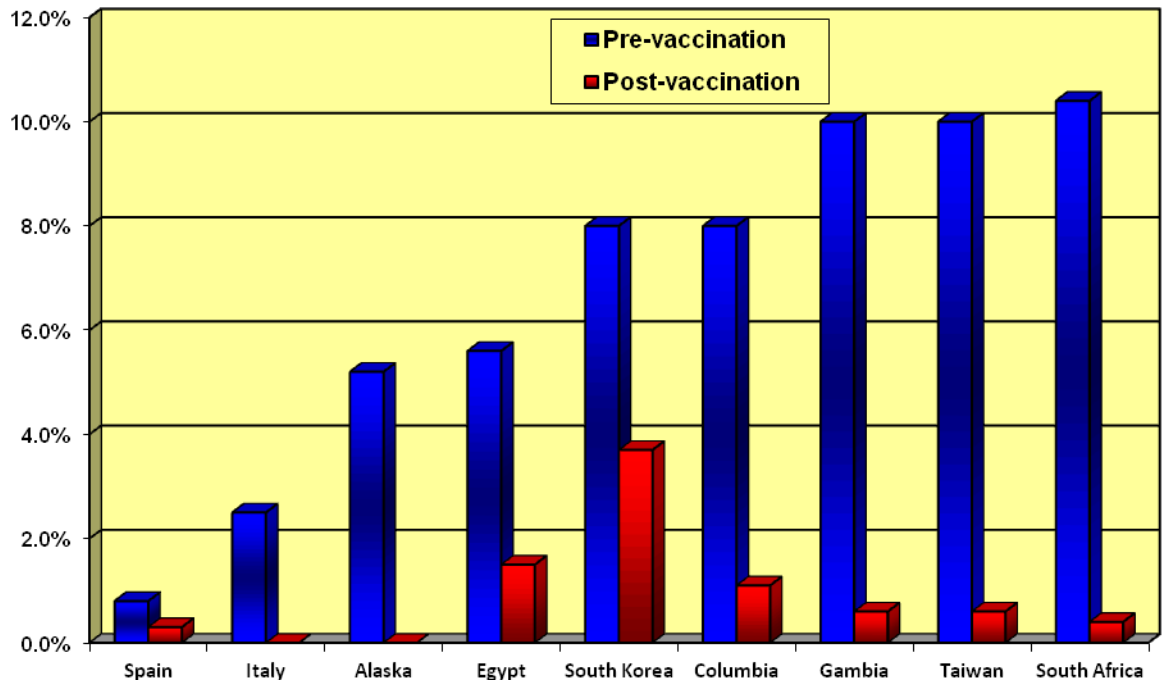


Figure 2.9: Graphical representation of the prevalence of HBsAg before and after the introduction of the hepatitis B vaccine in various regions

In view of the high prevalence (>10%) of childhood HBV infections in the country, the hepatitis B vaccine was introduced also into the South African EPI (EPI-SA) in as early as April 1995 (South African National Department of Health, 1995; Vardas *et al*., 1999). The first hepatitis B vaccine that was licensed for use within EPI-SA was a plasma-derived vaccine (Hepaccine B; Chiel Foods and Chemicals, South Korea) which was later replaced, as in most countries, by a recombinant DNA vaccine (Engerix B vaccine; GlaxoSmithKline, Belgium) in 1999 (Burnett *et al*., 2012). Within the EPI-SA schedule, the hepatitis B vaccine is administered to infants as a monovalent vaccine in 3 doses at 6, 10 and 14 weeks of age, along with the Diphtheria-Tetanus-acellular Pertussis, inactivated Polio and *Heamophilus influenzae* type b combined vaccine (DTaP-IPV/Hib) as well as the oral Polio (at 6 weeks)

and the Rotavirus and Pneumococcal vaccines (both at 6 and 14 weeks) (South African National Department of Health, 2012). Coverage of the hepatitis B vaccine 3rd dose (HepB3) in South Africa is continuously at an increase; currently reported at 97% by the WHO (2012b). Although this figure may be a slight overestimation as vaccine coverage estimates are not necessarily based on scientific surveys, the increase in vaccine coverage is evident in that there has been an observed decline in the incidence of early childhood HBV infections in the country as well as a significant reduction in hepatitis B chronic carriage rates from a previously reported >10% (Vardas *et al*., 1999), to under 1% in vaccinated cohorts (Table 2.2) (Tsebe *et al*., 2001; Schoub *et al*., 2002).

Table 2.2: Pre- and post- hepatitis B immunization data on immunity and chronic carriage of HBV in South African children

	Pre-immunization	**Post-immunization**	
Study	Vardas *et al*., 1999	Tsebe *et al*., 2001	Schoub *et al*., 2002
Age (years)	0-6	0.7-6	1.5
Immunity	-	86.8% (N = 519)	87.0% (N = 769)
Chronic carriage	10.4% (N=2 288)	0.0% (N = 578)	0.4% (N = 756)

2.11.2.2 Monitoring the long-term impact of hepatitis B vaccination

Hepatitis B serosurveys conducted within targeted vaccinated cohorts may not always report the holistic impact of the immunization programme especially in missed populations who did not receive the hepatitis B vaccine and remain susceptible to HBV infection (Advanced Immunization Management, 2009). Measuring population immunity and chronic carriage of HBV infection within a national representative population, regardless of vaccination status, allows for monitoring the long-term impact of the hepatitis B immunization programme (Advanced Immunization Management, 2009; WHO, 2011). The timing for monitoring the

long-term impact requires that the immunization programme should have been in effect long enough to measure the prevalence of chronic HBV infection and its related sequelae. The long-term indicator of the success of the hepatitis B immunization programme would therefore be an increase in immunity and a reduction in fatal chronic liver disease (including cirrhosis and HCC) as compared to when the programme was not in effect (Rani *et al*., 2009).

Quite a number of countries including Australia (Gidding *et al*., 2007), China (Liang *et al*., 2009), Cambodia (Soeung *et al*., 2009), Korea (Park *et al*., 2010), Mongolia (Davaalkham *et al*., 2007) and Italy (Coppola *et al*., 2012) have implemented such nationwide serosurveys in order to monitor the long-term impact of introducing the hepatitis B vaccine into their national EPIs, the output of which has advocated for the continuation of the hepatitis B immunization programme in the relevant countries. The nationwide serosurveys in China for example, revealed a reduction in the prevalence of HBsAg from 9.8% in 1995 to 7.2% in 2006 with the most significant impact noted in children <5 years of age; a 90% reduction (9.7% to 0.96%) in HBsAg prevalence in just over a decade of the hepatitis B immunization programme (Figure 2.10).

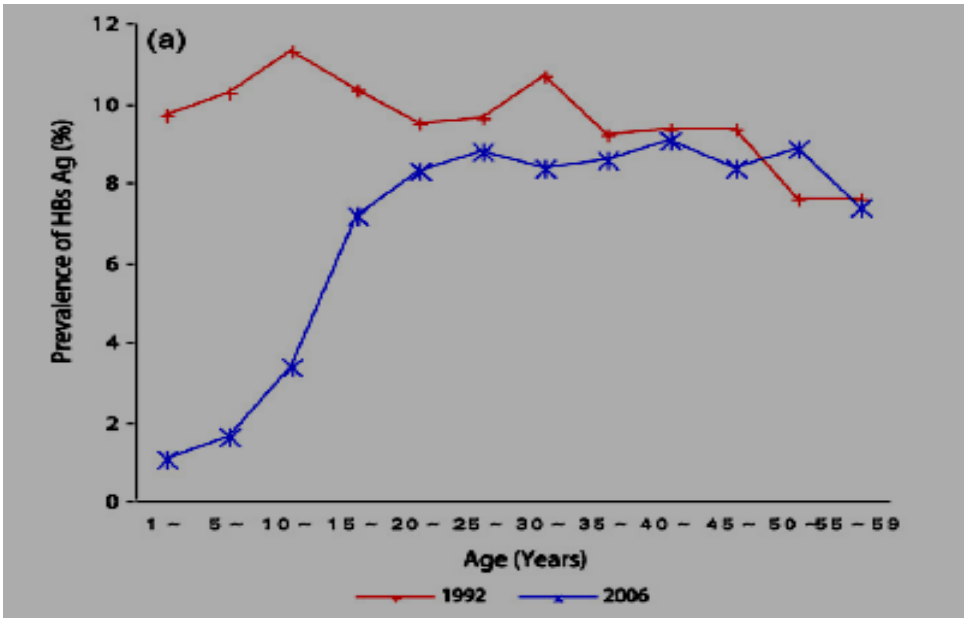


Figure 2.10: Comparison of the prevalence of HBsAg from two serosurveys conducted in China in 1992 and 2006 (Liang *et al*., 2009).

This reduction also meant that an estimated 16-20 million HBV carriers and 2.8-3.5 million future HBV-related deaths would be prevented in the future, backing the continuation of the programme and recommending an increase in hepatitis B vaccination coverage in the country (Liang *et al*., 2009).

2.11.3 Hepatitis B vaccination and HCC

Since chronic hepatitis B is associated with the development of HCC, it would be expected that a reduction in chronic carriage due to the implementation of universal hepatitis B immunization would subsequently influence a reduction in HCC cases and HCC-related mortality. Testing this hypothesis would require determining the prevalence of HCC in individuals at the peak age of HCC development in adulthood; that is 40-60 years after primary hepatitis B vaccination at infancy. However, universal hepatitis B immunization has not been in effect for as many years and as such the best correlate possible would be to investigate the impact of hepatitis B vaccination on childhood HCC cases (Chang, 2009; Chang, 2011). With this in mind, Wichajarn *et al* (2008) assessed the impact of hepatitis B vaccination on HBV-related HCC cases and reported the hepatitis B vaccine to have a positive impact on the incidence of HCC in vaccinated Thai children under the age of 18 years. A similar report emanated from Alaska, USA, recording a complete elimination of HBV-related HCC cases in individuals <20 years of age following a 25 year long programme of infant hepatitis B immunization and mass screening and immunization of all susceptible individuals (McMahon *et* al., 2011). A study in Taiwan also set out to investigate the effect of hepatitis B vaccination on childhood mortality rates resulting from HBV-related HCC cases and found that the introduction of infant hepatitis B immunization had significantly impacted HCC-related deaths in children aged 0 to 9 years (Figure 2.11) (Cheng and Ying, 1997).

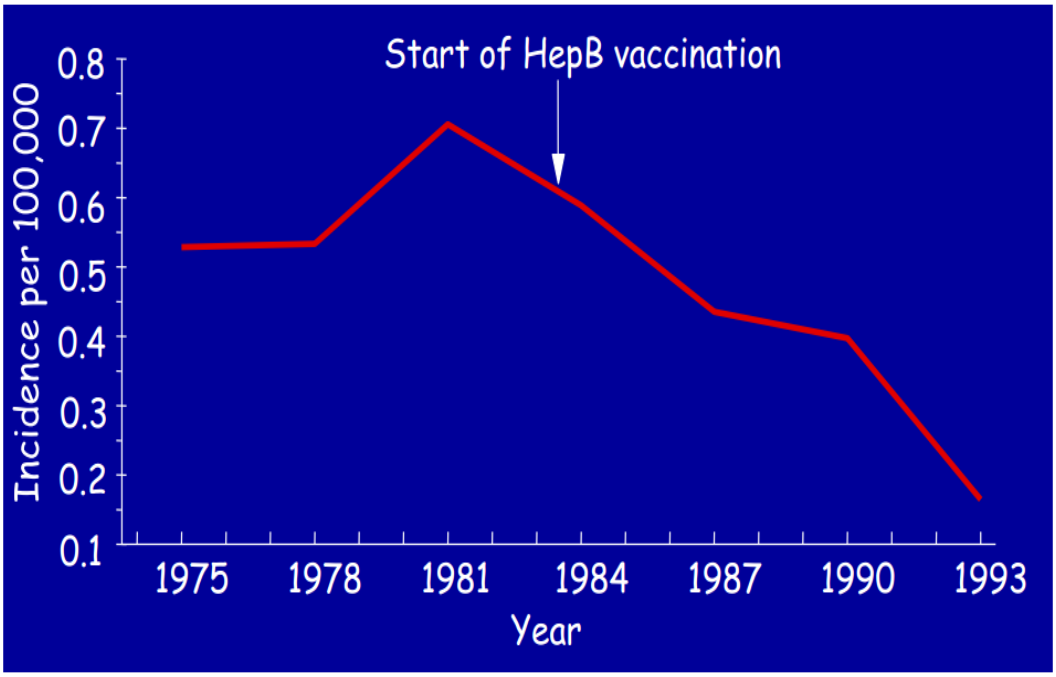


Figure 2.11: Graphical representation of HCC mortality rate ratios among 0-9 year old Taiwan children between 1974 and 1993 (adapted from Cheng and Ying, 1997)

All these studies reflect the success of hepatitis B vaccination in reducing the risk of developing HCC as well as reducing the incidence of HCC cases and HCC-related mortality rates. It is for this reason that the hepatitis B vaccine is regarded as the very first preventive vaccine against cancer (Chang, 2009).

The success of hepatitis B vaccination against HBV-related HCC cases has also been noted in the African region, particularly in South Africa (Burnett *et al*., 2012). An audit of malignant liver tumours in South African children under the age of 14 years, found HCC cases to have reduced proportionately to other malignant hepatic tumors including hepatoblastomas, liver sarcomas and vascular tumors (Figure 2.11), an improvement attributed to 10 years of the national hepatitis B immunization programme (Moore *et al*., 2004; Moore *et al*., 2008).

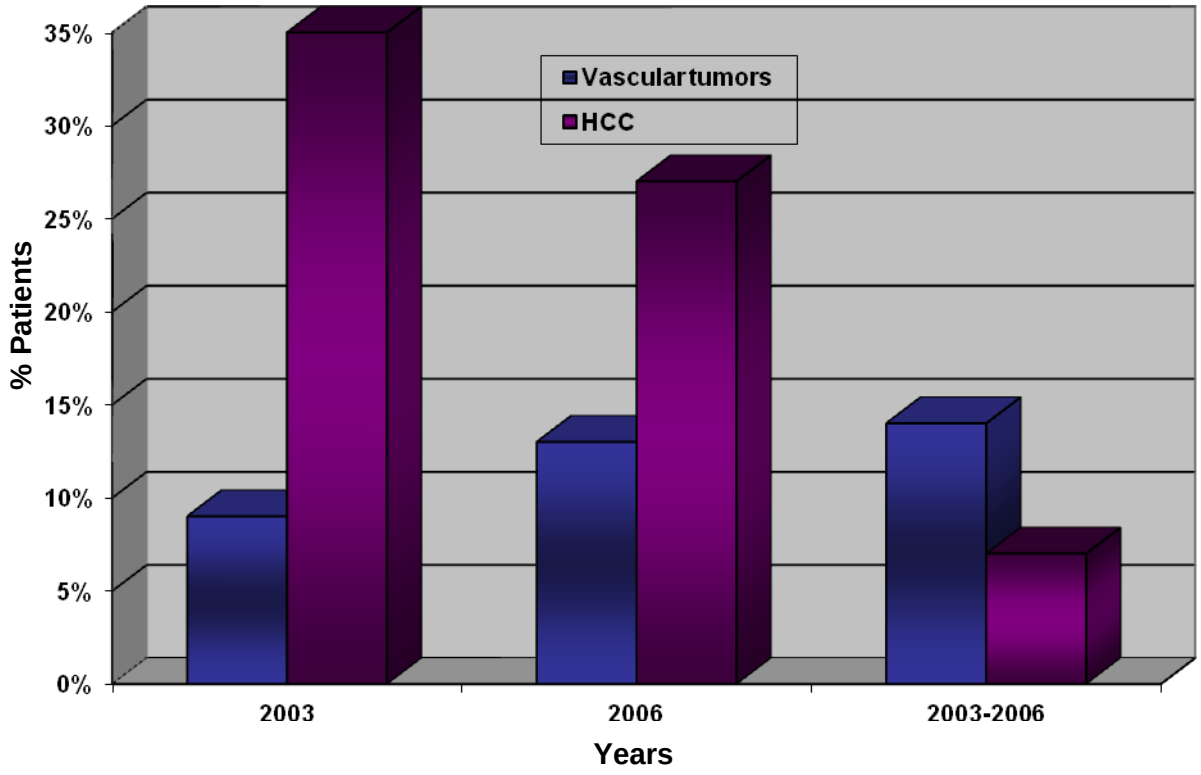


Figure 2.12: Increasing prevalence of vascular tumors and a decline in HCC prevalence rates during specific time periods in South Africa (adapted from Moore *et al*., 2008)

2.11.4 Hepatitis B vaccination and HIV

The increased risk of HBV infection in HIV infected individuals is a well established fact as is the rapid progression of chronic hepatitis B to HCC and end stage liver disease in HBV/HIV co-infected individuals as compared to HBV mono-infected individuals (Puoti *et* al., 2002; Thio, 2003; Cheruvu *et al*., 2007). It is thus imperative that those with an established HIV infection or those at high risk for an HIV infection, such as infants born to HIV positive mothers, be protected earlier on from chronic hepatitis B by timely vaccination (van den Berg *et al*., 2009; Kim *et al*., 2009). Generally, vaccines are safe and valuable for HIV infected individuals, however the immunogenicity of vaccines within this population is not comparable to that in fully immunocompetent individuals (Simani *et al*., 2008). Hyporesponsiveness (anti-HBs titre=10-100 mIU/mL) or non-response (anti-HBs titre <10 mIU/mL) to the hepatitis B vaccine occurs in 20-70% of HIV infected vaccinees and this is usually a result of uncontrolled HIV replication or high HIV viral load and lower $CD4^+$ T cell count (<200 cells/mL) (van den Berg *et al*., 2009). Measuring the anti-HBs titre post-vaccination is thus recommended for this population in order to assess vaccine response and administer

booster doses or re-vaccination when required, so as to achieve protective anti-HBs titres and provide long-term immunity to HBV infection (van den Berg *et al*., 2009; Kim *et al*., 2009).

Although the impact made in HIV uninfected individuals is more remarkable, the implementation of universal hepatitis B immunization is also influencing a potential decline in the number of new HBV/HIV co-infections, which will consequently avert a high number of deaths stemming from chronic hepatitis B-related complications in the HIV infected population (Simani *et al*., 2008; Burnett *et al*., 2012). Simani *et al* (2008) conducted a study in South Africa to deduce the level of protection conferred by the hepatitis B vaccine in HIV infected vaccinees <2 years of age. From this study, it was revealed that although the seroprotection rate was lower within this population compared to HIV uninfected children (78.1% versus 85.7% respectively), hepatitis B vaccination was still beneficial to the HIV infected population as it reduced chronic carriage rates to about 2.7% from >10% in the unvaccinated population (Simani *et al*., 2008).

2.11.5 Challenges in hepatitis B vaccination

A number of challenges exist that threaten the success of hepatitis B vaccination in preventing and potentially eliminating HBV infections. These challenges include imperfect hepatitis B vaccine coverage which could be a result of the inaccessibility of the hepatitis B vaccine particularly in resource-poor regions where government funding cannot afford to include the vaccine in national EPIs. It is for this reason that a number of organizations, such as the GAVI Alliance (formerly The Global Alliance for Vaccines and Immunization), have been set up to help finance and make vaccines available to these regions. Unfortunately not all resource-poor regions are currently eligible for this funding (François *et al*., 2008; GAVI Alliance, 2012). Other factors that affect hepatitis B vaccine coverage include non-adherence to the vaccination programme, either by those who delay receiving the vaccine or do not complete the recommended vaccine dosage, or by anti-vaccine lobbyists who boycott vaccination altogether for fear of apparent adverse effects from components within the vaccine even though the safety of the hepatitis B vaccine is widely established (Bardenheier *et* al., 2004; Zuckerman, 2006; Hammer *et al*., 2010).

Even when the vaccine is successfully administered some concerns still persist such as the waning of protective anti-HBs titres over time following primary hepatitis B vaccination at infancy, which questions the necessity for a booster dose later on in life. However with the vaccine reported to provide long-term (~20 years) protection, considered together with immunological memory which outlasts protective anti-HBs titres because of the luxury of an anamnestic response (rapid rise in antibody response) upon subsequent challenge with the virus or viral antigen, a vaccine booster dose is currently not advocated for immunocompetent vaccinees (Poovorawan *et al*., 2010; Chaves *et al*., 2012; Poovorawan *et al*., 2012). In cases of immune suppression, previously vaccinated individuals should be administered booster doses in order to prevent the development of HBV infection (Banatvala *et al*., 2000; Kane *et al*., 2000; Banatvala and van Damme, 2003). Vaccine non-response, or failure to develop seroprotective titres of anti-HBs after vaccination, is another major antagonist to hepatitis B vaccination, reported in up to 5% of hepatitis B vaccinees. This is said to be a result of the vaccinee's genetic or immune constitution, involving the presence of specific human leukocyte antigen-DR (HLA-DR) allele haplotypes (DRB1*0301 and DRB1*0701) and impaired T_h cell response to the vaccine respectively (Höhler *et al*., 2002; Zuckerman, 2006).

There are other general factors that may also contribute to suboptimal vaccine response, including the route of administration of the vaccine as well as the age, gender, and body mass of the recipient (Zuckerman, 2006). In those individuals who do not respond optimally to the primary hepatitis B vaccine dose, a subsequent 3 dose regimen is recommended to enhance the vaccine response. Since routine assessments of vaccine response are not a common place task in clinical practice, non-responders are unfortunately not easily identified until they develop an HBV infection. Vaccine failure may also occur as a result of infection with HBV vaccine escape mutants (discussed under section 2.7.2.4) which allow for the development of breakthrough HBV infections in previously vaccinated individuals (Carman, 1997; Zuckerman and Zuckerman, 2003). Altogether, these challenges pose the need for assessment of the impact of hepatitis B immunization programmes in order to identify any arising discrepancies and implement timely interventions so as to ensure that its efficacy is maintained.

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Ethical consideration

This study was approved by the Medunsa Research and Ethics Committee (MREC) (project number: MREC/P/22/2012: PG). Only biodemographic data of critical importance for analysis were recorded during collection of study samples and any personal identifiers were removed from the research data base in order to maintain patient confidentiality. The study was linked to samples received and tested by National Health Laboratory Service (NHLS) Diagnostic Laboratory in the Department of Virology, University of Limpopo (Medunsa campus). This laboratory provides diagnostic services for various health care facilities from different provinces. Following routine testing, samples are stored for a maximum period of 5 years as a control measure before being discarded.

For the purpose of this study, the principal researcher sought permission to use blood samples from the afore-mentioned laboratory after routine testing had been completed. Samples were then blinded and stratified according to age (1-15 years and 16-25 years) and HIV status (where results were available). The samples collected were then tested for the following HBV serological markers; HBsAg, anti-HBc, anti-HBs and IgM anti-HBc. Samples were also screened for HBV DNA and those that tested positive (with detectable viral loads) were subjected to molecular characterization of HBV surface (S) and polymerase (*pol*) genes.

3.2 Study design

This was an explorative and descriptive retrospective, cross-sectional study based on recently tested and stored blood samples from the NHLS Diagnostic Laboratory in the Department of Virology. In order to assess population immunity and chronic carriage of HBV almost 17 years after the introduction of the hepatitis B vaccine into EPI-SA, two representative populations were targeted based on the year (i.e. 1995) the hepatitis B vaccine was introduced into EPI-SA; a post-vaccination population comprising blood samples from individuals aged 1-15 years and a pre-vaccination population comprising blood samples from individuals aged 16-25 years. The study assumed that the majority of the

study subjects in the post-vaccination population had received the hepatitis B vaccine while the majority of those in the pre-vaccination population had not received the vaccine. To mimic population-based hepatitis B serosurveys, the hepatitis B vaccination status of all study subjects was not verified. The post-vaccination population was also stratified by age as follows; 1-5, 6-10 and 11-15 years, in order to assess immunity and chronic carriage of HBV across the different age groups. Study subjects with records of their HIV status were pooled for subset analyses so as to investigate the influence of HIV infection on immunity and chronic carriage of HBV. All the study samples were then subjected to a number of laboratory tests, following which results were obtained and analyzed.

3.3 Study population and sampling

3.3.1 Sample size

The sample size was 850, calculated at 95% confidence with a power of 80% using the statistical package available on EPI Info version 3.5.3 (CDC, USA). The calculation assumed a 10% prevalence rate of HBV infection in the South African general population (n=532) while that for the post-vaccination population was estimated at 2% (n=318: 106 per age stratum; 1-5, 6-10 and 11-15 years).

However, a total sample size of 1206 blood samples was obtained within the period allocated for sample collection (September 2011-September 2012). Of this total, 605 blood samples represented the post-vaccination population while 601 blood samples represented the pre-vaccination population (Table 3.1).

3.3.2 Demographics of the study population

The study population comprised of more females (65.4%) than males (33.7%), however 0.8% (10/1206) of the study subjects had no record of their gender. Gender disparities between the post- and pre-vaccination populations were found to be statistically significant with a p value <0.001. In terms of the ages of study subjects, the overall mean age of the study population was 15 years (Table 3.1). Study subjects originated from a total of five of the nine provinces in South Africa, the majority hailing from the Gauteng province (57.1%)

followed by North West (33.8%), Mpumalanga (5.2%), Limpopo (3.7%) and Northern Cape provinces (0.1%).

Table 3.1: A summary of the demographic data of study subjects

Study Populations	Mean Age (standard deviation)	Sex (%)			Provinces (No. of samples)
		Male	Female	Unknown*	
Post-vaccination 1-15 years (n=605)	8.7 years (4.8)	266 (44.0%)	330 (54.5%)	9 (1.5%)	Gauteng (689) North West (408) Mpumalanga (63) Limpopo (45) Northern Cape (1)
Pre-vaccination 16-25 years (n=601)	21.6 years (2.7)	141 (23.5%)	459 (76.4%)	1 (0.2%)	
Total 1-25 years (N=1206)	14.9 years (7.7)	407 (33.7%)	789 (65.4%)	10 (0.8%)	

*Study subjects' gender not recorded

3.3.3 Sampling procedure

This study employed a purposive sampling method. Blood samples from patients who presented at health care facilities in various provinces in the country and had requested for diagnosis of blood-borne infections through the NHLS Diagnostic Laboratory were included in this study after routine testing had been completed.

3.3.3.1 Inclusion criteria

The following criteria were used in selecting samples for inclusion into the study:

- Blood samples tested by the NHLS Diagnostic Laboratory.

- Blood samples from individuals between the ages of 1 and 25 years.

- Blood samples of enough quantity required for most laboratory tests to be performed.

3.3.3.2 Collection of samples

Assisted by patient records, blood samples which met the study inclusion criteria were selected and serum fractions aliquoted into 2 mL micro tubes. Serum samples were then stored at -70˚C at the HIV and Hepatitis Research Unit (HHRU) in the Department of Virology, University of Limpopo (Medunsa Campus), until required for laboratory testing.

3.4 Laboratory methods

3.4.1 Hepatitis B serological testing

All serum samples were tested for the following primary serological markers; HBsAg, anti-HBc and anti-HBs. Samples which tested positive for either HBsAg or anti-HBc or both markers, indicating some form of exposure to the virus, were further tested for IgM anti-HBc to determine the presence of an acute HBV infection. All serological tests were performed on the Elescys® 2010 Immunoassay Analyzer (Roche, Hitachi, Japan) using Elecsys® test kits (Roche Diagnostics, Penzberg, Germany) for each respective marker in compliance with the manufacturer's instructions. The principle of the Elecsys® Immunoassay Analyzer and the test kits used are described in the sections that follow.

3.4.1.1 Elecsys® 2010 Immunoassay Analyzer

Immunoassays are an indispensable component to the field of laboratory medicine and for this reason form an integral part of diagnostics. To date, there is a vast repertoire of immunoassays which measure results using various principles and one of such is the electrochemiluminescence or electrogenerated chemiluminescence (ECL) method; a process in which highly reactive species are generated from stable precursors at the surface of an electrode. Luminescent molecules which upon electrical excitation generate light include compounds of rhenium, ruthenium and osmium, making them very useful in ECL systems and devices (Mathew *et al*., 2005)

The Elescys® 2010 Immunoassay Analyzer is a software-controlled system consisting of fully automated, random access auto-analyzers and standalone instruments used to perform heterogeneous immunoassays by employing the unique ECL principle for qualitative and quantitative *in vitro* determinations. With a throughput estimated at 86 tests per hour, an assay on the Elescys® 2010 Immunoassay Analyzer comprises at least three basic test components (test sample, reagent and streptavidin-coated microparticles) which are subjected to a number of pipetting steps, at least one incubation period and a measuring step to determine the intensity of light emitted in direct proportion to the amount of antigen or antibody present within the test sample.

3.4.1.2 Elecsys® Anti-HBs Immunoassay

The Elecsys® Anti-HBs Immunoassay test kit uses a mixture of HBsAg subtypes *ad* and *ay*, purified from HBV-infected human serum to quantitatively detect antibodies (anti-HBs usually of the IgG isotype) in the test sample. The Elecsys® anti-HBs Immunoassay reagent pack consists of a set of three bottles with colour-coded caps; a transparent-capped bottle (M) which contains 6.5 mL of streptavidin-coated microparticles (at a concentration of 0.72 mg/mL), a grey-capped bottle (R1) which contains 10 mL of HBsAg-biotin [biotinylated HBsAg (*ad*/*ay*) from human serum (>0.5 mg/L) in MES (2-morpholino-ethane sulphonic acid) buffer (85 mmol/L at pH 6.5)] and finally a black-capped bottle (R2) containing 8 mL of HBsAg-$Ru(bpy)^{2}{}_{3}^{+}$ [HBsAg (*ad*/*ay*) from human serum labelled with ruthenium complex (>0.35 mg/L) in MES buffer (85 mmol/L and pH 6.5)]. A set of reagent calibrators (Cal1 and Cal2) also accompany the reagent pack and contain 1.3 mL of anti-HBs (purified from human serum) in human serum, which are run on the Elescys® 2010 Immunoassay Analyzer (at least once per reagent kit) prior to sample testing.

3.4.1.2.1 Principle of the Elecsys® Anti-HBs Immunoassay

This assay is based on the sandwich immunoassay principle and consists of two incubation periods in a total testing time of 18 minutes per test. During the first incubation step, anti-HBs (if present within the test sample) and the biotinylated HBsAg (*ad*/*ay*) labelled with ruthenium complex [Tris (2,2'-bipyridyl)ruthenium(II)-complex; ($Ru(bpy)^{2}{}_{3}^{+}$)] react to form a sandwich complex. After the addition of streptavidin-coated microparticles the reaction is incubated for a second time during which period the sandwich complex becomes bound to the solid phase via interaction between the biotin and streptavidin. The reaction mixture is

aspirated into a measuring cell where the microparticles are magnetically captured onto the surface of an electrode. Unbound reaction components that may obscure results are washed-off with a System Buffer (ProCell). A voltage is then applied to the electrode which induces chemiluminescent emission measured by a photomultiplier (a transducer). Results are determined via a calibration curve which is instrument-specifically generated by a 2-point calibration as well as a master curve provided by the reagent barcode.

3.4.1.2.2 Quality control

For quality control purposes, Elecsys® PreciControl Anti-HBs was used to monitor the accuracy of the Elecsys® Anti-HBs Immunoassay. It consists of a set of controls (PC A-HBS1 and PC A-HBS2) containing control serum (based on human serum in the negative and positive concentration range), which were run as single determinations at least once every 24 hours when the test was in use, once per reagent kit and after every calibration. The first control, PC A-HBS1, contains 1.3 mL of control serum (Human serum) negative for anti-HBs (target concentration range for anti-HBs: ≤5 IU/L), while the second control, PC A-HBS2, contains 1.3 mL of control serum, Anti-HBs antibodies (human) ~100 IU/L in human serum (target concentration range for anti-HBs: 60-150 IU/L). The control intervals and limits were adapted to the laboratory's requirements (reported at a standard deviation of 2 for all Elecsys® Immunoassay controls used at the HHRU laboratory).

3.4.1.3 Elecsys® HBsAg II Immunoassay

This immunoassay is intended for the *in vitro* qualitative determination of HBsAg in human serum and plasma. The reagent pack contains a set of three colour-coded reagent bottles; a transparent-capped bottle (M) which contains 6.5 mL of streptavidine-coated microparticles at a concentration of 0.72 mg/mL, a grey-capped bottle (R1) which contains 8 mL of anti-HBsAg-Ab-biotin [two biotinylated monoclonal anti-HBsAg antibodies (purified from mouse serum) (>0.5 mg/L) in phosphate buffer (100mmol/L and a pH of 7.5)] and finally a black-capped bottle (R2) which contains 7 mL of anti-HBsAg-Ab~Ru(bpy)$^{2}_{3}{}^{+}$ [monoclonal anti-HBsAg antibody (purified from mouse serum), polyclonal anti-HBsAg antibodies (purified from sheep serum) labelled with ruthenium complex (>1.5 mg/L) in phosphate buffer (100 mmol/L at pH 8.0)]. A calibrator set (Cal1 and Cal2) is also included in the reagent pack. Cal1 the negative calibrator, contains 1.3 mL of human serum while Cal2 which is the positive calibrator, contains 1.3 mL of HBsAg (~0.5 IU/mL) in human serum.

3.4.1.3.1 Principle of the Elecsys® HBsAg II Immunoassay

Similar to the Elecsys® Anti-HBs Immunoassay, the Elecsys® HBsAg II Immunoassay follows a sandwich principle. The test sample is first incubated together with the two biotinylated monoclonal anti-HBsAg antibodies and a mixture of monoclonal anti-HBsAg antibodies and polyclonal anti-HBsAg antibodies labelled with a ruthenium complex, resulting in the formation of a sandwich complex. The steps that follow are similar to what has been previously described for the Elecsys® Anti-HBs Immunoassay (section 3.4.1.2.1).

3.4.1.3.2 Quality control

The Elecsys® PreciControl HBsAg II was used for monitoring the accuracy of Elecsys® HBsAg II Immunoassays. The kit contains a set of controls; PC HBSAGII1 containing 1.3 mL of control serum (human serum), negative for HBsAg with a target range for the cut-off index falling between 0.0-0.80 and PC HBSAGII2 containing 1.3 mL of control serum; HBsAg (human) (~0.2 IU/mL) in human serum with a target range for the cut-off index between 2.6-5.0. The results obtained for each test were interpreted according to the ranges specified by the controls.

3.4.1.4 Elecsys® Anti-HBc Immunoassay

This assay is used for the *in vitro* qualitative determination of total IgM and IgG antibodies to HBcAg in human serum and plasma. The assay reagent pack contains four colour-coded reagent bottles; a transparent-capped bottle (M) containing 6.5 mL of streptavidin-coated microparticles at a concentration of 0.72 mg/mL, a white-capped bottle (R0) containing 5 mL of DTT [1,4-dithiothreitol (110 mmol/L) in citrate buffer (50 mmol/L)], a gray-capped bottle containing 8 mL of HBcAg [HBcAg (E. coli, rDNA) (>25 ng/mL) in phosphate buffer (100mmol/L and a pH of 7.4)] and finally a black-capped bottle which contains 8 mL of Anti-HBcAg-Ab~biotin; anti-HBcAg-Ab~$Ru(bpy)^{2}{}_{3}^{+}$ [biotinylated monoclonal anti-HBc antibody (mouse) (>800 ng/mL); monoclonal anti-HBc antibody (mouse) labelled with ruthenium complex (>130 ng/mL) all in phosphate buffer (100 mmol/L with a pH of 7.4)]. The Elecsys® Anti-HBc Immunoassay calibrators are Cal1; the negative calibrator containing 1.0 mL of human serum and Cal2; the positive calibrator which contains 1.0 mL of Anti-HBc (human) [>8 PEI (Paul-Ehrlich-Institute) U/mL] in human serum.

3.4.1.4.1 Principle of the Elecsys® Anti-HBc Immunoassay

The test is based on a competitive immunoassay principle with three incubation stages and a total assay time of 27 minutes per test. The first incubation involves pre-treatment of 40 µL of sample with the DTT (a reducing agent). The HBcAg is then added to the sample and the reaction mixture incubated, allowing the formation of a complex between the HBcAg and the anti-HBc antibodies in the sample. In the third and final incubation step, biotinylated antibodies and ruthenium complex-labelled antibodies specific for HBcAg together with streptavidin-coated microparticles are added to the reaction mixture resulting in the previously free binding sites on the HBcAg becoming occupied. The entire complex becomes bound to the solid phase due to interactions between the biotin and streptavidin. To induce and measure chemiluminescence, the reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode (unbound reactants are removed with ProCell) to which a voltage is applied. Chemiluminescent emission is then measured by a photomultiplier and results are determined automatically by the Elecsys® software. This is done by comparing the electrochemiluminescence signal obtained from the reaction product of the sample with the signal of the cut-off value obtained by anti-HBc calibration.

3.4.1.4.2 Quality control

For quality control purposes, Elecsys® PreciControl Anti-HBc containing control serum based on human serum in the negative and positive concentration ranges was used to monitor the accuracy of the Elecsys® Anti-HBc Immunoassays. The controls are PC A-HBC1 (negative control) containing 1.3 mL of control serum (Human serum) negative for anti-HBc with a target range for the cut-off index falling between 1.05-3.0 and PC A-HBC2 (positive control) which contains 1.3 mL of control serum; Anti-HBc antibodies (human) (~1 PEI-U/mL) in human serum with a target range cut-off index between 0.14-0.87.

3.4.1.5 Elecsys® Anti-HBc IgM Immunoassay

The Elecsys® Anti-HBc IgM Immunoassay is used for the *in vitro* qualitative determination of IgM antibodies to HBcAg (IgM anti-HBc) present in human serum and plasma. The reagent pack consists of three colour-coded reagent bottles; a transparent-capped bottle (M) containing 6.5 mL of streptavidin-coated microparticles at a concentration of 0.72mg/mL, a

gray-capped bottle (R1) containing 10 mL of pre-treatment anti-HBc IgM [anti-human-Fd_γ-antibody (sheep) (>0.05mg/mL) in phosphate buffer (100 mmol/L)] and finally a black-capped bottle (R2) which contains 10 mL of Anti-h-IgM-Ab~biotin; HBcAg~$Ru(bpy)^{2+}_3$ [biotinylated monoclonal anti-h-IgM antibody (mouse) (>600 ng/mL); HBcAg (E. coli, rDNA), labelled with ruthenium complex (>200 ng/mL) in phosphate buffer (100 mmol/L at a pH of 7.4)]. The Elecsys® Anti-HBc IgM calibrators include Cal1 which is the negative calibrator and contains 1.0 mL of human serum and Cal2, the positive calibrator, which contains 1.0 mL of anti-HBc IgM (human) (>100 PEI-U/mL) in human serum.

3.4.1.5.1 Principle of the Elecsys® Anti-HBc IgM Immunoassay

This assay follows a μ-Capture test principle with a total assay time of 18 minutes per test. The first incubation step involves pre-treatment of the test sample (automatically pre-diluted 1:400 with Elecsys® Diluent Universal) with anti-Fd_γ reagent to block specific IgG that may be present in the sample. In the second incubation step, biotinylated monoclonal h-IgM-specific antibodies, HBcAg labelled with ruthenium complex and streptavidin-coated microparticles are added to the pre-treated sample. IgM anti-HBc present in the sample reacts with the ruthenium-labelled HBcAg and the biotinylated anti-h-IgM to form a complex bound to the solid phase. The reaction mixture is then aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode (unbound reactants are washed-off with ProCell). By applying a voltage to the electrode, chemiluminescence emission is induced and this can be measured by a photomultiplier after which the results are determined automatically by the Elecsys® software.

3.4.1.5.2 Quality control

Elecsys® PreciControl Anti-HBc IgM was used to monitor the accuracy of the Elecsys® Anti-HBc IgM immunoassay. The reagent pack contains a set of controls; PC A-HBCIGM1 which contains 1.0 mL of control serum (human serum) negative for IgM anti-HBc having a target range for the cut-off index between 0-0.3 and the positive control, PC A-HBCIGM2 which contains 1.0 mL of control serum; Anti-HBc IgM antibodies (human) (>130 PEI-U/mL) in human serum with a target range for the cut-off index of 1.1-2.5.

3.4.2 Hepatitis B molecular testing

The presence of HBV DNA in patient serum is a confirmation of an active HBV infection, while the concentration of HBV DNA detected gives an indication of viral replication and infectivity. As HBV DNA is never completely cleared even after resolution of an infection, the distinction between a current and past HBV infection after detection of low levels of HBV DNA should be accompanied by serological profile. Direct detection of HBV DNA can be achieved through various molecular assays including real time and conventional PCR assays which were used in this study (Pawlotsky, 2002).

Hepatitis B virus DNA was determined in all samples with serological evidence of either exposure to or infection with the virus. A quantitative real time PCR (qPCR) assay was employed for the quantitative detection of HBV DNA in the selected samples. Samples with detectable HBV DNA concentrations were also subjected to a nested PCR assay to amplify HBV S and *pol* genes by making use of specific primers.

3.4.2.1 HBV DNA extraction

In order to isolate viral DNA and make them available for detection by the PCR assays, an extraction procedure was carried out to extract HBV DNA from serum samples using the Nucleospin® Viral Nucleic Acid Isolation kit (Macherey-Nagel, Düren, Germany). The Nucleospin® Nucleic Acid Isolation kit comes complete with Lysis Buffer (RAV1 buffer), Wash Buffers RAW and RAV3 (concentrate), RNase-free H_2O, Elution Buffer (RE buffer; 5 mM Tris/HCl, pH 8.5), Carrier RNA (lyophilized), Proteinase Buffer (PB buffer), Proteinase K (lyophilized), Nucleospin® Dx Virus Columns, Collection Tubes, Lysis Tubes, and Elution Tubes.

Before starting the DNA extraction process working buffers were first prepared; PB buffer was added to the vial containing lyophilized Proteinase K to dissolve it and prepare a ready-to-use liquid formulation, lyophilized carrier RNA was dissolved in RAV1, and 100% ethanol was added to the concentrated RAV3. The first step of the extraction process was to lyse host cells and this was performed by mixing 600 µL of lysis buffer (RAV1 buffer containing carrier RNA) and 20 µL of liquid Proteinase K with 150 µL of serum sample within a Lysis Tube. This mixture was incubated for 5 minutes at 70°C on an Eppendorf® Thermomixer

Compact (Eppendorf, USA) after which 600 µL of 100% ethanol was added to each tube in order to clear the lysate. Each lysate was then loaded onto a Nucleospin® Dx Virus Column placed in a Collection Tube and centrifuged for a minute at 8000 rpm to allow binding of nucleic acids within the lysate to the membrane of the column while the filtrate collected in the flow-through of the Collection Tube. With the flow-through discarded, each column was then washed with RAW and RAV3 buffers to remove any unbound contaminants present, such as cell debris, host and viral proteins and RNA. To unbind and elute DNA from the membrane, 50 µL of pre-warmed elution buffer (RE buffer) was loaded onto each column placed in elution tubes. These tubes were then centrifuged for a minute at 11000 rpm, causing the DNA to collect in the eluent. Positive (HBV positive serum) and negative (RNase free H_2O) controls were included during each extraction process. All extracts were then stored at -20°C until required for further testing.

3.4.2.2 HBV quantitative real time PCR (qPCR) assay

The qPCR assay is a highly sensitive nucleic acid detection method which allows for the simultaneous amplification and quantification of as little as a single copy of DNA within a test sample. Detection of DNA is reported during the course of the amplification process and as such results are accessible in 'real time', eliminating the need for post-PCR processing and thus saving time and resources. 'Real time' reporting is achieved by the use of fluorescent reporter dyes and probes which are designed to fluoresce only after binding specifically to DNA (Arya *et al*., 2005). Thus emission of fluorescence confirms the presence of DNA while a measure of the fluorescence emitted during the binding process correlates with the amount of DNA present within the test sample. A wide range of these DNA binding molecules are available, including double-stranded DNA binding dyes (SYBR® Green 1 and EvaGreen®), fluorescent probes (hydrolysis probes, dual hybridization probes and scorpion probes) and molecular beacons (Arya *et al*., 2005).

For the purpose of this study, qPCR was performed using the LightCycler® 2.0 Real-Time PCR System (Roche, Germany) which comprises documentation (user manual and guides), hardware (LightCycler® 2.0 instrument with a mounted sample carousel, personal computer, mouse and power cables), the system software (LightCycler® Software 4.1) installed on the computer, LightCycler® 2.0 Centrifuge Adapters, Capping Tools and Capillary Releaser, and consumables (LightCycler® 20 µL and 100 µL capillaries).

Real time PCR reactions were performed for all extracts using the 5X HOT FIREPol® EvaGreen® qPCR Mix Plus (no ROX) (Solis BioDyne, Estonia). A tube of the 5X HOT FIREPol® EvaGreen® qPCR Mix Plus (no ROX) contains the major components required for a qPCR assay, including HOT FIREPol® DNA Polymerase, ultrapure dNTPs, $MgCl_2$ and EvaGreen® dye (a DNA binding dye). Real time PCR amplification was performed in a 20 µL reaction mixture, containing the 5X HOT FIREPol® EvaGreen® qPCR Mix Plus (no ROX) at a final concentration of 1X which was added to 10 pmol/µL each of a set of primers (Table 3.2) targeting the basal core promoter (BCP) region of the HBV core gene (Ho *et al*., 2003) as well as 50 ng/µL of the DNA extract (template). The final volume was then made up by adding 9 µL of PCR grade H_2O (water) (Fermentas, Burlington, Canada) to the qPCR reaction mixture. Each reaction mixture was then transferred to individual LightCycler® capillaries (20 µL) which were then capped and loaded onto the sample carousel of the LightCycler® 2.0 instrument. Cycling conditions (Table 3.3) were programmed onto the computer to perform the qPCR assay.

Table 3.2: Primers used for qPCR targeting the BCP region of the HBV core gene

Primer	Sequence	Polarity	Amplicon size
BcP1	5' -ACCACCAAATGCCCCTAT- 3'	Sense	130bp
BcP2	5' -TTC TGC GAC GCG GCG A- 3'	Anti-sense	

Table 3.3: HBV qPCR cycling conditions using 5X HOT FIREPol® EvaGreen® qPCR Mix Plus (no ROX) (Solis BioDyne, Estonia)

Cycling conditions	Initial denaturation	Amplification			Cooling	Melting curve
		Denaturation	Annealing	Elongation		
Target (°C)	95°C	95°C	60°C	72°C	40°C	95°C
Cycles	-	50			-	-
Acquisition mode	None	None	None	Single	None	Continuous
Hold (mm:ss)	10:00	00:10	00:40	00:10	02:00	-

Since the EvaGreen® dye may bind to any dsDNA present in the reaction mixture including non-specific DNA products, a DNA melting curve analysis, which allows for specific product characterization, was performed for each qPCR assay (Ririe *et al*., 1997). To ensure the validity of qPCR results, positive and negative controls were included in every qPCR assay performed. The results of qPCR were generated and analysed by the LightCycler® Software 4.1. The results obtained were then recorded and captured into the research database.

3.4.2.3 HBV nested PCR assay

The nested PCR assay is a conventional PCR method used for the amplification of DNA in a two step reaction process, using two pairs of nested primers targeting the desired gene region. The PCR reaction is automated; performed on a thermal cycler which is programmed to apply different temperature ranges to a reaction mixture in order to amplify multiply copies of DNA form a single template, with the assistance of a thermostable polymerase enzyme. This nested PCR assay was used to amplify the HBV S and *pol* genes (primers shown in Table 3.4) in order to generate DNA products for molecular characterization.

Table 3.4: Primers targeting HBV surface (S) and polymerase (*pol*) genes

S gene primers	Sequence (5'-3')	Purpose	T_A	Product
S1	CCTGCTGGTGGCTCCAGTTC	1st round	48°C	947bp
S2Na	CCACAATTCK(G/T)TTGACATACTTTCCA			
S6Bs	GATCCGAGGACTGGGGAC	2nd round	55°C	750bp
S7Ps	GGTTAGGGTTTAAATGTATAC			
Pol gene primers				
P1	GTCTGCGGCGTTTTATCA	1st round	50°C	901bp
P2	GGAGTTCCGCACTATGGATCGG			
P3	GGTATGTTCCCGTTTGTCC	2nd round	52°C	645bp
P4	GGCGAGAAAGTGAAAGCCT			

3.4.2.3.1 Sensitivity of the nested PCR assay

Nested PCR assays are less sensitive when compared to qPCR assays in that they are incapable of detecting very low DNA concentrations which are easily detectable by qPCR. Thus the sensitivity of the nested PCR assay used for this study was assessed in order to determine the lowest detectable HBV DNA concentration. To do this, duplicate serial dilutions of an HBV positive control (HBV infected human serum with known viral DNA concentration) were prepared and HBV DNA extracted from each dilution. The extracts were then subjected to a nested PCR assay using primers specific for the HBV S and *pol* genes (Table 3.4). The first round PCR reaction was carried out in a 25 µL reaction mixture containing 25 mM dNTP mix (Fermentas, Burlington, Canada), a 10X reaction buffer (Bioline, Luckenwalde, Germany), 50 mM $MgCl_2$ (Bioline, Luckenwalde, Germany), 10 pmol/mL of each primer set (Table 3.4), 5 U/µL of Taq DNA polymerase (Bioline, Luckenwalde, Germany) and 50 ng/µL of the HBV DNA extract (template). The final volume of each reaction mixture was made up by adding PCR grade H_2O (Fermentas, Burlington, Canada). Reaction mixtures were then transferred into individual 0.5 mL eppendorf tubes which were then loaded onto a thermal cycler (Vacutec, USA) where nested PCR cycling conditions (Table 3.5) were programmed.

Table 3.5: Nested PCR cycling condition for amplification of HBV surface (S) and polymerase (*pol*) genes

Cycling conditions	S gene PCR		*Pol* gene PCR		Hold (mm:ss)
	1st round	2nd round	1st round	2nd round	
Initial denaturation	95°C	95°C	94°C	94°C	02:00
No. of cycles	35	27	35	30	-
Denaturation	95°C	95°C	94°C	94°C	00:20
Annealing	55°C	60°C	50°C	50°C	00:30
Extension	72°C	72°C	72°C	72°C	01:00
Final extension	72°C	72°C	72°C	72°C	10:00
Store	4°C	4°C	4°C	4°C	Infinity

After the first round PCR programme was completed, a similar reaction mixture was prepared for the second round PCR reaction, this time replacing the first round primers with the second round primers (Table 3.3) and using 10 ng/µL of each first round PCR product as template.

3.4.2.3.2 Detection of nested PCR products

An amount of 3 µL of each second round nested PCR product was subjected to a gel electrophoresis procedure which involved loading the PCR products (mixed with a 6X DNA loading dye; Fermentas, Burlington, Canada) into wells created in a 2% agarose gel (Fermentas, Burlington, Canada) stained with 0.5 µL/100mL of ethidium bromide (Merck, Darmstadt, Germany); a DNA intercalating agent which fluoresces upon exposure to ultraviolet (UV) light, consequently allowing for visualization of the DNA product in the form of bands containing multiple copies of the desired gene fragment. The loaded agarose gel was then subjected to 100 Volts of a unidirectional (from the anode to the cathode) electrical current for a duration of 30 minutes, while submerged in a 1X Tris-acetate-EDTA (TAE) buffer (Qiagen, Hilden, Germany). The gel was then exposed to UV light with the use of a transilluminator (Vacutec, USA) in order to view bands which were measured against a 0.1 µg/µL 100bp DNA molecular weight maker (Fermentas, Burlington, Canada).

3.4.2.3.3 Amplification of HBV surface (S) and polymerase (*pol*) genes

After assessing the sensitivity of the nested PCR used in this study, extracts of study samples for which qPCR had been performed and HBV DNA concentrations determined, were selected for the amplification of HBV S and *pol* genes (where necessary), following the nested PCR protocol described under section 3.4.2.3.1. Second round PCR products were also subjected to an agarose gel electrophoresis procedure (described under section 3.4.2.3.2) to confirm the presence of the desired gene fragments.

3.5 Sequencing of HBV surface and polymerase genes

Second round nested PCR products which were confirmed by agarose gel electrophoresis to be positive for the desired gene fragments were sent for sequencing at Inqaba Biotechnological Industries (South Africa). Inqaba Biotechnological Industries is a company

which provides commercial sequencing services for various laboratories using the SpectruMedix SCE 2410 Genetic Analysis System (SpectruMedix LLC, PA). Briefly, a dideoxy terminator sequencing reaction was performed on the second round nested PCR products, making use of the second round primers for each respective gene (Table 3.4) and incorporating fluorescent chain termination dideoxynucleotides (ddNTPs). This was followed by exposure of the sequencing reaction products to a high resolution polyacrilamide electrophoresis reaction to separate short gene fragments (oligonuleotides). Using laser detection, the different wavelengths of the fluorescence emitted by each of the ddNTPs (ddATP, ddTTP, ddCTP, ddGTP) used were observed and then analyzed by a Spectrumedix computer software programme to generate sequenced data.

3.6 Data analysis

3.6.1 Bias

Consideration was given to sampling bias for which this study was subject to. In order to minimize the biased effect of the purposive sampling method utilized, a larger sample size would have been required. However financial and time constraints influenced the sample size employed in this study and was noted as a limitation of the study.

3.6.2 Reliability and validity

To ensure validity of the study, internal controls (positive and negative controls) were included in all laboratory tests as appropriate. Where test kits were used, manufacturers' instructions were adhered to. In terms of reliability of the study, the NHLS Diagnostic department that was linked with this study is a SANAS (South African National Accreditation System) accredited laboratory that routinely participates in external quality assurance programs for HBV testing.

3.6.3 Analysis of serology results

Briefly, the detection of both HBsAg and anti-HBc is indicative of a past HBV infection while the presence of the IgM isotype of anti-HBc confirms an acute HBV infection. The presence of both anti-HBc and anti-HBs on the other hand may give an indication of recovery from a previous infection or immunity due to a natural infection, while anti-HBs alone is indicative of

immunity as a result of vaccination against HBV infection. Geometric mean titres (GMT) of anti-HBs values were determined with the use of the statistical tools available on Microsoft Excel, 2003. Serological results were also statistically analysed by determining the odds ratio and relative risks. The chi-square test and Fisher's exact test were used, where appropriate, to assess the statistical significance of serological results using Epi Info version 3.5.3 (CDC, USA).

3.6.4 Analysis of gene sequences

The HBV S gene as well as the overlapping *pol* gene were amplified and analyzed for mutations. Gene sequence analysis was performed using various computer software programmes including BioEdit Sequence Alignment Editor (Hall, 1999), ChromasPro version 1.49 (Technelysium Pty. Ltd.) and MEGA version 4.1 (Tamura *et al*., 2007) programmes. The ChromasPro programme was first used to edit each consensus nucleotide sequence in order to correct any ambiguities which were automatically displayed by the software. The edited nucleotide sequences were then aligned, together with HBV published reference sequences, (retrieved from GenBank; http://www.ncbi.nlm.nih.gov/genbank/) using the BioEdit programme (Hall, 1999). Alignment was performed so as to compare the study sequences with the HBV reference sequences for any nucleotide and amino acid variations. In order to determine the genotypic lineage of the study sequences, the Neighbour-Joining method on the MEGA programme was used to generate a bootstrap consensus phylogenetic tree out of a 1000 bootstrap replicates, comparing the study sequences with the HBV references sequences which already had established genotype lineages (Tamura *et al*., 2007).

CHAPTER 4

4. RESULTS

4.1 Overview and validation of laboratory methods

4.1.1 Hepatitis B serology

In brief, all samples were serologically screened for the following primary markers; HBsAg, anti-HBc and anti-HBs, in order to determine the prevalence of HBV infection and exposure as well as immunity to HBV infection respectively. For those samples with serological evidence of HBV exposure or infection, a further assessment for the presence of an acute HBV infection was carried out by testing for IgM anti-HBc. Serology was performed on the Elecsys® 2010 Immunoassay Analyzer using Elecsys® reagents which also included reagent controls and calibrators. Any HBsAg, anti-HBc, anti-HBs or IgM anti-HBc present in a test sample was quantified (mIU/mL) and compared to cut-off values of positive and negative reagent controls.

4.1.2 Hepatitis B qPCR

A qPCR assay was used to screen for the presence of HBV DNA in all samples that had serological evidence of HBV exposure or infection in order to determine the presence of active HBV infection. The assay employed a set of primers specifically targeting the BCP region of the HBV genome. During the qPCR assay, results were reported in “real-time” in the form of amplification curves (Figure 4.1) showing the amount of fluorescence emitted, which was directly proportional to the amount of HBV DNA present within the test samples. In this way, any HBV DNA present was quantified and reported in copies per mL (copies/mL). Where HBV DNA was not present within a test sample, no fluorescence was emitted and thus no peaks generated for that sample within the graph showing the amplification curves. The results obtained were also compared with positive (PC) and negative (NC) controls which were included in all qPCR assays performed.

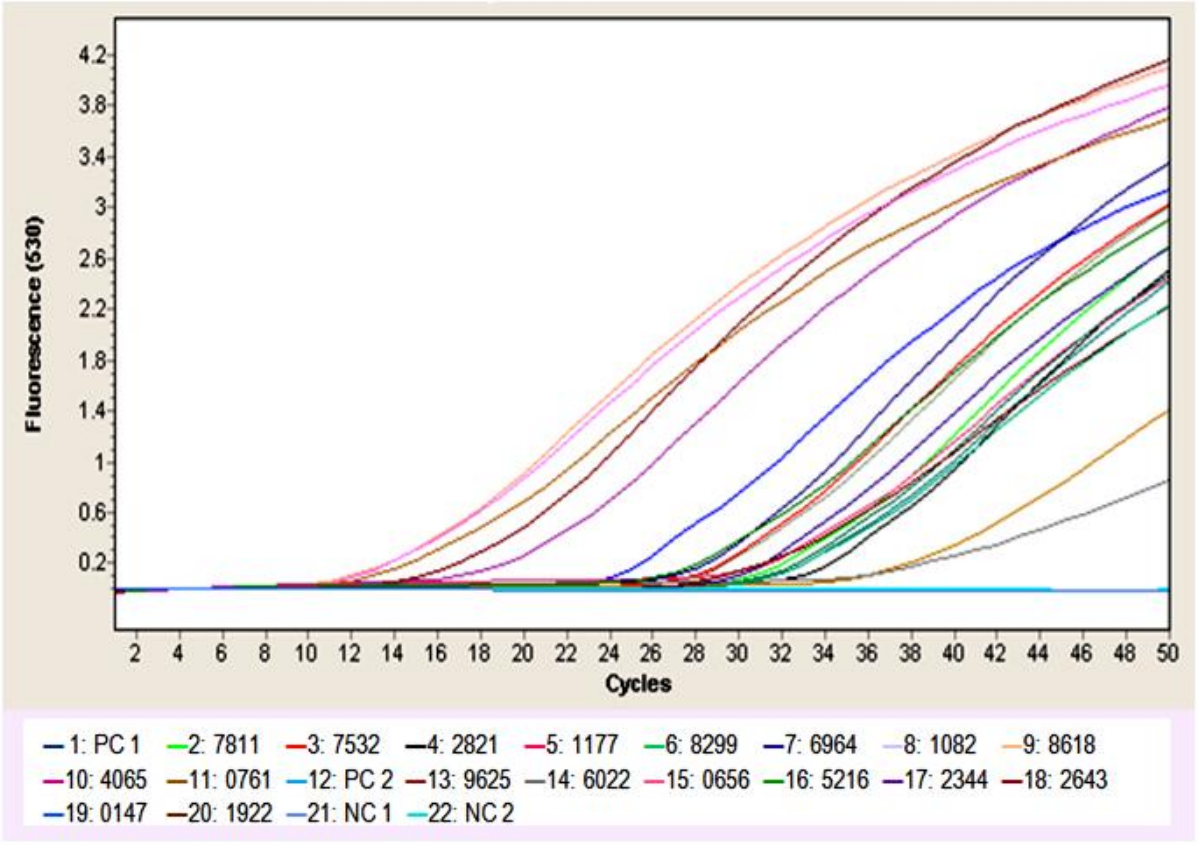


Figure 4.1: Graphical representation of amplification curves during qPCR assay (PC=Positive Control; NC=Negative Control)

To ensure specificity of the qPCR assay performed, a melting curve analysis was included during each run so as to confirm that the fluorescence generated was from the desired HBV DNA fragment and not a non-specific qPCR product. This was reported at the end of each assay in the form of a graph (Figure 4.2) plotting the fluorescence as a function of temperature. The melting peaks of test samples were also compared to that of positive controls (PC1 and PC2). All melting peaks at a melting temperature (T_m) of 2°C±85°C confirmed the amplified products to be the HBV BCP region while any melting peaks outside this temperature rage were regarded as non-specific qPCR products.

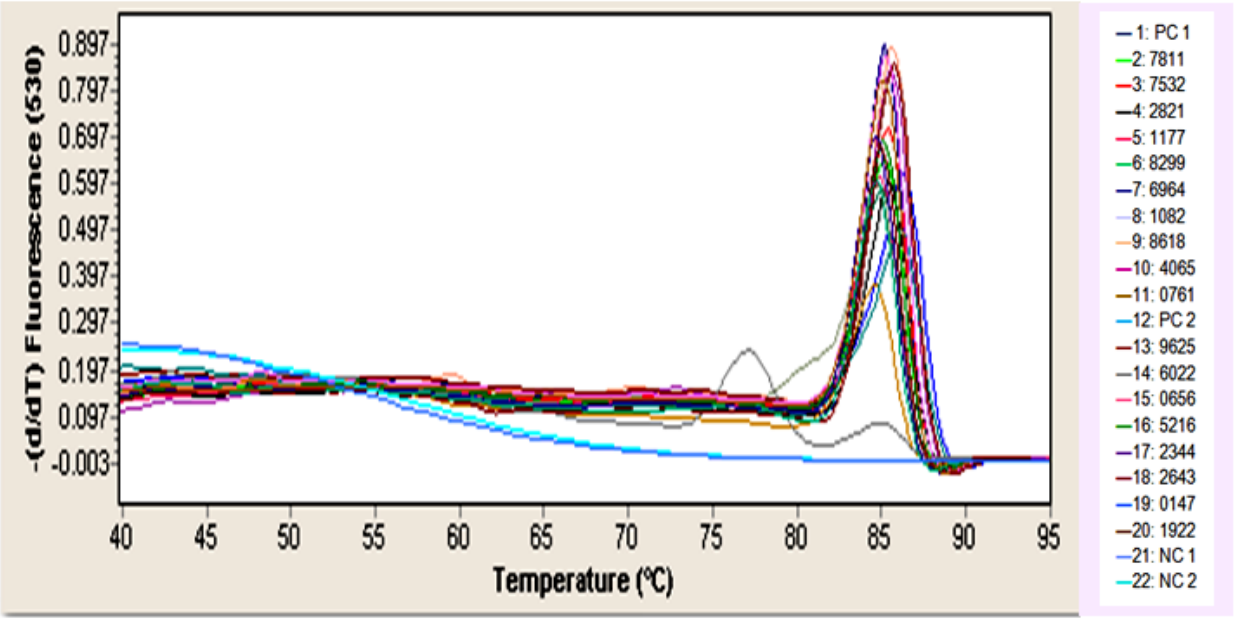


Figure 4.2: Graphical representation of melting peaks generated after melting curve analysis of qPCR products (PC=Positive Control; NC=Negative Control)

4.1.3 Nested PCR for HBV DNA detection

The detection of an active HBV infection; that is the presence of HBV DNA, especially within the post-vaccination population could imply that the study subjects simply did not receive the hepatitis B vaccine or may have received the vaccine but did not mount protective levels of anti-HBs. To identify any HBsAg mutations, the HBV S gene as well as the overlapping *pol* gene (when necessary) was amplified, sequenced and analyzed.

A nested PCR assay was thus employed in this study to amplify the viral S and *pol* genes. The sensitivity of the nested PCR assay used was first determined by performing duplicate serial dilutions (dilutions A and B) of an HBV positive control (human serum positive for HBV DNA at a concentration of 10^6 copies/mL) and running a PCR assay on all dilutions, amplifying both the S and *pol* genes. All PCR products were then subjected to an agarose gel electrophoresis so as to allow visualization of amplicons through UV illumination of the gel. As an example, Figure 4.3 shows the gel documentation of S gene amplicons for the duplicate serial dilutions (lanes A and B) following gel electrophoresis. The size of the amplicon, which was measured against a 100bp DNA molecular weight marker (MW), was found to be about 750 bp for the S gene (Figure 4.3) while that for the *pol* gene was about 645 bp. The highest dilution detectable was consistent for both A and B sets of dilutions, found to be 10^3 or 1000 copies/mL of HBV DNA by both the S and *pol* gene PCR assays.

Dilutions of 10^2 copies/mL or less were not detectable and as such only study samples determined by qPCR to have HBV DNA concentrations >10^2 copies/mL were selected for nested PCR. Positive (PC) and negative (NC) controls were also included in all nested PCR assays performed.

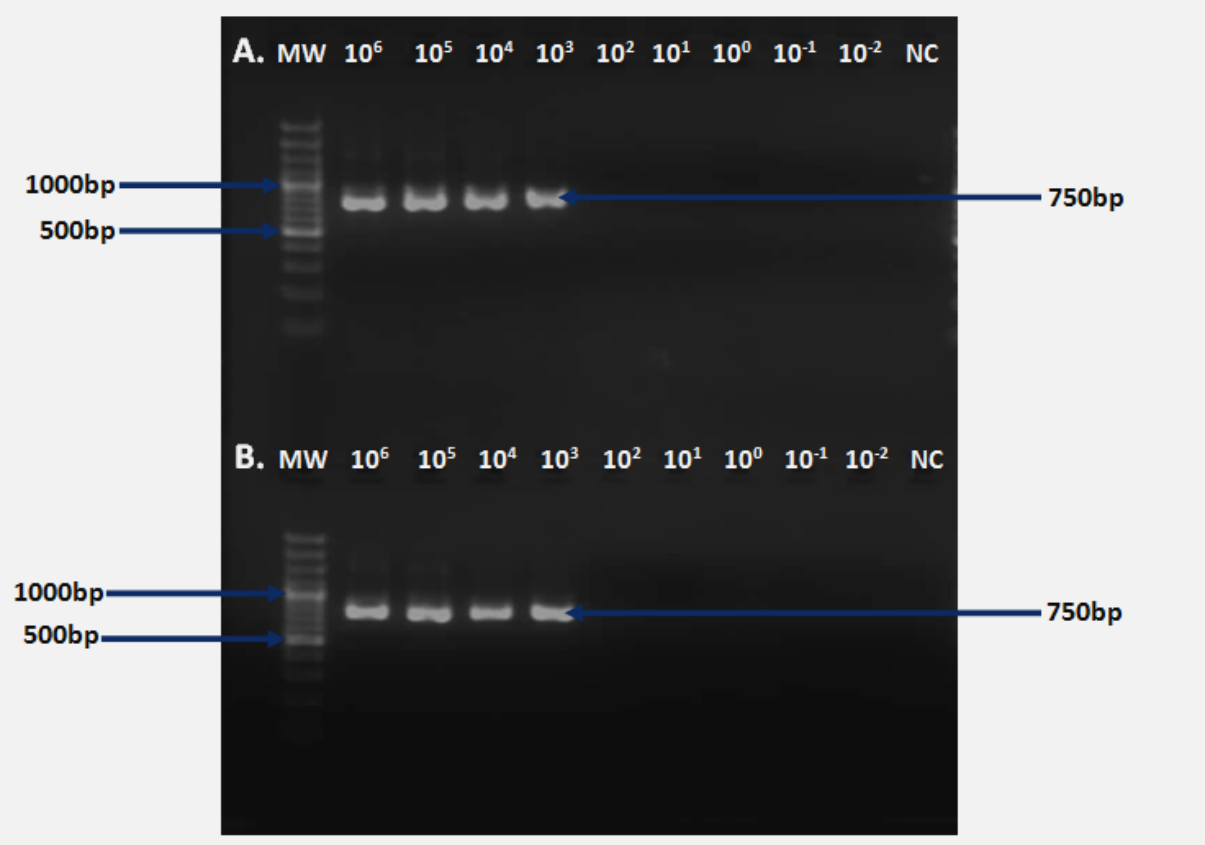


Figure 4.3: Gel documentation of S gene amplicons (750 bp) for duplicate serial dilutions (panels A and B) of HBV positive control. The highest dilution detectable in both dilutions was 10^3 copies/mL of HBV DNA. The DNA concentrations of 10^2 copies/mL or less were not detectable [MW=100bp DNA Molecular Weight Marker; NC=Negative Control]

4.2 Overview of results

4.2.1 Hepatitis B serology and qPCR results

All 1206 study samples were screened for primary serological markers; HBsAg, anti-HBc and anti-HBs. Of the 1206 samples screened, 201 (16.7%) had serological evidence of either past exposure to HBV (168/201) or HBV infection (33/201). Due to insufficient sample volume, only 133/201 (37/61 for the post-vaccination population and 97/140 for the pre-vaccination population) samples could be tested for IgM anti-HBc in order to determine the

presence of acute HBV infection. The presence of active HBV infection determined by HBV DNA positivity was also investigated within this population of serologically exposed or infected subjects. Table 4.1 shows a summary of the results obtained for serology and qPCR.

Table 4.1: Summary of HBV serology and qPCR results for both pre- and post-vaccination populations

Profiles	Profiles	Post-vaccination population				Pre-vaccination population
		Age strata			Total	
		1-5 yrs (%)	6-10 yrs (%)	11-15 yrs (%)		
Immunity	Anti-HBs positive alone	159/209 (76.1%)	77/154 (50.0%)	107/242 (44.2%)	343/605 (56.7%)	93/601 (15.5%)
Susceptibility	Negative for all primary markers[1]	33/209 (15.8%)	66/154 (42.9%)	102/242 (42.1%)	201/605 (33.2%)	368/601 (61.2%)
Infection	HBsAg positive alone	0/209 (0.0%)	0/154 (0.0%)	3/242 (1.2%)	3/605 (0.5%)	3/601 (0.5%)
	HBsAg and anti-HBs positive	1/209 (0.5%)	0/154 (0.0%)	0/242 (0.0%)	1/605 (0.2%)	1/601 (0.2%)
	HBsAg and anti-HBc positive	0/209 (0.0%)	1/154 (0.6%)	2/242 (0.8%)	3/605 (0.5%)	20/601 (3.3%)
	Positive for all primary markers[1]	0/209 (0.0%)	1/154 (0.6%)	1/242 (0.4%)	2/605 (0.3%)	0/601 (0.0%)
Past exposure	Anti-HBc positive alone	3/209 (1.4%)	3/154 (1.9%)	13/242 (5.4%)	19/605 (3.1%)	43/601 (7.2%)
	Anti-HBc and anti-HBs positive	13/209 (6.2%)	6/154 (3.9%)	14/242 (5.8%)	33/605 (5.5%)	73/601 (12.1%)
Total Exposure[2]	**$HBsAg^+/anti\text{-}HBc^+$**	**17/209 (8.1%)**	**11/154 (6.9%)**	**33/242 (13.6%)**	**61/605 (10.1%)**	**140/601 (23.3%)**
Acute infection*	IgM anti-HBc positive	0/10 (0.0%)	0/7 (0.0%)	0/20 (0.0%)	0/37* (0.0%)	14/96* (14.6%)
Active infection**	HBV DNA positive	12/17 (70.1%)	10/11 (90.9%)	21/33 (63.6%)	43/61 (70.5%)	106/140 (75.7%)

[1]Primary markers: HBsAg, anti-HBc and anti-HBs

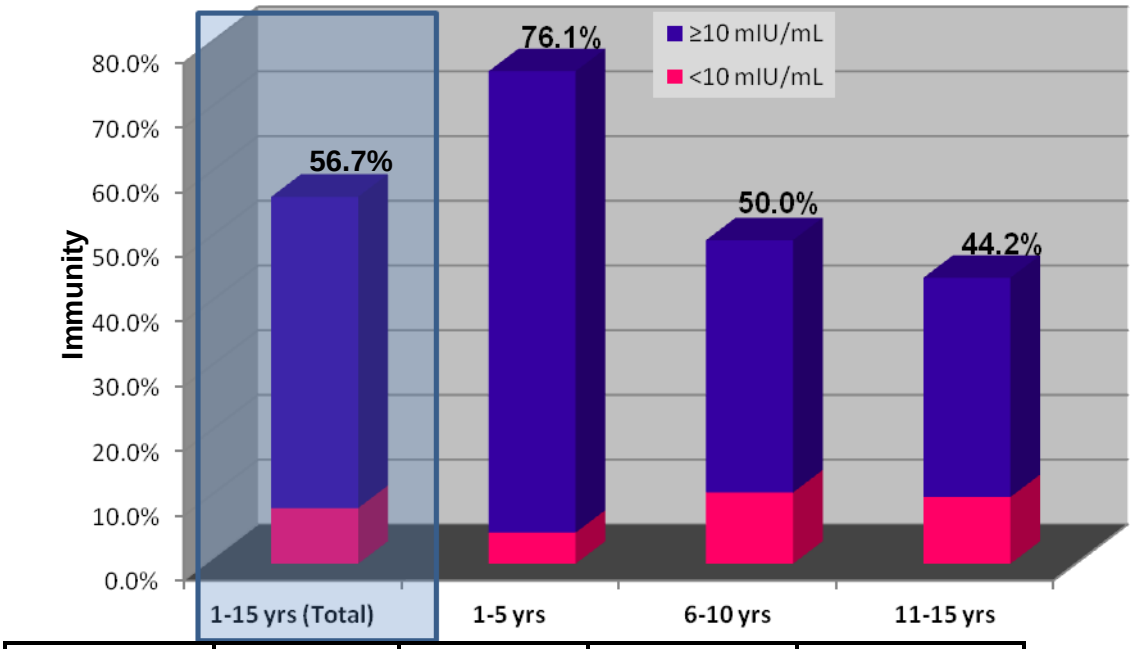
[2]Total exposure: HBsAg positive and/or anti-HBc positive. Subjects with at least one marker of total exposure were further tested for IgM anti-HBc and HBV DNA

* Not all samples with at least one marker of total exposure could be tested for IgM anti-HBc due to insufficient sample volume

[Note**: Viral loads of HBV DNA positive subjects, together with their serological profiles, were reported to the NHLS Diagnostic Department for future patient management]

4.2.1.1 Immunity to HBV infection

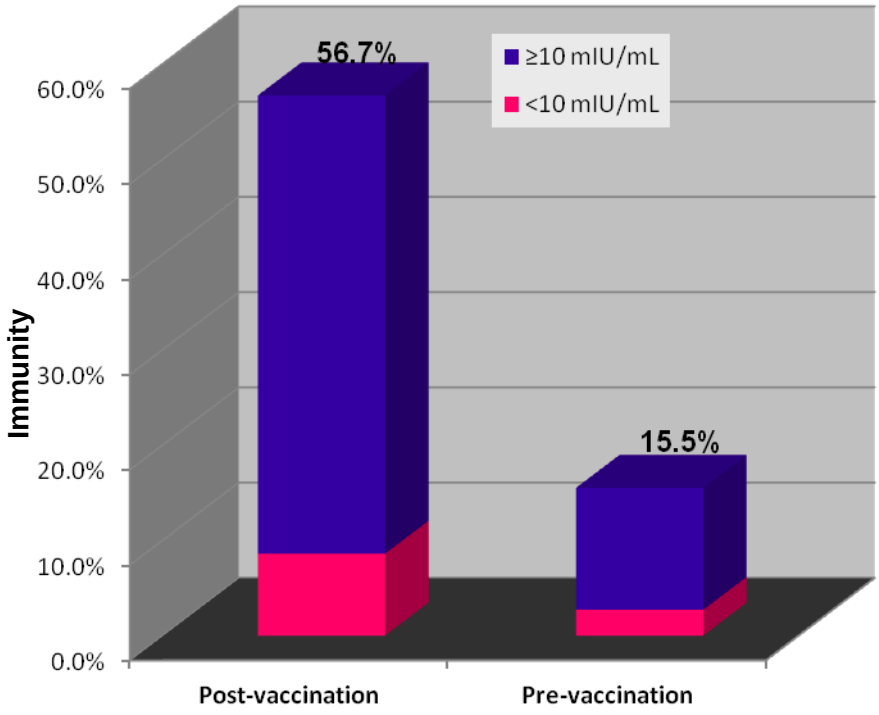
The detection of anti-HBs in the absence of all other serological markers is typically an indication of immunity to HBV infection as a result of exposure to the hepatitis B vaccine and not the virus itself. Thus, the detection of anti-HBs alone was taken as an indication of those within the population who may have received the hepatitis B vaccine. The titre of anti-HBs detected was also used as a measure of vaccine response within the study population. In assessing all age strata within the post-vaccination population alone, immunity was found to be higher (76.1%) in the youngest age group (1-5 years) and waned with increasing age to 50.0% in those 6-10 years of age and 44.2% in those 11-15 years of age (Figure 4.4), clearly supporting the study hypothesis that the majority of the individuals in this population should have received the hepatitis B vaccine. In terms of vaccine response, the majority of study subjects in each age stratum had anti-HBs titres ≥10 mIU/mL with just under 10% of this whole population having titres below the seroprotective level (<10 mIU/mL). As expected, the percentage of study subjects with anti-HBs titres <10 mIU/mL increased with increasing age; from 4.8% in those 1-5 years to 10.3% in those 11-15 years, a result of waning immunity. Geometric mean titres (GMT) of the anti-HBs detected were determined and were also found to wane with increasing age; from 110.2 mIU/mL in the 1-5 years age group to 40.98 mIU/mL in the 11-15 years age group (Figure 4.4).



GMT (mIU/mL)	**63.96**	**110.20**	**38.59**	**40.98**

Figure 4.4: Immunity to HBV infection within the post-vaccination population as per age strata

Overall, there was a significant (p<0.001) difference in immunity between the post-vaccination population and the pre-vaccination population with 56.7% and 15.5% respectively (Figure 4.5). The GMT of anti-HBs detected was also found to be marginally higher in the pre-vaccination population (73.38 mIU/mL) as compared to the post-vaccination population (63.96 mIU/mL). In assessing the difference in immunity rates between male and female study subjects, no statistically significant association could be made, with p values of 0.27 and 0.83 for the post- and pre-vaccination populations respectively.



GMT (mIU/mL)	**63.96**	**73.38**

Figure 4.5: Immunity to HBV infection within the post- and pre-vaccination populations

4.2.1.2 Susceptibility to HBV infection

Susceptibility to infection was determined as seronegativity for all primary serological markers; HBsAg, anti-HBc and anti-HBs. Although almost half of those within the 6-10 and 11-15 years age groups had serological evidence of immunity, the other half of these two groups (42.9% of those 6-10 years and 42.1% of those 11-15 years) were negative for all three primary markers and thus were susceptible to HBV infection (Table 4.1). On the other hand, only 15.8% of those in the 1-5 years age group were susceptible to HBV infection. In

comparing the two main study populations, susceptibility was notably higher (p<0.001) in the pre-vaccination population (61.2%) as compared to the post-vaccination population (33.2%).

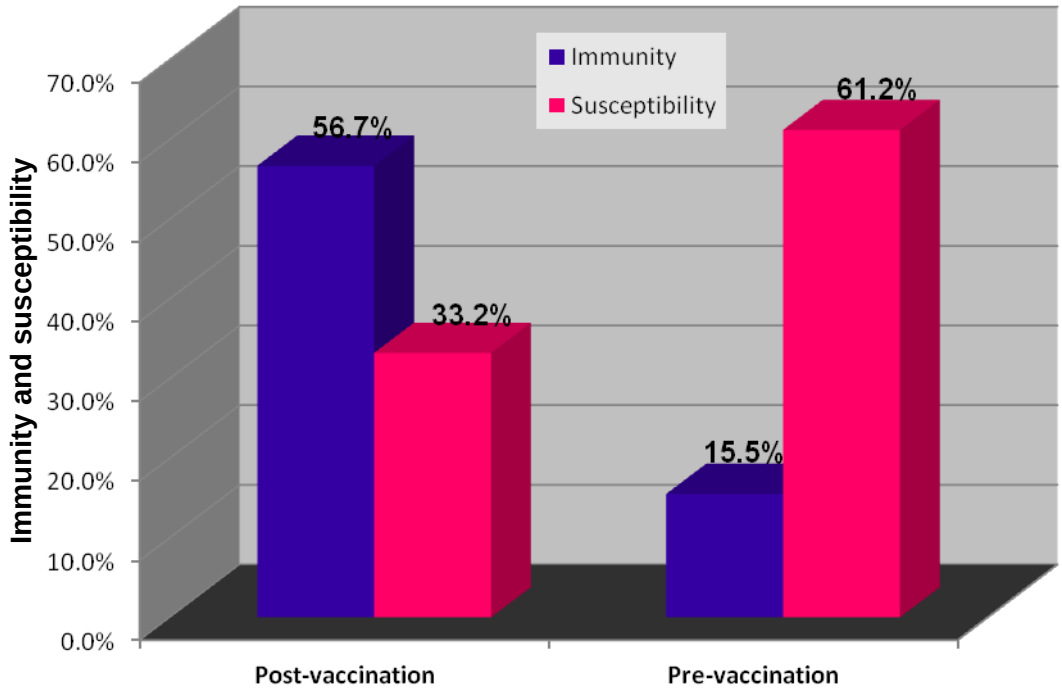


Figure 4.6: Immunity and susceptibility to HBV infection within the post- and pre-vaccination populations

An association was thus made between high immunity rates and reduced susceptibility to HBV infection within the post-vaccination population (Figure 4.6).

4.2.1.3 Prevalence of HBV chronic carriage

Chronic carriage was determined by the prevalence of HBsAg within the population. Within the post-vaccination population, the chronic carriage rate was at 1.5% (9/605), found to be largely driven by the HBsAg prevalence rates within the 6-10 (1.3%) and 11-15 (2.5%) years age groups. Only 1 (0.5%) study subject within the 1-5 years age group was found to be HBsAg positive. Thus in associating immunity and chronic carriage within the different age strata, it was found that while immunity waned with increasing age, chronic carriage appeared to increase with increasing age (Figure 4.7).

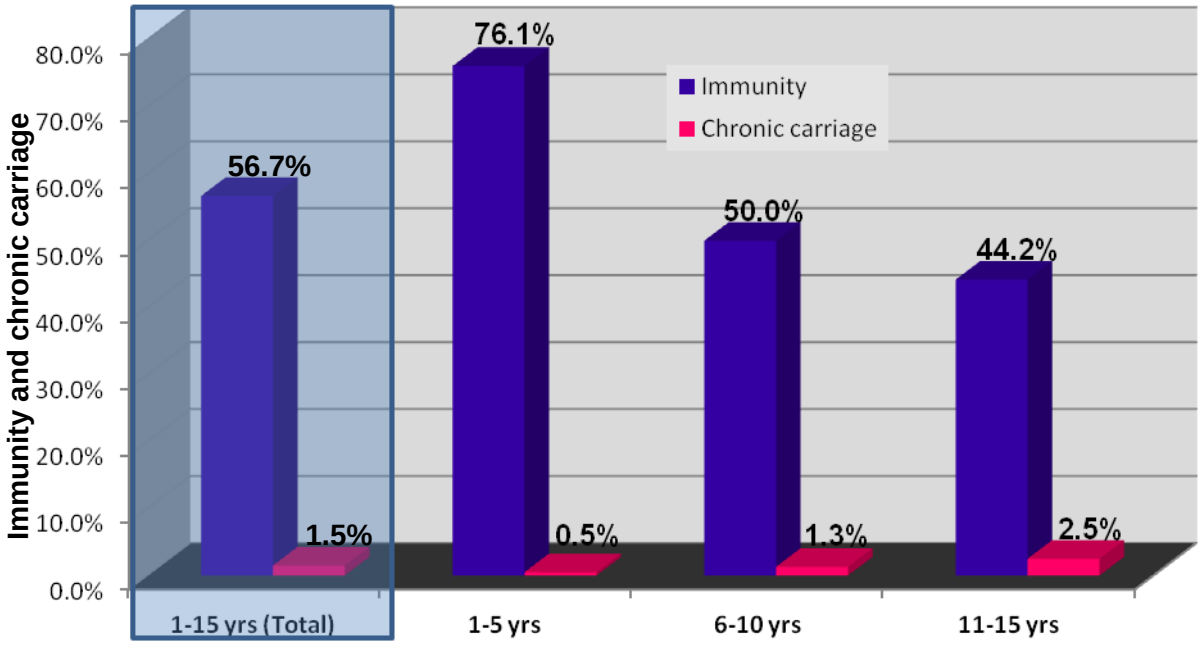


Figure 4.7: Immunity and chronic carriage within the post-vaccination population as per age strata

The difference in chronic carriage rates between the post- and pre-vaccination populations was found to be statistically significant (p=0.008), being higher in the pre-vaccination population (4.0%) than in the post-vaccination population (1.5%). Within the pre-vaccination population alone, chronic carriage was significantly (p=0.03) higher in females (2.2%) than males (1.7%), while the difference between the genders in the post-vaccination population was not statistically significant (p=0.49).

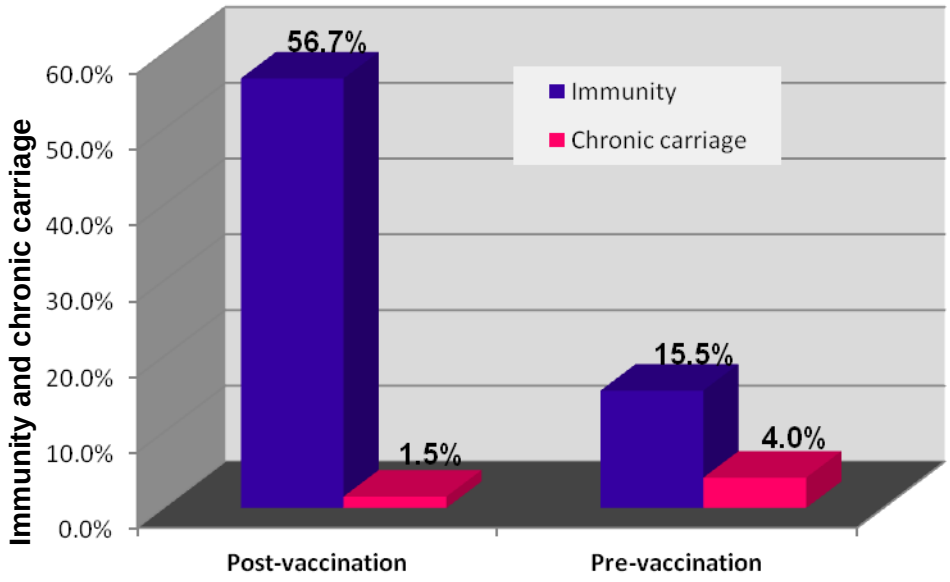


Figure 4.8: immunity and chronic carriage within the post- and pre-vaccination populations

4.2.1.4 Incidence of acute HBV infection

Of the 37 post-vaccination samples that had serological evidence of exposure (anti-HBc positive) or infection (HBsAg positive) to HBV, none were positive for IgM anti-HBc. Thus the exposures and infections detected within this 37 study subjects were confirmed to be of a chronic nature and not acute. In contrast, an IgM anti-HBc prevalence rate of 14.6% (14/96) was found for the pre-vaccination population (Table 4.1).

4.2.1.5 Prevalence of active HBV infection

Of the 201 serologically exposed or infected samples screened for HBV DNA, 149 (43 from the post-vaccination population and 106 from the pre-vaccination population) tested positive for HBV DNA and thus carried an active HBV infection. The prevalence of active HBV infection was higher in the pre-vaccination population than in the post-vaccination population (Table 4.1). Among male and female study subjects, the difference in the prevalence of active HBV infection was not statistically significant, with *p* values of 0.37 and 0.22 for the post- and pre-vaccination populations respectively. Of the 149 overall HBV DNA positive subjects, only 33 (22.1%) were HBsAg positive, leaving a total of 116 (77.9%) HBsAg negative subjects. Thus, the majority (116/149 or 77.9%) of HBV DNA positive study subjects had an occult HBV infection; 34/116 (29.3%) accounted for by the post-vaccination population (1-5 years=11/34, 6-10 years=8/34 and 11-15 years=15/34 occult HBV infections).

4.2.1.6 Total HBV exposure

Total HBV exposure, was measured by the total number of study subjects positive for either HBsAg or anti-HBc, or both serological markers.

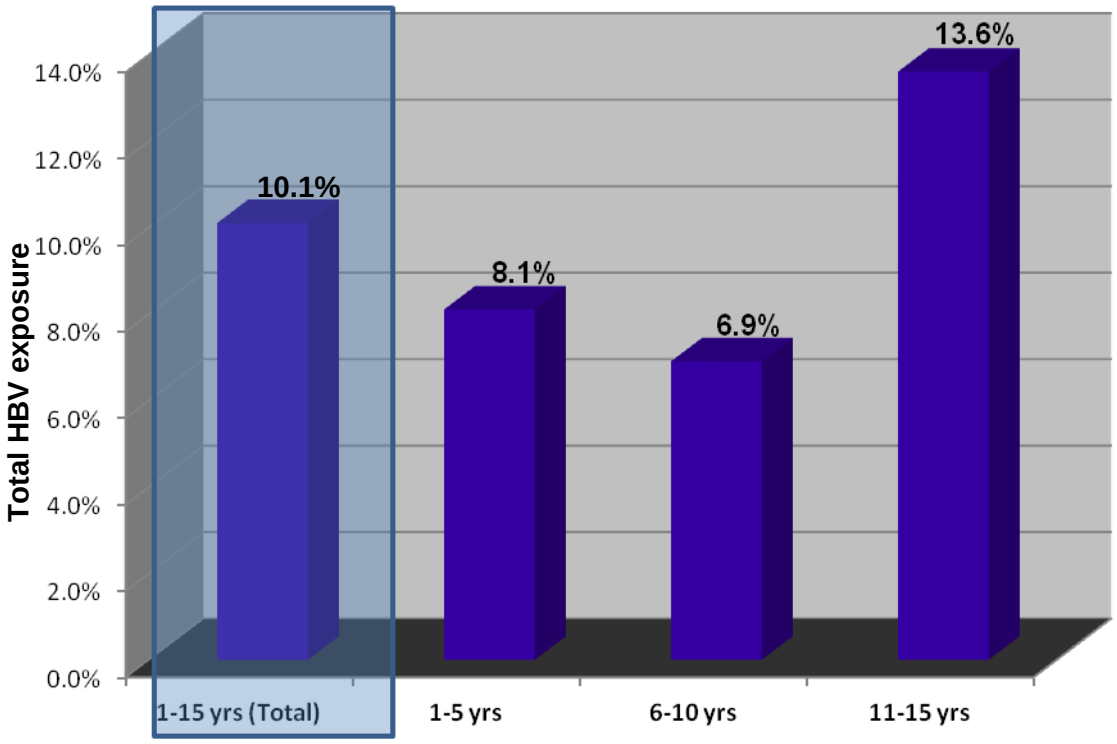


Figure 4.9: Total HBV exposure within the post-vaccination population as per age strata

Total HBV exposure was found to be higher within the oldest age group (13.6% in 11-15 year olds) as compared to the other two age strata within the post-vaccination population (Figure 4.9). Overall, total exposure was higher (p<0.001) in the pre-vaccination population than the post-vaccination population with 23.3% and 10.1% respectively (Figure 4.10).

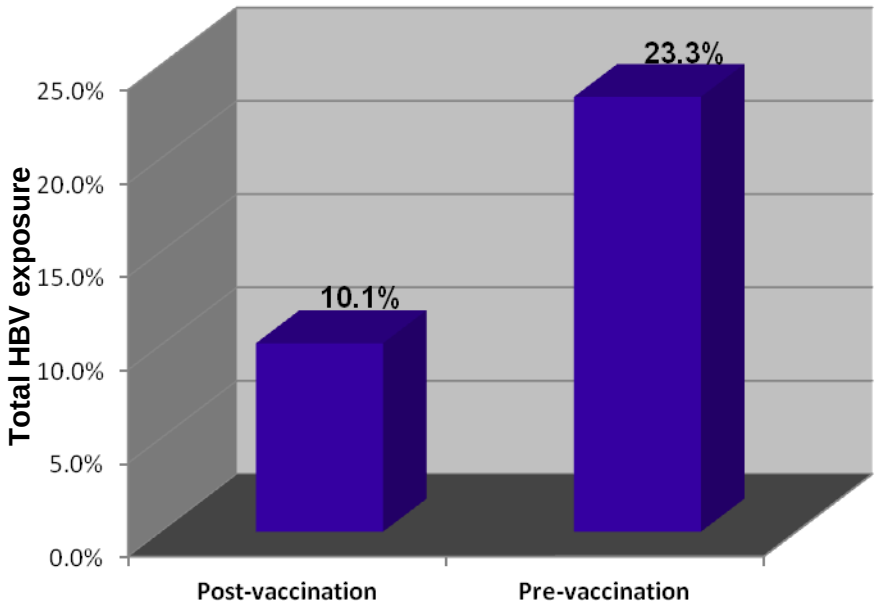


Figure 4.10: Total HBV exposure within the post- and pre-vaccination populations

4.2.2 Subset analysis of study subjects with HIV records

Records of HIV status, either HIV infected or uninfected, were obtained for a total of 346 (28.7%) of the 1 206 study subjects; 230 (66.5%) for the post-vaccination population and 116 (33.5%) for the pre-vaccination population. All the 346 samples were stratified by HIV status as HIV infected (HIV+) or uninfected (HIV-), and hepatitis B serology and qPCR results analyzed within this subset. Table 4.2 gives a summary of the serology and qPCR results for the HIV subset of both the post- and pre-vaccination populations.

Table 4.2: Summary of hepatitis B serology and qPCR results for the HIV subset of the post-and pre-vaccination populations

Profiles	Profiles	Post-vaccination population								Pre-vaccination population	
		Age strata						Total			
		1-5 yrs		6-10 yrs		11-15 yrs					
		HIV+	HIV-	HIV+	HIV-	HIV+	HIV-	HIV+	HIV-	HIV+	HIV-
Immunity	Anti-HBs positive alone	6/20 (30.0%)	80/89 (90.9%)	5/20 (25.0%)	24/32 (75.0%)	2/19 (10.5%)	37/50 (74.0%)	13/59 (22.0%)	141/171 (82.5%)	0/49 (0.0%)	18/67 (26.7%)
Susceptibility	Negative for all primary markers[1]	8/20 (40.0%)	6/89 (6.7%)	12/20 (60.0%)	5/32 (15.6%)	14/19 (73.7%)	9/50 (18.0%)	34/59 (57.6%)	20/171 (11.7%)	24/49 (49.0%)	39/67 (58.2%)
Infection	HBsAg positive alone	0/20 (0.0%)	0/89 (0.0%)	0/20 (0.0%)	0/32 (0.0%)	0/19 (0.0%)	0/50 (0.0%)	0/59 (0.0%)	0/171 (0.0%)	1/49 (2.0%)	0/67 (0.0%)
	HBsAg and anti-HBs positive	1/20 (5.0%)	0/89 (0.0%)	0/20 (0.0%)	0/32 (0.0%)	0/19 (0.0%)	0/50 (0.0%)	1/59 (1.7%)	0/171 (0.0%)	0/49 (0.0%)	0/67 (0.0%)
	HBsAg and anti-HBc positive	0/20 (0.0%)	0/89 (0.0%)	0/20 (0.0%)	0/32 (0.0%)	0/19 (0.0%)	0/50 (0.0%)	0/59 (0.0%)	0/171 (0.0%)	1/49 (2.0%)	1/67 (1.5%)
	Positive for all primary markers[1]	0/20 (0.0%)	0/89 (0.0%)	0/20 (0.0%)	0/32 (0.0%)	0/19 (0.0%)	0/50 (0.0%)	0/59 (0.0%)	0/171 (0.0%)	0/49 (0.0%)	0/67 (0.0%)
Past exposure	Anti-HBc positive alone	0/20 (0.0%)	1/89 (1.1%)	3/20 (15.0%)	0/32 (0.0%)	3/19 (15.8%)	2/50 (4.0%)	6/59 (10.2%)	3/171 (1.8%)	13/49 (26.5%)	3/67 (4.5%)
	Anti-HBc and anti-HBs positive	5/20 (25.0%)	2/89 (2.2%)	0/20 (0.0%)	3/32 (9.4%)	0/19 (0.0%)	2/50 (4.0%)	5/59 (8.5%)	7/171 (4.1%)	10/49 (20.4%)	6/67 (9.0%)
Total exposure[2]	**HBsAg$^+$/anti-HBc$^+$**	**6/20 (30.0%)**	**3/89 (3.4%)**	**3/20 (15.0%)**	**3/32 (9.4%)**	**3/19 (15.8%)**	**4/50 (8.0%)**	**12/59 (20.3%)**	**10/171 (5.8%)**	**25/49 (51.0%)**	**10/67 (14.9%)**
Acute infection*	IgM anti-HBc positive	0/5 (0.0%)	0/3 (0.0%)	0/3 (0.0%)	0/3 (0.0%)	0/3 (0.0%)	0/4 (0.0%)	0/9 (0.0%)	0/5 (0.0%)	0/13 (0.0%)	1/9 (11.1%)
Active infection**	HBV DNA positive	6/6 (100.0%)	2/3 (66.7%)	3/3 (100.0%)	3/3 (100.0%)	2/3 (66.7%)	2/4 (50.0%)	11/12 (91.7%)	7/10 (70.0%)	17/25 (68.0%)	9/10 (90.0%)

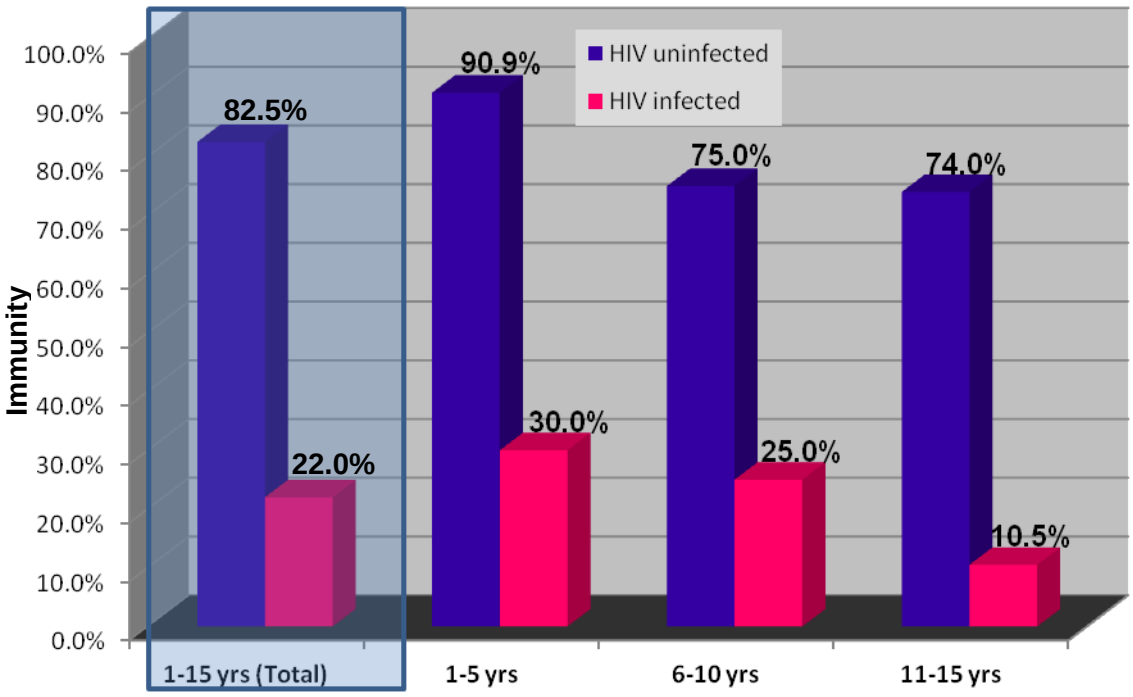
[1]Primary markers: HBsAg, anti-HBc and anti-HBs

[2]Total exposure: HBsAg positive and/or anti-HBc positive. Subjects with at least one marker of total exposure were further tested for IgM anti-HBc and HBV DNA

* Not all samples with at least one marker of total exposure could be tested for IgM anti-HBc due to insufficient sample volume

[Note**: Viral loads of HBV DNA positive subjects, together with their serological profiles, were reported to the NHLS Diagnostic Department for future patient management]

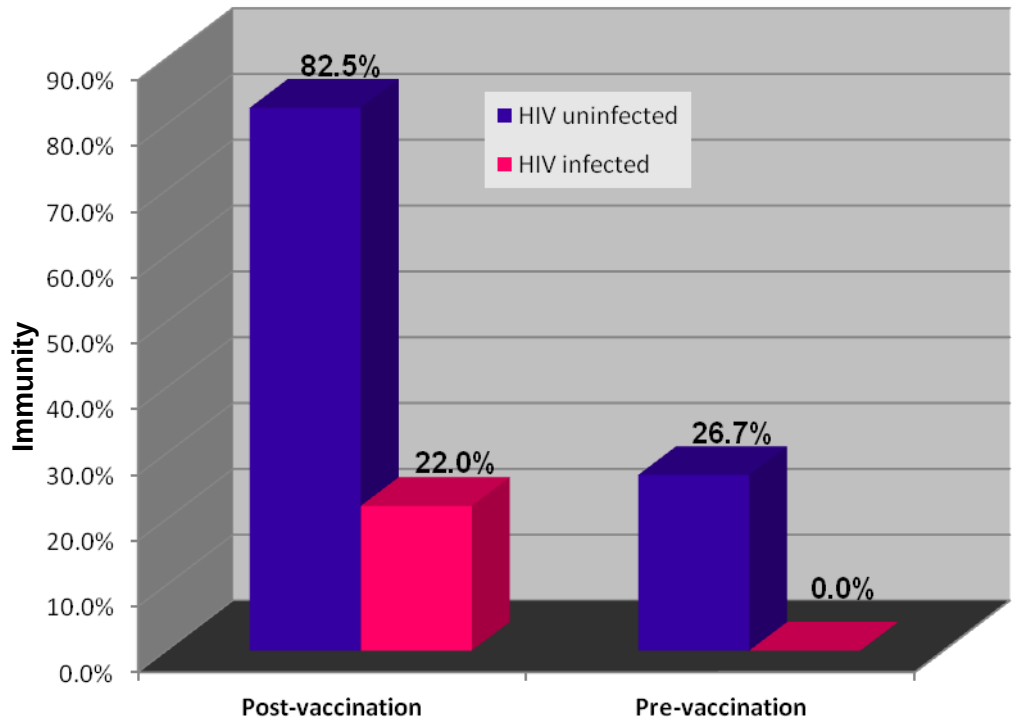
4.2.2.1 Immunity to HBV infection



GMT (mIU/mL)	**72.05**	**26.95**	**118.23**	**61.95**	**33.99**	**13.22**	**40.20**	**13.15**

Figure 4.11: Immunity to HBV infection within the HIV infected and uninfected subsets as per age strata

Within all age strata, immunity and GMT of anti-HBs were consistently higher in the HIV uninfected population as compared to the HIV infected population (Figure 4.11). Within both HIV infected and uninfected populations, immunity to HBV infection appeared to wane with increasing age as did the GMT of the anti-HBs detected. In comparing the post- and pre-vaccination populations, immunity was higher within the post-vaccination population compared to the pre-vaccination population regardless of HIV status. When considering HIV status, immunity was significantly (p<0.001) higher in the HIV uninfected populations compared to the HIV infected populations within both post- and pre-vaccination populations, with no anti-HBs detected in the HIV infected subset of the pre-vaccination population (Figure 4.12).



GMT (mIU/mL)	**72.05**	**26.95**	**56.71**	**-**

Figure 4.12: Immunity to HBV infection within the HIV infected and uninfected subsets

4.2.2.2 Susceptibility to HBV infection

Within the post-vaccination population, susceptibility to HBV infection was found to be significantly (p<0.001) higher in the HIV infected population (57.6%) as compared to the HIV uninfected population (11.7%), and also increased with increasing age within the different age strata (Table 4.2). Thus a high rate of immunity in the HIV uninfected population was associated with reduced susceptibility to infection within the post-vaccination population. On the contrary, even though immunity was higher in the HIV uninfected subset of the pre-vaccination population, susceptibility to infection was rather high (although not statistically significant; p=0.37) as compared to the HIV infected subset (Table 4.3).

Table 4.3: Immunity and susceptibility to HBV infection within the HIV subset

Profiles	Post-vaccination		Pre-vaccination	
	HIV+	**HIV-**	**HIV+**	**HIV-**
Immunity	13/59 (22.0%)	141/171 **(82.5%)**	0/49 (0.0%)	18/67 **(26.7%)**
Susceptibility	34/59 (57.6%)	20/171 **(11.7%)**	24/49 (49.0%)	39/67 **(58.2%)**

4.2.2.3 Prevalence of HBV chronic carriage

Chronic carriage, as measured by the prevalence of HBsAg, was only established in 1 (1.7%) HIV infected study subject within the post-vaccination population (Table 4.2). Within the pre-vaccination population, chronic carriage was higher in the HIV infected population than in the HIV uninfected population with 4.1% and 1.5% respectively (Figure 4.13).

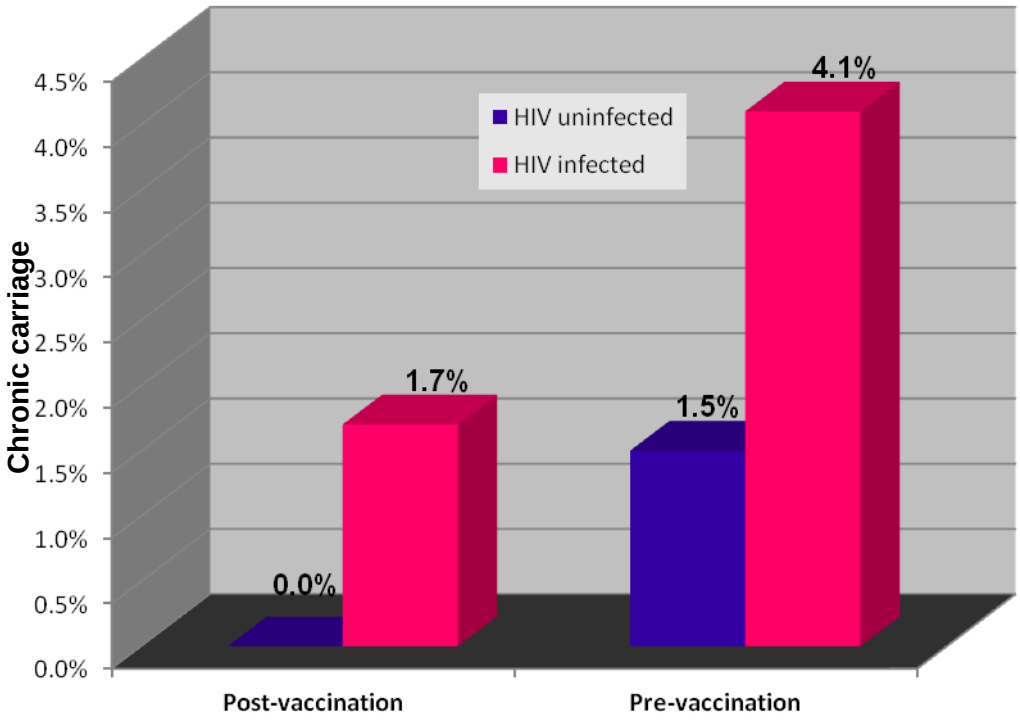


Figure 4.13: Prevalence of HBV chronic carriage within the HIV infected and uninfected subsets

4.2.2.4 Incidence of acute HBV infection

No acute HBV infections were detected within the HIV subset of the post-vaccination population. Within the pre-vaccination population however, acute HBV infection was only detected in 1 HIV uninfected study subject (Table 4.2).

4.2.2.5 Prevalence of active HBV infection

Of the 57 serologically exposed or infected ($HBsAg^+$ and/or anti-HBc^+) study subjects screened for HBV DNA within the overall subset, 34 (18 from the post-vaccination population and 26 from the pre-vaccination population) tested positive and thus had an active HBV infection. Of the 18 HBV DNA positive subjects from the post-vaccination population, only 1 was HBsAg positive while 3 out of the 26 HBV DNA positive subjects from the pre-vaccination population were HBsAg positive. The prevalence of HBV DNA within the post-vaccination population was higher (but not significant, p=0.22) in those who were HIV infected than in those who were HIV uninfected. Within the pre-vaccination population however, HBV DNA was more prevalent (p=0.23) in the HIV uninfected population than in the HIV infected population (Table 4.2).

4.2.2.6 Total HBV exposure

Total HBV exposure within the post-vaccination population was found to increase with increasing age within the HIV uninfected population. In the HIV infected population however, total HBV exposure appeared to decrease with increasing age, with the highest rate found in those 1-5 years of age (30.0%) (Figure 4.14).

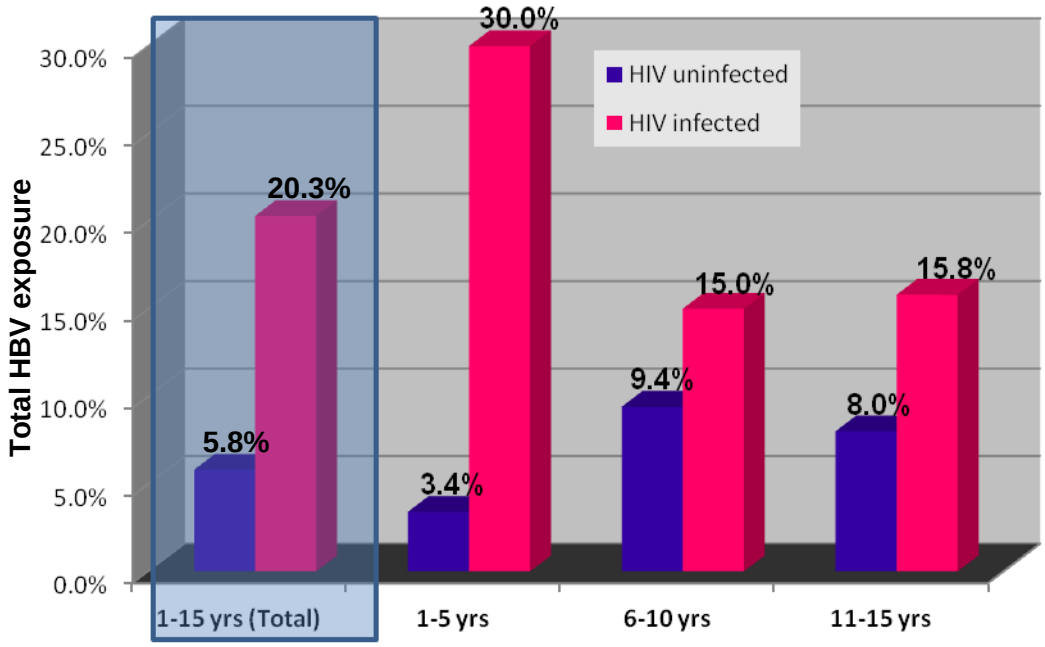


Figure 4.14: Total HBV exposure within the HIV infected and uninfected subsets of the post-vaccination population

Total HBV exposure was higher in the HIV infected population as compared to the HIV uninfected population in both the post- (p=0.001) and pre-vaccination (p<0.001) populations (Figure 4.15).

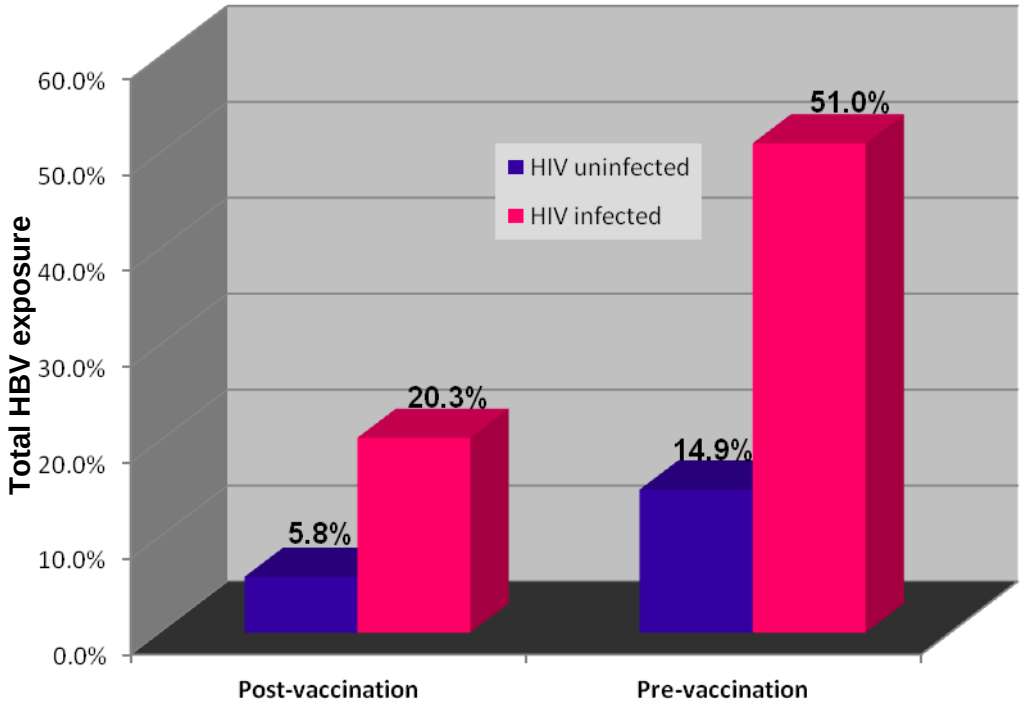


Figure 4.15: Total HBV exposure within the HIV infected and uninfected subsets

4.3 Molecular characterization of HBV S gene

4.3.1 Detection of HBV DNA

A total of 47 samples with HBV DNA concentrations >10^2 copies/mL were selected for nested PCR to detect and amplify the HBV S gene. Of the 47 samples for which the HBV S gene was amplified, 10 of them were undetectable. Figure 4.16 shows an example of an agarose gel documentation of S gene amplicons.

Figure 4.16: Agarose gel documentation of HBV S gene amplicons [MW=100bp DNA Molecular weight marker, PC=Positive Control, NC=Negative Control, 1-20=study samples]. The HBV S amplicon was undetectable in sample 9.

Of the 37 HBV DNA carriers for whom the S gene was successfully amplified, 9 were from the post-vaccination population while the remaining 28 were from the pre-vaccination population. All 37 HBV S gene amplicons were sequenced and nucleotide and amino acid sequences analyzed.

4.3.2 Phylogenetic analyses

A phylogenetic tree was generated for all 37 nucleotide sequences as shown in Figure 4.17. The predominant genotype was genotype A, with only one study sequence (sample 6964) clustering with genotype D reference sequences. None of the study sequences clustered with reference sequences for genotypes B, C, E, F, G, or H.

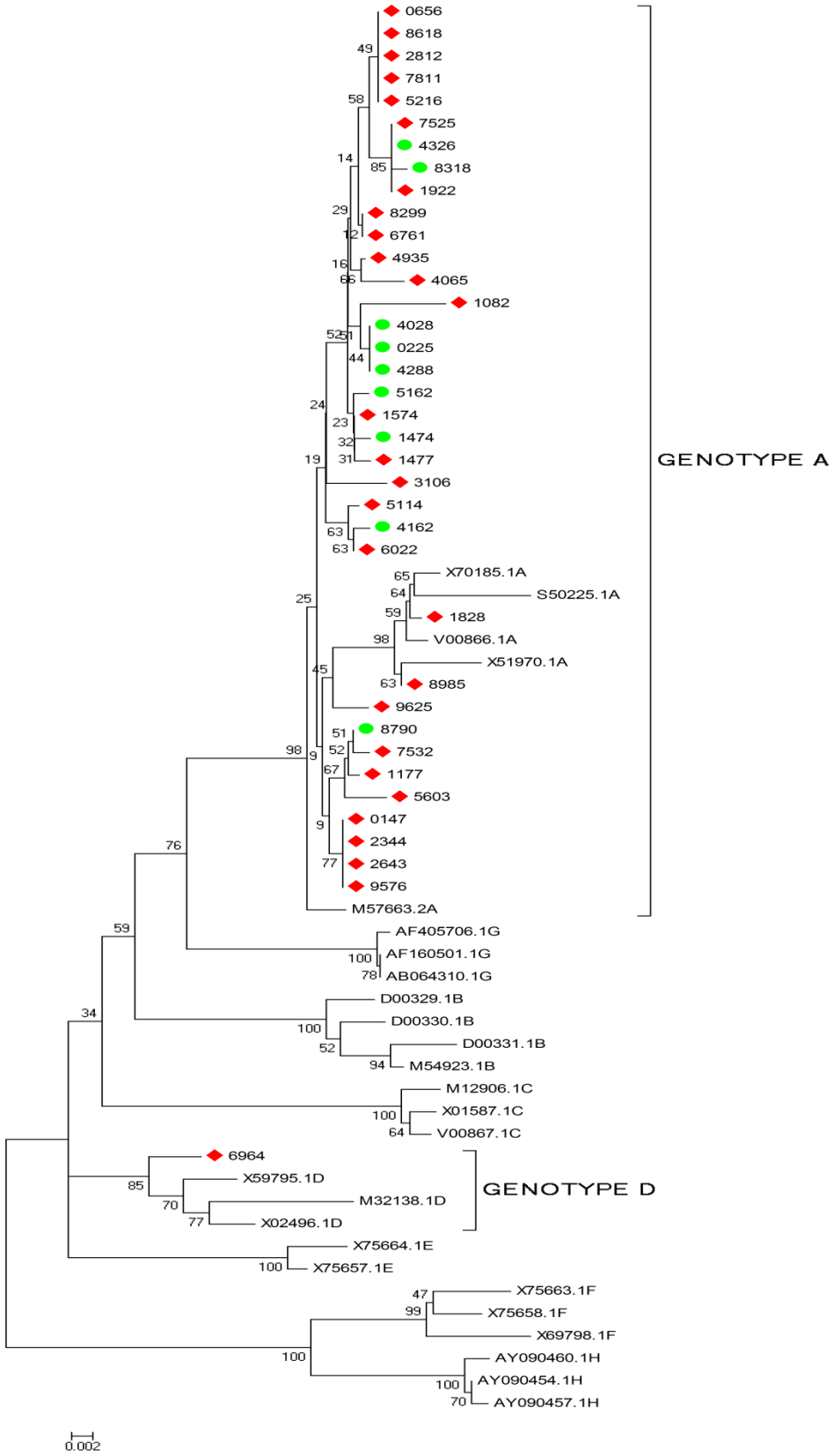


Figure 4.17: Phylogenetic analyses of HBV S gene nucleotide sequences. Study sequences are labelled ◆ for the pre-vaccination population and ● for the post-vaccination population, while reference sequences from GenBank are unlabelled.

4.3.3 Nucleotide sequence variations

All nucleotide sequences were aligned with relevant reference sequences, either genotype A or D reference sequences, in order to determine the presence of any nucleotide variations. Aligned sequences are shown in Figures 4.18 and 4.19. All nucleotide variations detected for genotype A sequences are summarized in Table 4.4.

Table 4.4: Nucleotide variations detected within genotype A sequences

Variations	Nucleotide Positions	Significance
Nucleotide substitutions	T22C, G132C, G132A, G132T, T133C, T146G, C192T, C209A, G282A, T288A, T300C, G309A, A339C, C348G, T351C, A357T, A360C, A360T, A365G, A366G, A427T, T432C, T438C, C444T, G462A, C465T, G475C, G492C, T503C, T513C, A534C, C581T, T600C, T600A, A612T, G620A, G625T, T647A, C650T, T655C, C666T, T678A.	**Missense mutations**: T22C, G132C + T133C, T146G, C209A, G309A, A365G + A366G, A427T, G475C, G492C, T503C, A612T, G620A, G625T, C650T, T655C.
		Nonsense mutation: T600A, T647A,
		Silent mutations: G132A, G132T, C192T, G282A, T288A, T300C, A339C, C348G, T351C, A357T, A360C, A360T, T432C, T438C, C444T, G462A, C465T, T513C, A534C, C581T, T600C, C666T, T678A.

The nucleotide variations detected within the genotype D (sample 6964) sequence were also nucleotide substitutions. The significance of these nucleotide substitutions are shown in Table 4.5.

Table 4.5: Nucleotide variations detected within the genotype D sequence

Variation	Nucleotide Positions	Significance
Nucleotide substitutions	T339A, A360C, G366A, C390A, A392C, C420A, C432T, T438C, T465C, C513T, C671T.	**Missense mutations**: A392C and C671T.
		Silent mutations: T339A, A360C, G366A, C390A C420A, C432T, T438C, T465C, C513T.

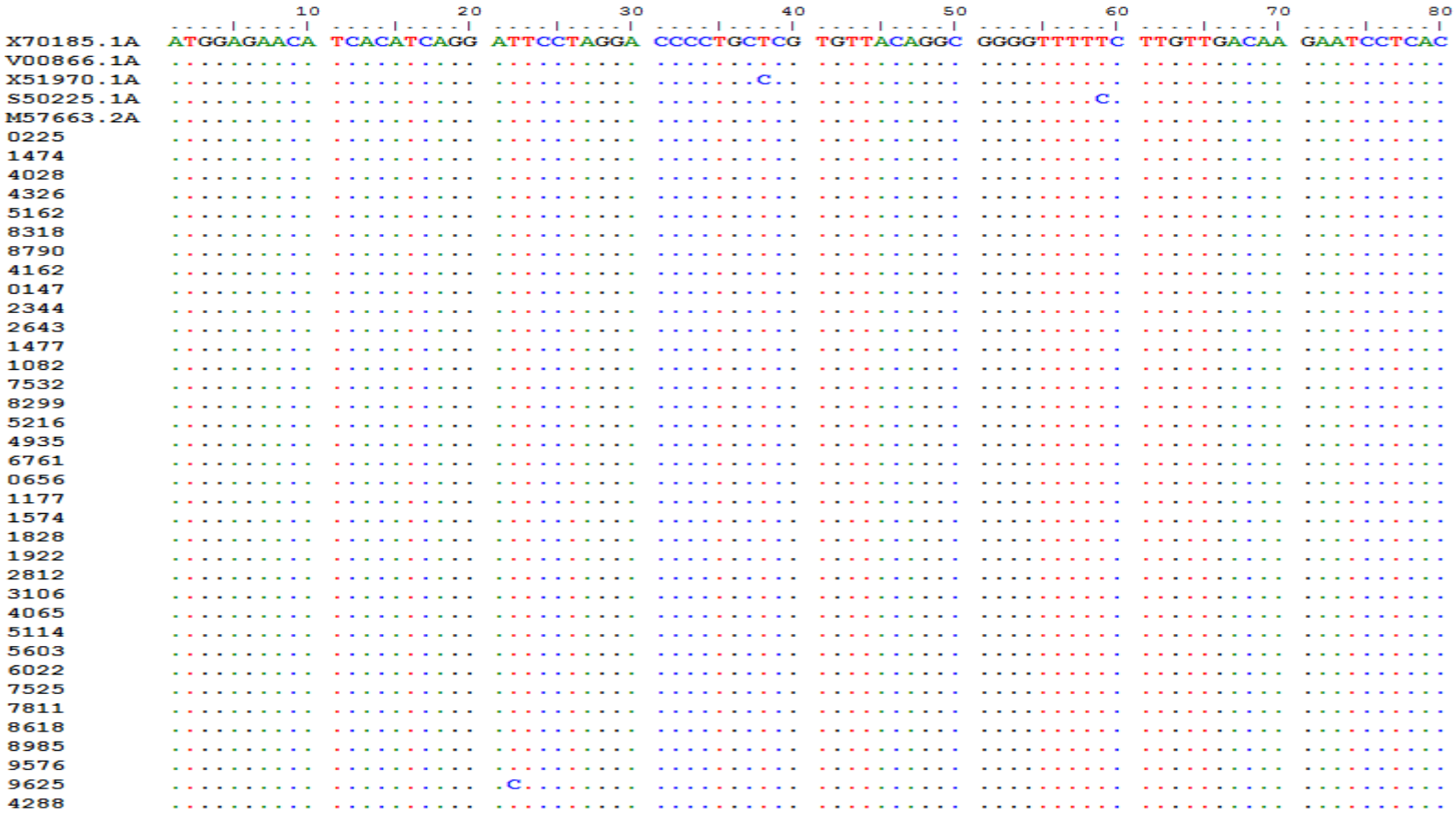


Figure 4.18: Nucleotide sequence alignment of genotype A isolates [Genotype A reference sequences: X20185.1, V00866.1, X51970.1, S50225.1 and M57663.2]

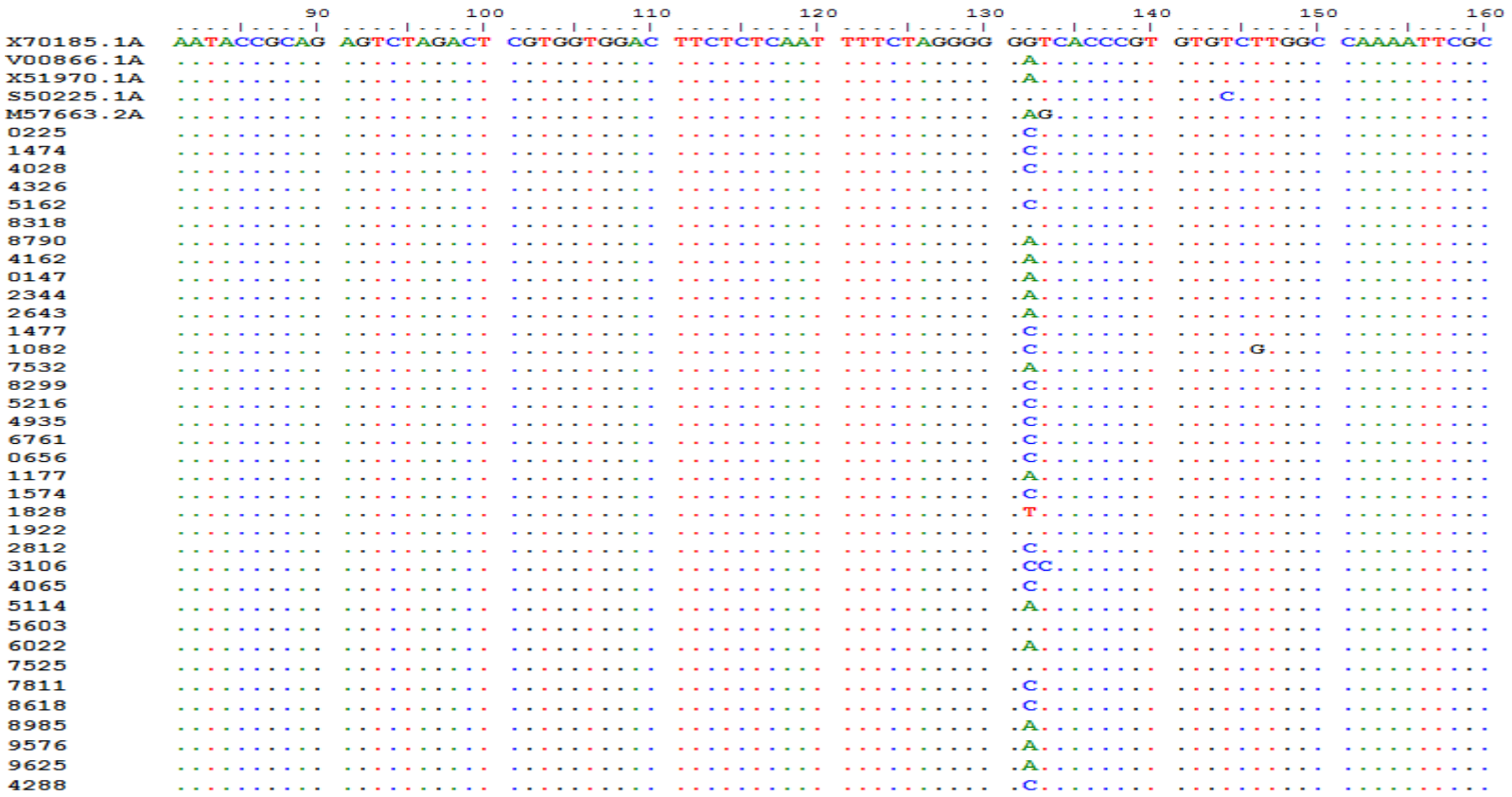


Figure 4.18: Nucleotide sequence alignment of genotype A isolates [Genotype A reference sequences: X20185.1, V00866.1, X51970.1, S50225.1 and M57663.2]

Figure 4.18: Nucleotide sequence alignment of genotype A isolates [Genotype A reference sequences: X20185.1, V00866.1, X51970.1, S50225.1 and M57663.2]

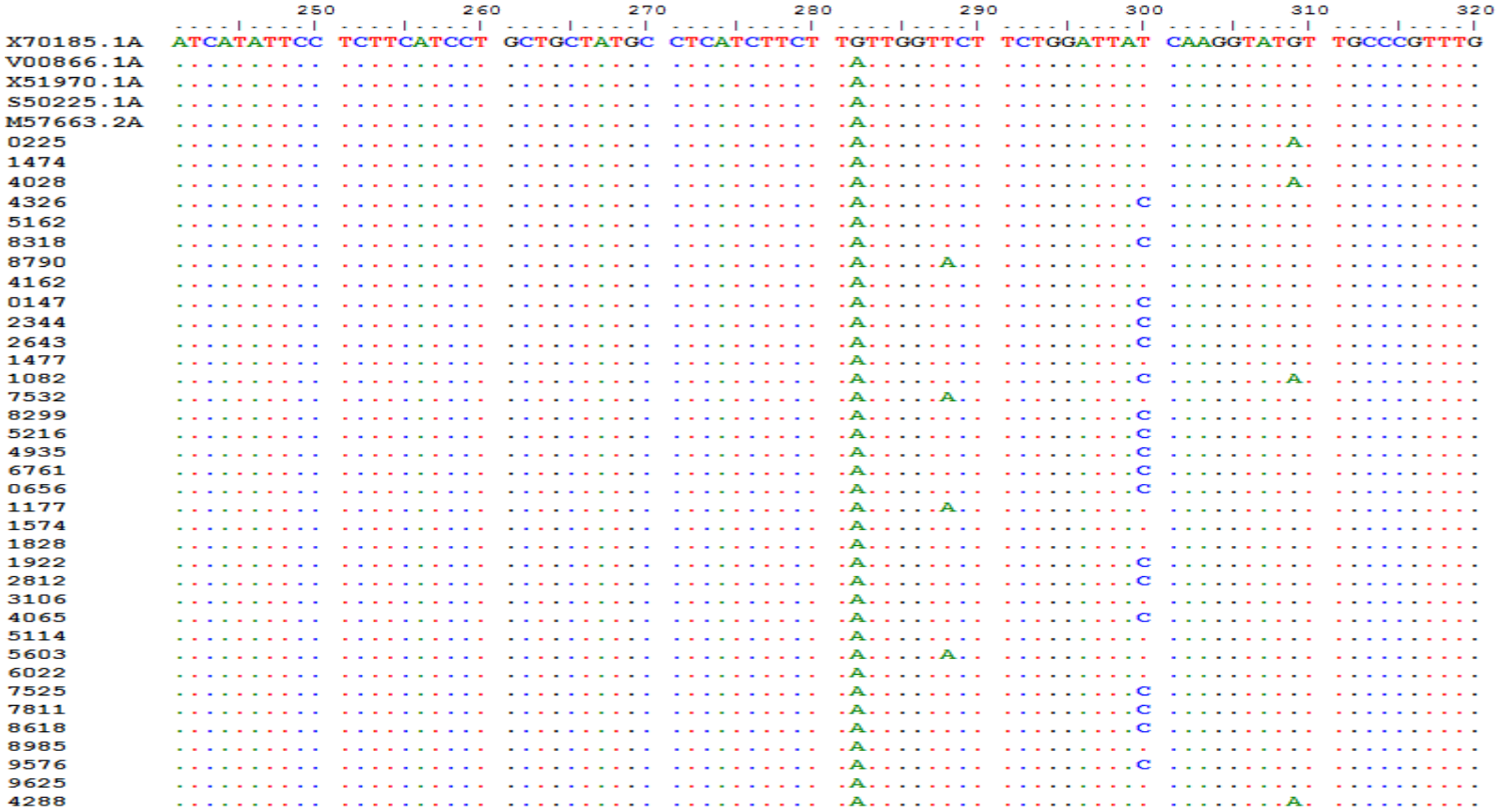


Figure 4.18: Nucleotide sequence alignment of genotype A isolates [Genotype A reference sequences: X20185.1, V00866.1, X51970.1, S50225.1 and M57663.2]

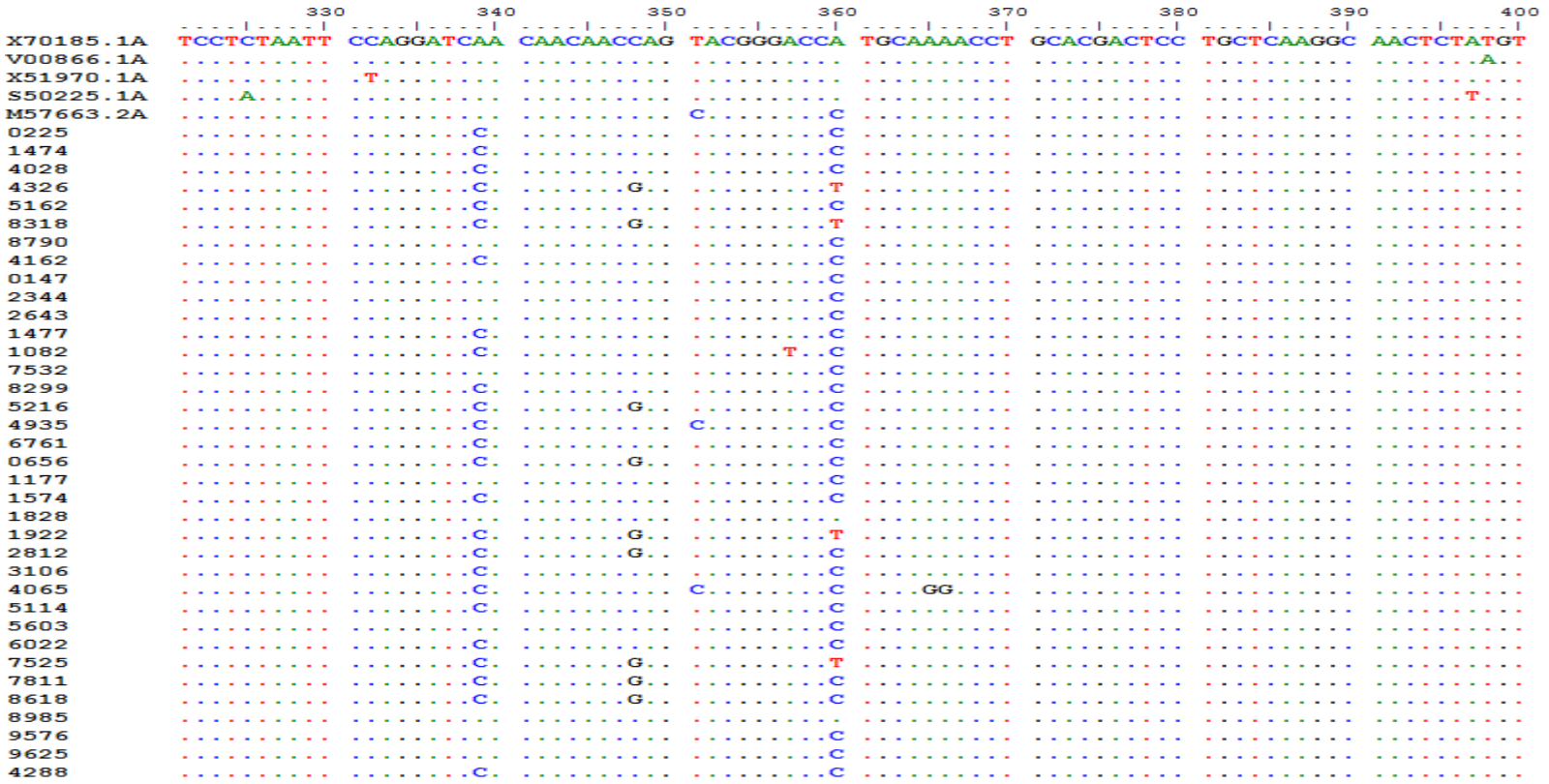


Figure 4.18: Nucleotide sequence alignment of genotype A isolates [Genotype A reference sequences: X20185.1, V00866.1, X51970.1, S50225.1 and M57663.2]

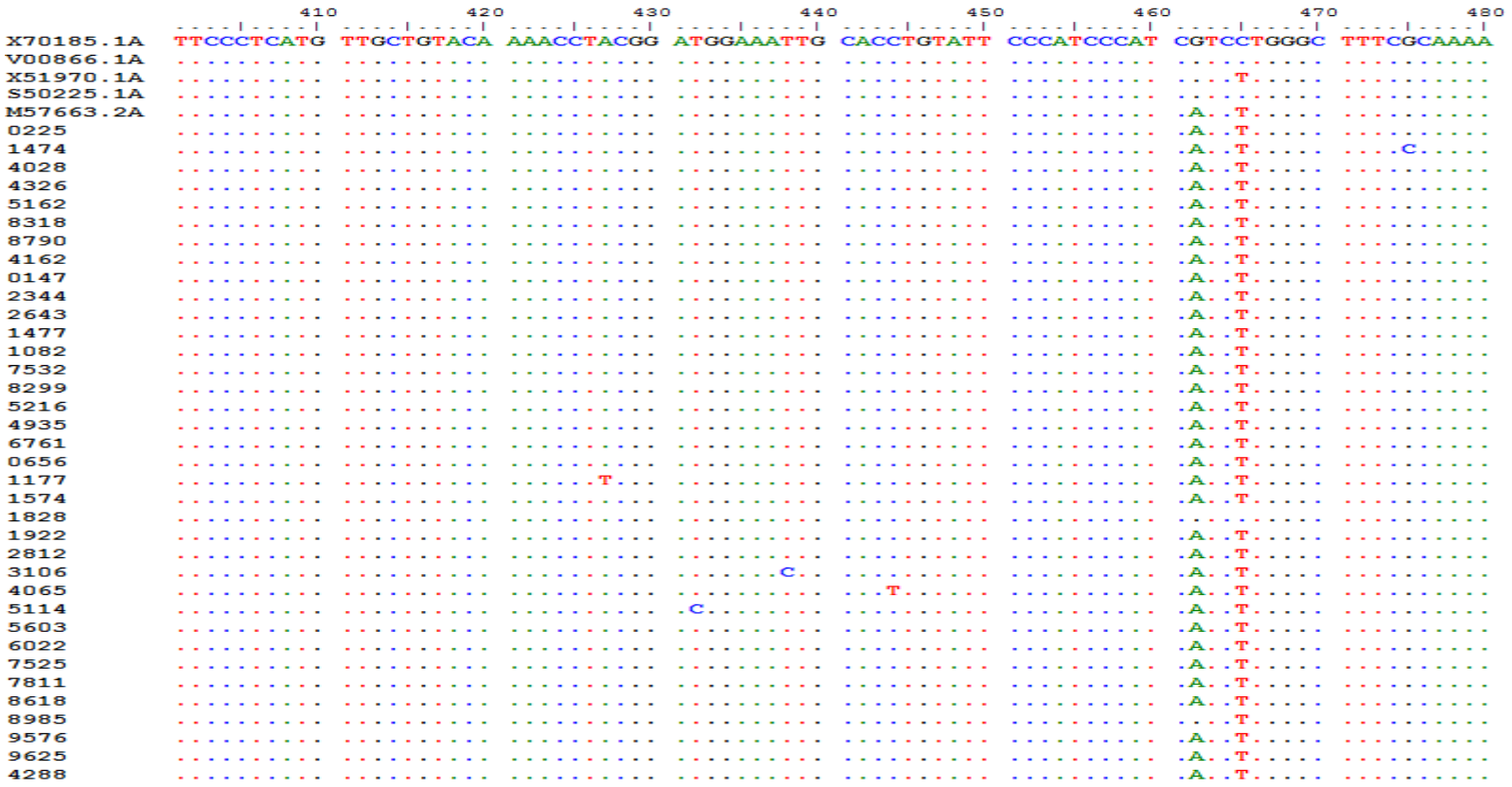


Figure 4.18: Nucleotide sequence alignment of genotype A isolates [Genotype A reference sequences: X20185.1, V00866.1, X51970.1, S50225.1 and M57663.2]

Figure 4.18: Nucleotide sequence alignment of genotype A isolates [Genotype A reference sequences: X20185.1, V00866.1, X51970.1, S50225.1 and M57663.2]

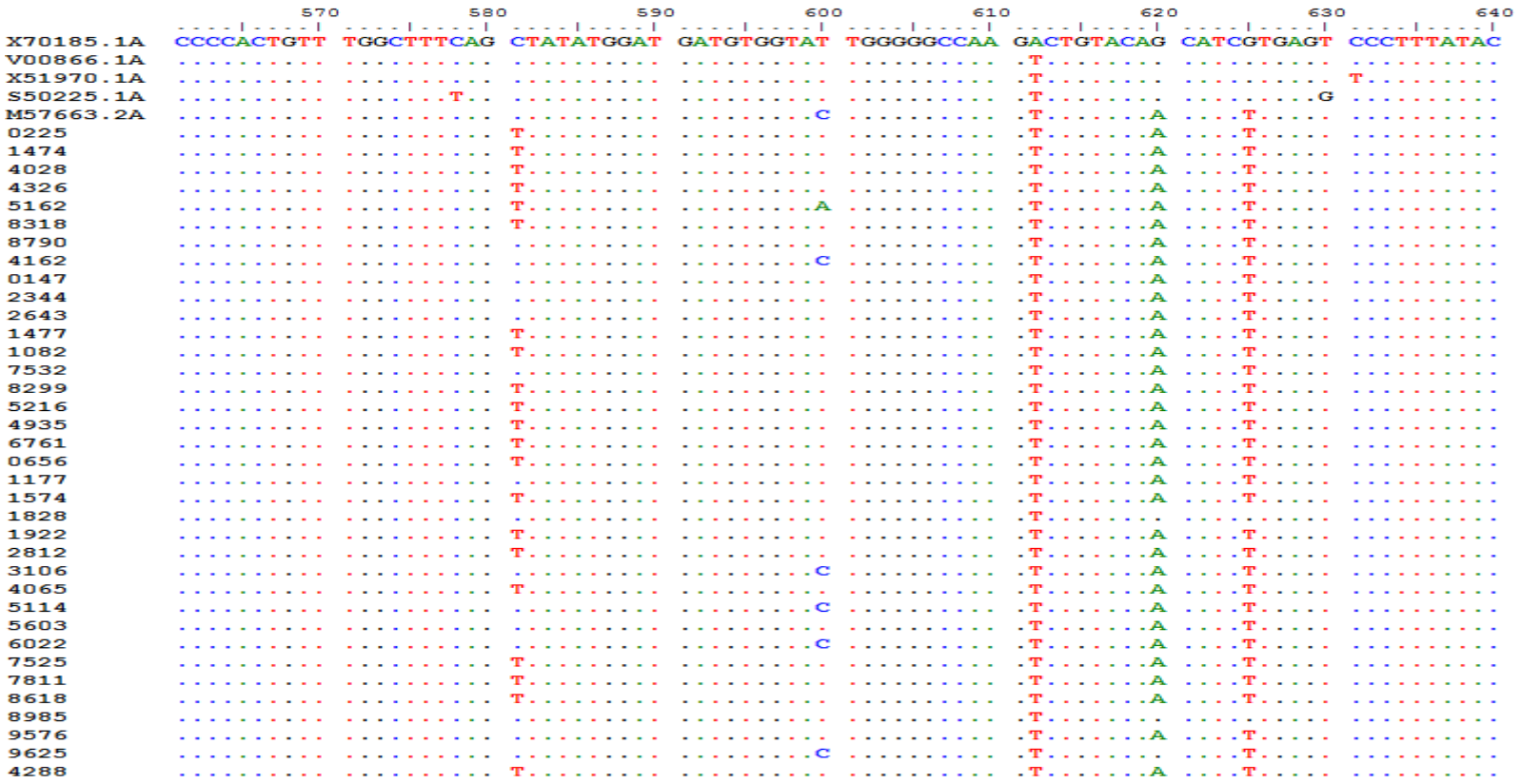


Figure 4.18: Nucleotide sequence alignment of genotype A isolates [Genotype A reference sequences: X20185.1, V00866.1, X51970.1, S50225.1 and M57663.2]

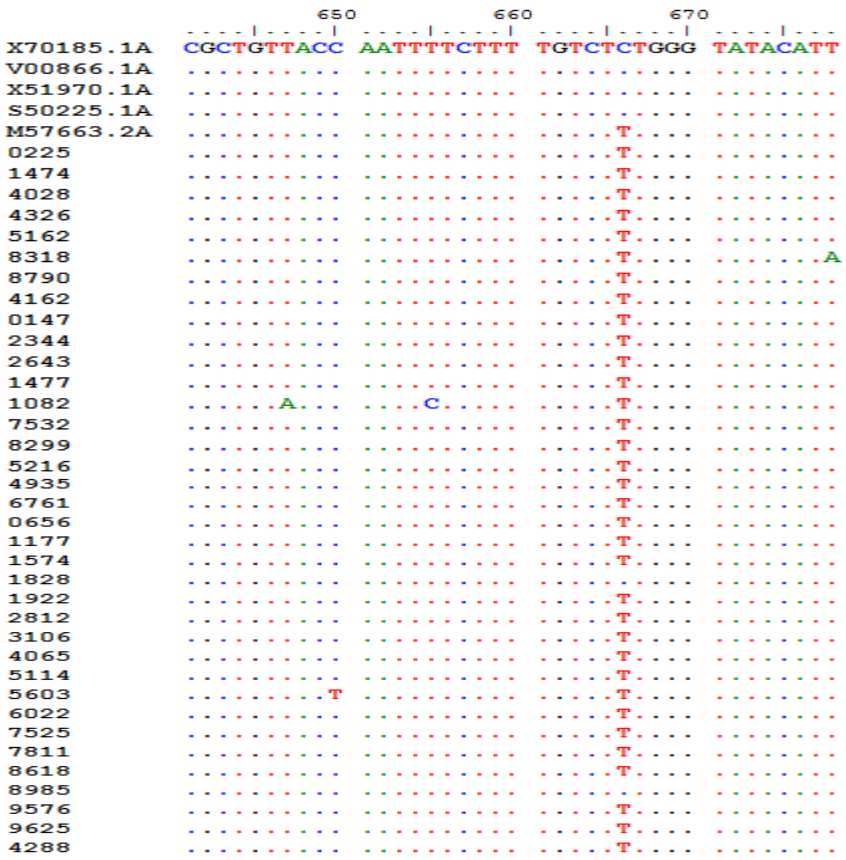


Figure 4.18: Nucleotide sequence alignment of genotype A isolates [Genotype A reference sequences: X20185.1, V00866.1, X51970.1, S50225.1 and M57663.2]

Figure 4.19: Nucleotide sequence alignment of genotype D isolates [Genotype D reference sequences: X59795.1, M32138.1 and X02496.1]

4.3.4 Amino acid sequence variations

The aligned nucleotide sequences were translated into amino acid sequences to allow for determination of any variations within the S protein (Figures 4.20 and 4.21). Amino acid variations detected within the genotype A sequences are summarized in Table 4.6.

Table 4.6: Amino acid variations within genotype A sequences

Amino Acid Variations	Amino Acid Positions	Significance
Amino acid substitutions	F8L, S45P, L49R, P70H, M103I, K122R, T143S, A159P, E164D, V168A, A194V, Y200*, R204S, S207N, V209L, L216*, P217L, F219L.	**Missense mutations**: F8L, S45P, L49R, P70H, M103I, K122R, T143S, A159P, E164D, V168A, A194V, R204S, S207N, V209L, P217L, F219L. **Nonsense mutation**: Y200*, L216*

Overall, a total of 18 amino acid substitutions were detected within the genotype A sequences, this including some notable variations such as the F8L (sample 9625), M103I (samples 0225, 4028, 1082 and 4288), K122R (sample 4065), T143S (sample 1177) and V168A (sample 5603) mutations. A stop codon (TAA) was inserted by a T600A and a T647A nucleotide substitution for the viral isolates from samples 5162 and 1082 respectively. This resulted in a premature truncation of the surface protein of both viral isolates. This truncation appeared not to affect the integrity of the viral surface protein (possibly due to the location of the stop codons near the end of the protein), as sample 5162 and 1082 were positive for HBsAg when tested during serology.

Within the genotype D sequence, two amino acid variations (substitutions) were observed namely N131T and A224V (Figure 4.21).

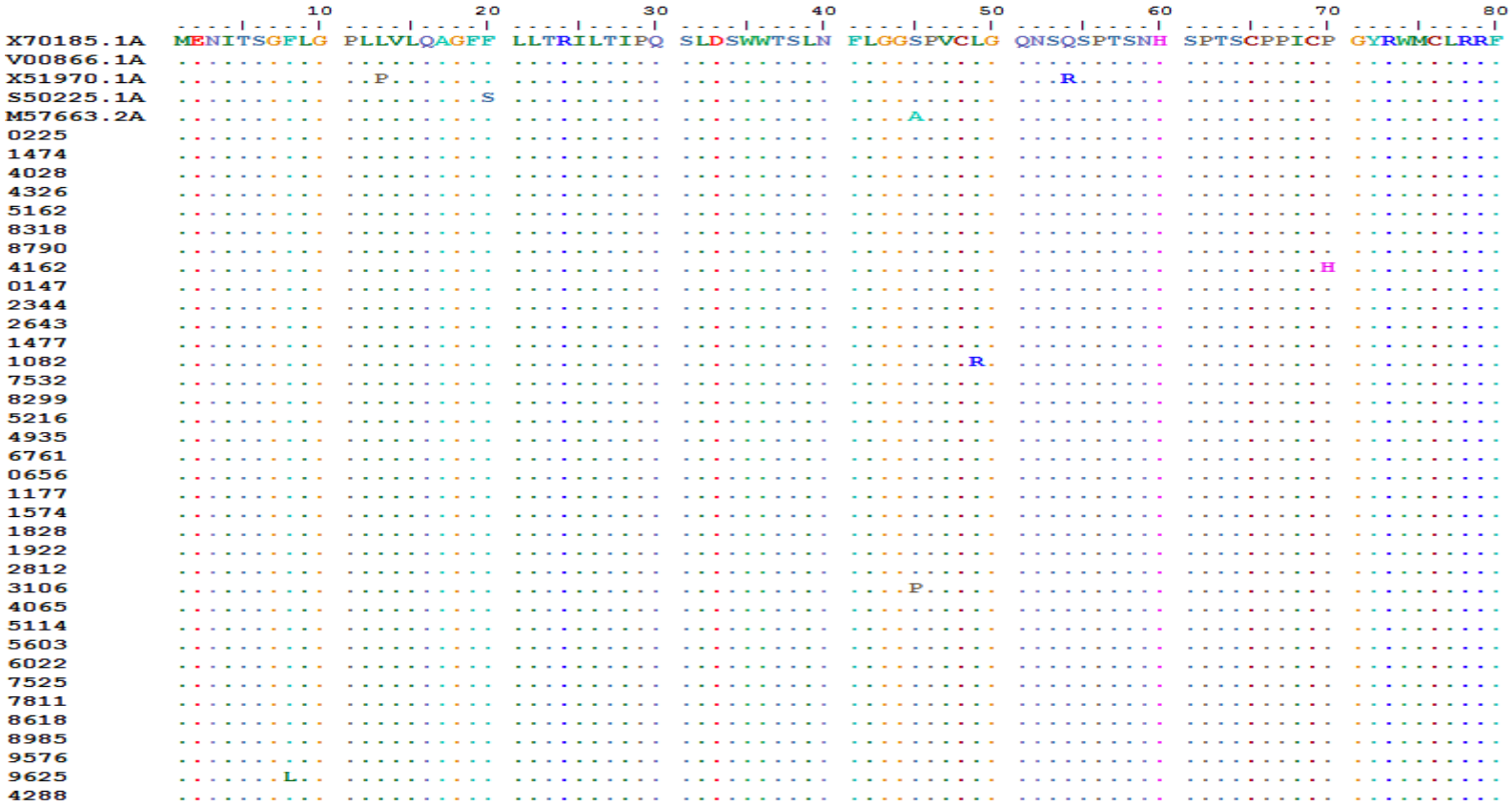


Figure 4.20: Amino acid sequence alignment of genotype A isolates [Genotype A reference sequences: X20185.1, V00866.1, X51970.1, S50225.1 and M57663.2]

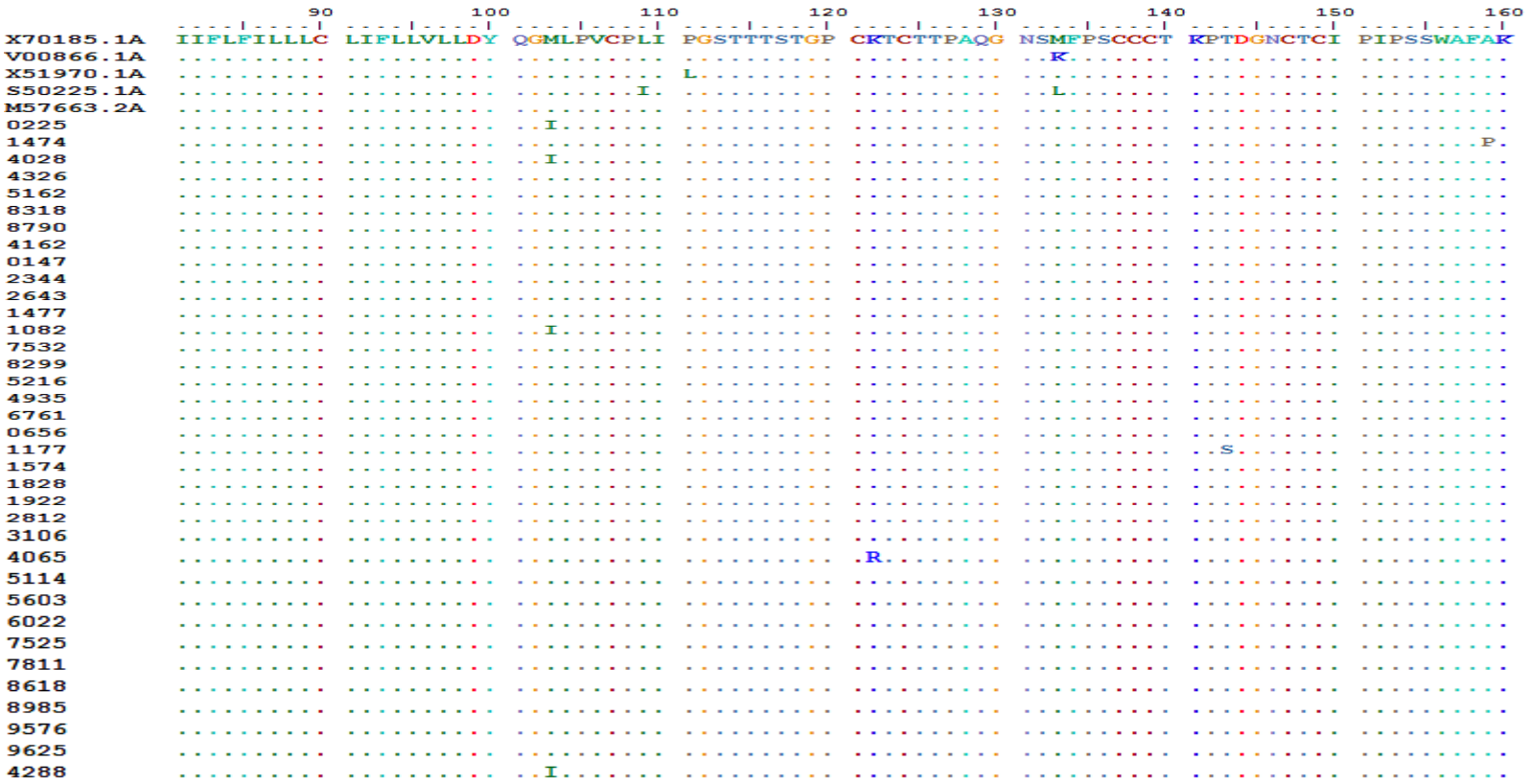


Figure 4.20: Amino acid sequence alignment of genotype A isolates [Genotype A reference sequences: X20185.1, V00866.1, X51970.1, S50225.1 and M57663.2]

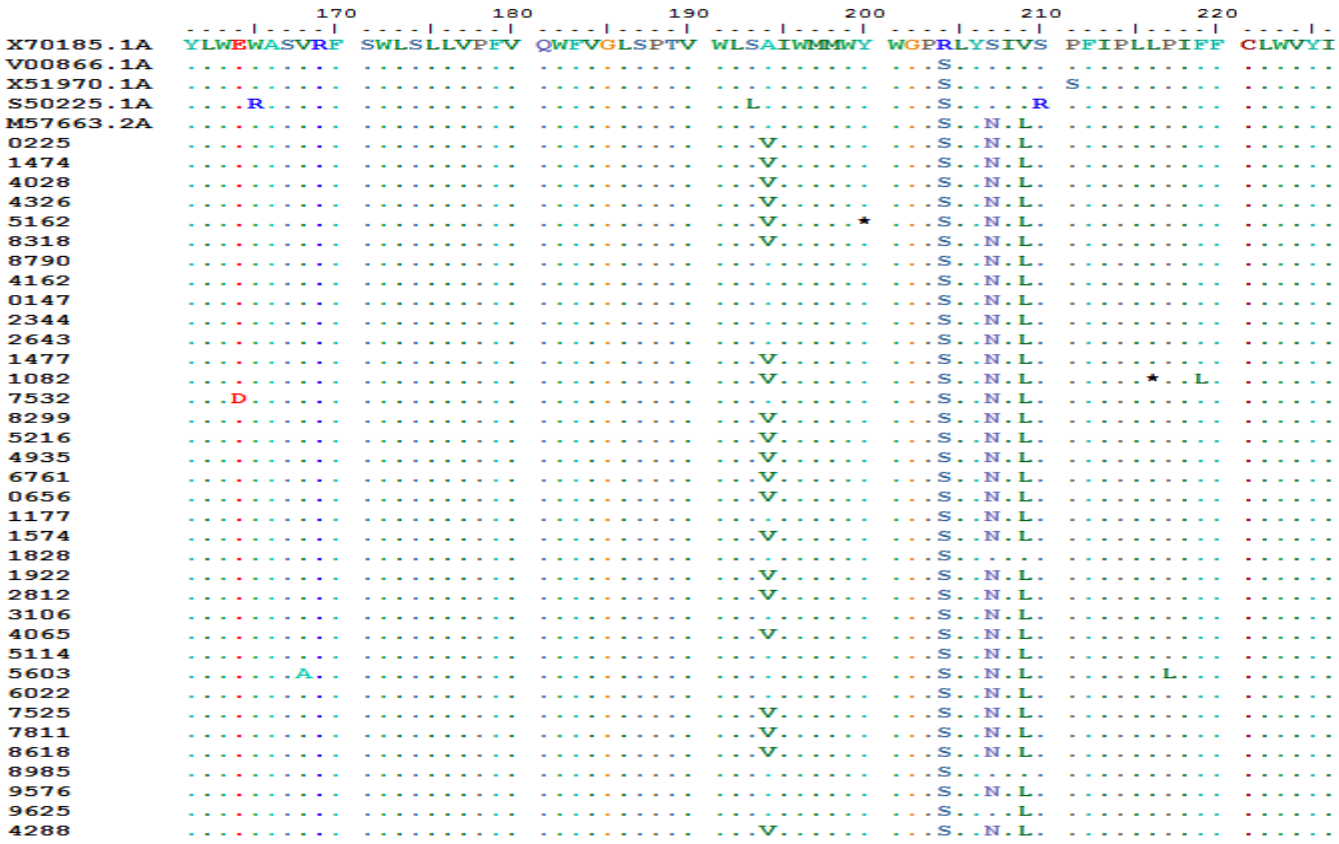


Figure 4.20: Amino acid sequence alignment of genotype A isolates [Genotype A reference sequences: X20185.1, V00866.1, X51970.1, S50225.1 and M57663.2]

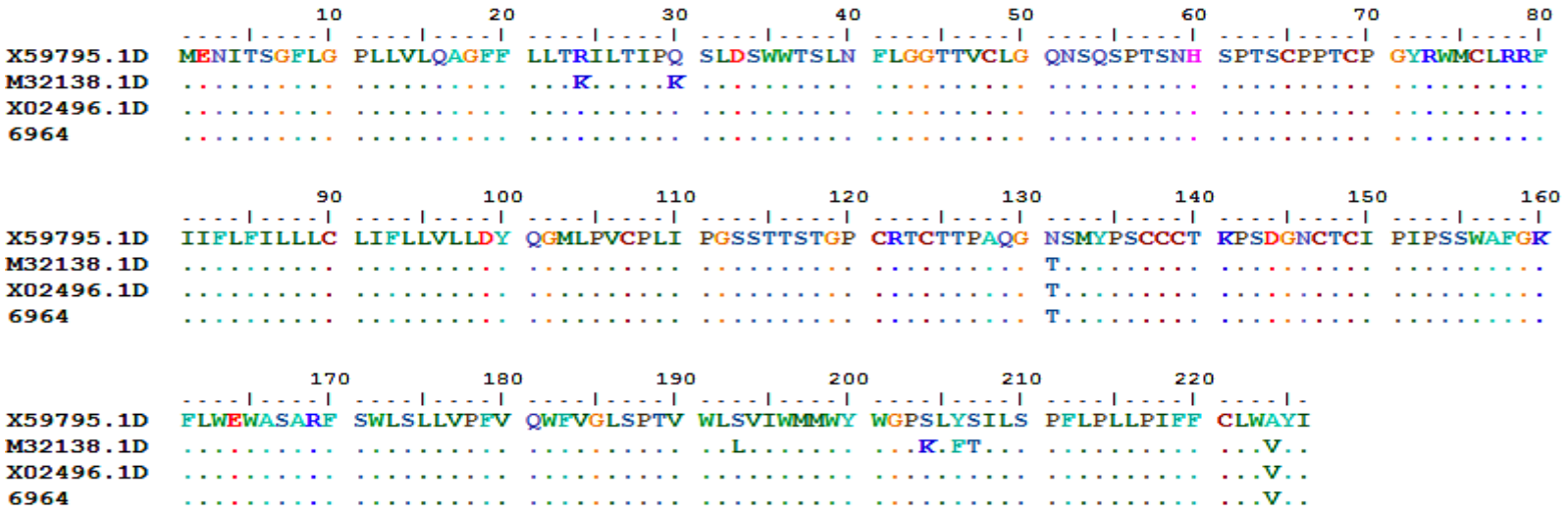


Figure 4.21: Amino acid sequence alignment of genotype D isolates [Genotype D reference sequences: X59795.1, M32138.1 and X02496.1]

4.3.4.1 Analyses of amino acid variations within the HBsAg

Variability within the antigenic region of the HBsAg could result in a conformational change to the antigenic epitope and this may interfere with binding of anti-HBs. The MHR (major hydrophilic region; aa110-aa165) of the HBsAg represents the major antigenic region and contains the viral "α" determinant or epitope (aa124-aa147). Figure 4.22 shows the sequence analyses of the MHR of all study sequences.

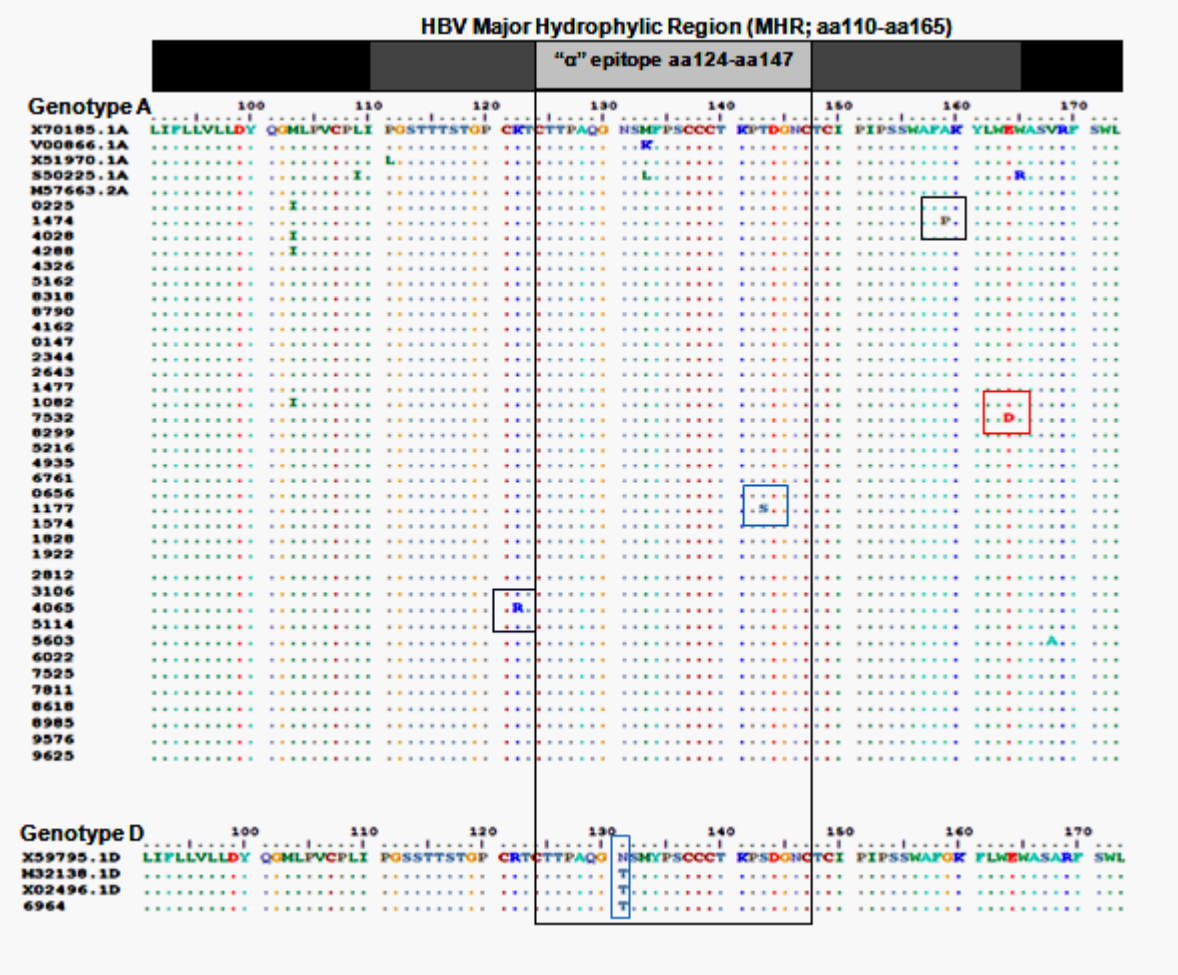


Figure 4.22: Analyses of amino acid variations within the HBV MHR (aa110 to aa165). [Genotype A reference sequences: X70185.1, V00866.1, X51970.1, S50225.1, M57663.2; Genotype D reference sequences: X59795.1, M32138.1, X02496.1).

Within the MHR (aa110-aa165), a K122R substitution; a polymorphism typically linked with determining *d*/*y* sub-serotype specificity, was detected for sample 4065 (pre-vaccination population). Two other mutations were detected within the "α" epitope; an N131T (sample 6964; pre-vaccination population) substitution also involved in *d*/*y* sub-serotype specificity

and a T143S (sample 1177; pre-vaccination population) substitution associated with genotype variation. Further on in the MHR, an A159P substitution was observed for sample 1474 (post-vaccination population). Finally an E164D mutation which is linked with reduced binding of anti-HBs was observed for sample 7532 (pre-vaccination population). Selection of the E164D surface mutation is associated with Lamivudine therapy, selected for by a V173L drug-related mutation within the overlapping *pol* protein. To confirm selection of this mutation, the *pol* gene was amplified for sample 7532 and the nucleotide (Figure 23) and amino acid (Figure 24) sequences analyzed. A total of 29 nucleotide substitutions were detected within the *pol* gene of sample 7532 alone, the majority of them being silent mutations (Table 4.7).

Table 4.7: Nucleotide variations within the *pol* gene of sample 7532

Nucleotide Variations	Nucleotide Positions	Significance
Nucleotide substitutions	A385C, G487A, C490T, G517C, A637T, G645A, G650T, C691T, A720T, A723G, C747T, T752G, C756T, C757G, A774T, G780A, T789C, G804A, C811T, A812G, T834C, C837T, G840A, T846C, T891C, T903A, A912T, T915C, T951A.	**Missense mutations:** A385C, G487A, G517C, G650T, T752G, C757G, C811T + A812G.
		Silent mutations: C490T, A637T, G645A, C691T, A720T, A723G, C747T, C756T, A774T, G780A, T789C, G804A, T834C, C837T, G840A, T846C, T891C, T903A, A912T, T915C, T951A.

Figure 4.23: Nucleotide sequence alignment for the *pol* gene [Genotype A reference sequences: X20185.1, V00866.1, X51970.1, S50225.1 and M57663.2]

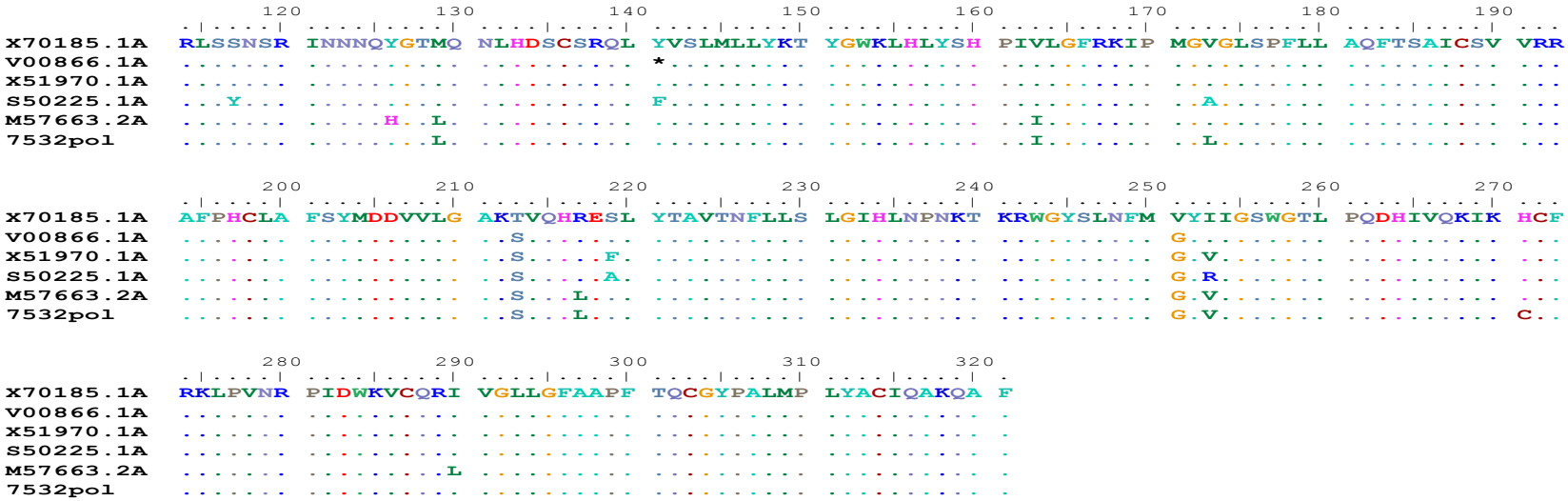


Figure 4.24: Amino acid sequence alignment for the *pol* gene [Genotype A reference sequences: X20185.1, V00866.1, X51970.1, S50225.1 and M57663.2]

The presence of the V173L mutation within the *pol* protein of the viral isolate from sample 7532 was confirmed. Seven other amino acid variations were also observed within the *pol* protein of the same isolate: M129L, V163I, T213S, R217L, V251G, I253V and H271C. Some of these variations are drug-related, such as T213S and I253V, while M129L, V163I and R217L are compensatory mutations involved in restoration of viral fitness.

Of the 5 mutations (4 in the pre-vaccination population and 1 in the post-vaccination population) observed within the MHR of study sequences, none were confirmed to have a direct association with hepatitis B vaccine escape.

CHAPTER 5

5. DISCUSSION

Implementation of universal hepatitis B immunization programmes in various parts of the world has seen a clear decline in the global hepatitis B burden (Zanetti *et al*., 2008; Hwang and Cheung, 2011). Due to its reported success, maintaining hepatitis B immunization programmes is of utmost importance for hepatitis B prevention and control. The use of serosurveys in monitoring the impact made by hepatitis B immunization programmes has been proposed as one of the most effective tools in ensuring that the success of the programme is maintained and improved when necessary (Advanced Immunization Management, 2009; WHO, 2011). Like several other countries endemic for hepatitis B, South Africa included the hepatitis B vaccine into its national EPI (EPI-SA), and this has been in effect for almost 17 years (Burnett *et al*., 2012). Unlike other countries with established hepatitis B immunization programmes, like China and Korea, South Africa has not conducted nationwide serosurveys to monitor the impact of the hepatitis B vaccine within the framework of EPI-SA (Liang *et al*., 2009; Park *et al*., 2010). In place of such nationwide serosurveys, a number of field and laboratory studies have been conducted in the country and have been the main advice on the impact made by the hepatitis B vaccine, particularly in vaccinated children within the first 5 years of life (Tsebe *et al*., 2001; Schoub *et al*., 2002; Simani *et al*., 2008).

With the national hepatitis B immunization programme nearing its second decade since its introduction, this study looked to contribute to the data available on the impact made by the hepatitis B immunization programme in South Africa. For this reason, this study identified two target populations based on the year of introduction of the hepatitis B vaccine into EPI-SA; individuals born after 1995 representing a post-vaccination population and those born before 1995 representing a pre-vaccination population. Viral (HBsAg) and host (anti-HBc, anti-HBs and IgM anti-HBc) serological markers were evaluated in all study subjects and HBV DNA was screened and quantified in those with serological evidence of HBV exposure or infection. On account of the “co-burden” of HIV infection in South Africa, subset analyses were also performed in study subjects with records of HIV status (either HIV infected or uninfected) in order to assess the influence of HIV infection on the impact of the national hepatitis B immunization programme. Molecular characterization of HBV surface and

overlapping polymerase genes was performed in HBV DNA carriers to determine the presence of HBsAg mutations within the study population.

Briefly, results from this study showed that immunity to HBV infection was significantly (p<0.001) higher (56.7%) in the post-vaccination population as compared to the pre-vaccination population (15.5%), while chronic carriage was significantly (p=0.008) reduced in the post-vaccination population with a 1.5% HBsAg prevalence as compared to the 4.0% detected in the pre-vaccination population. While no acute HBV infection was detected within the post-vaccination population, a 14.6% prevalence rate of acute HBV infection was detected in the pre-vaccination population. Furthermore, subset analyses revealed that immunity was significantly (p<0.001) higher in the HIV uninfected population as compared to the HIV infected population which also had a higher rate of HBV chronic carriage. Finally, molecular characterization revealed a number of notable amino acid variations within the antigenic region of the HBsAg of viral isolates. All these major outcomes of the study are further discussed in terms of the short- and long-term impact of hepatitis B vaccination as identified within this study population.

5.1 Short-term impact of hepatitis B vaccination

5.1.1 Prevalence of new HBV infections

The short-term indicator of the success of a hepatitis B immunization programme is a reduction in the prevalence of new or acute HBV infections among vaccinated cohorts, especially those under the age of 5 years, which is the peak age range for contracting HBV infection in South Africa (Vardas *et al*., 1999; Kew, 2008;). A limitation to some of the previous South African and African studies is the lack of IgM anti-HBc testing within this age group (<5 year olds) (Tsebe *et al*., 2001; Schoub *et al*., 2002). In this current study, IgM anti-HBc was tested in subjects with serological evidence of infection or exposure to HBV, and of those tested none were positive for IgM anti-HBc, indicating a reduction in acute HBV cases in those <5 years (Table 4.1). Similar investigations of the incidence of acute HBV infection have been conducted in other regions in the world since the start of universal hepatitis B immunization, where a decline in new HBV cases have also been recorded (USA recorded a decline from <2 to 0 cases in 100 000 persons, CDC, 2004; Hawaii from 4.5 to 0 cases in 100 000 persons, Perz *et al*., 2006; and Italy from 5.1 to 1.3 cases in 100 000 persons, Mele *et al*., 2008) (Zanetti *et al*., 2008).

5.1.2 Reduction in HBV chronic carriage

The success of a hepatitis B immunization programme should also be apparent in a reduction in HBV chronic carriage rates (Zanetti *et al*., 2008). In investigating the prevalence of HBsAg among those under the age of 5 years, this study found a chronic carriage rate of 0.5% (1/209 subjects; Figure 4.7); a remarkable result, taking into account the fact that the vaccination status of study subjects was unknown. This chronic carriage rate is highly comparable to that reported by Schoub *et al* in a study published in 2002 which found HBV chronic carriage at 0.4% (3/756) among 18 month old, vaccinated infants originating from rural districts of all nine provinces in South Africa. Tsebe and colleagues (2001) on the other hand reported a total elimination (0/578) in chronic carriage among <5 year olds from the Limpopo province of South Africa, in a field study conducted to assess the impact of the national immunization programme after the first 5 years of its implementation. Thus hepatitis B chronic carriage rates found in this current study and previous South African studies are a stark contrast to the 10.4% reported in pre-immunization data for the country, revealing a 10% decline since the start of the immunization programme in April 1995 (Vardas *et al*., 1999). Reduction in chronic carriage rates has also been noted by other African countries such as Egypt (<6% to 1.5%; Sherbini *et al*., 2006) and The Gambia (10% to 0.6%; Viviani *et al*., 1999), as well as in regions outside the Sub-continent including Alaska, USA (>6% to 0%; McMahon *et* al., 2011), Taiwan (10% to 0.6%; Ni *et al*., 2007; Ni and Chen, 2010), and South Korea (8% to 3.7%; Park *et* al., 2010).

5.1.3 Increased immunity to HBV infection

The efficacy of the hepatitis B vaccine, as defined by increased immunity to HBV infection, was determined by quantifying anti-HBs in sera of study subjects, in the absence of all other serological markers. The lowest detectable titre of anti-HBs by the Elecsys® 2010 Immunoassay System (Roche, Hitachi, Japan; Roche Diagnostics, Penzberg, Germany) used in this study was 2 mIU/mL. It has already been established that anti-HBs titres below the stipulated seroprotection level of 10 mIU/mL are still protective due to primed immune memory cells which effect an anamnestic response upon subsequent challenge with viral antigen (Chavez *et al*., 2012; Zhu *et al*., 2011). Thus for the purpose of this study, all subjects with detectable anti-HBs titres (≥2 mIU/mL) were recognized as being protected against HBV infection.

The overall rate of immunity to HBV infection in study subjects under the age of 5 years was found to be 76.1% with 4.8% having anti-HBs titres between 2 and 10 mIU/mL (Figure 4.4). Immunity to HBV infection within vaccinated cohorts under the age of 5 years has been previously reported at 86.8% and 87.0% from two of the most recent South African studies (Tsebe *et al*., 2001; Schoub *et al*., 2002). Considering that the vaccination status of study subjects from this current study was unknown, the 76.1% immunity rate observed remains comparable to that obtained from the previous studies; a notable finding. In comparing this finding with others emanating from the African region, some parallels can be drawn with countries like Nigeria (Odusanya *et al*., 2011) and The Gambia (Viviani *et al*., 1999) which reported hepatitis B vaccine protection rates of 84.7% and 83% respectively. In a 2007 health facility-based study conducted in Tanzania after the first 5 years of hepatitis B vaccine introduction into the country's EPI, a total of 69.3% of vaccinated children (<5 years of age) were found to be immune to HBV infection (Metodi *et al*., 2010). Egypt on the other hand, reported a 54% immunity rate after 2 decades of universal hepatitis B immunization in the country (Sherbini *et al*., 2006). Such varied rates in immunity to HBV infection have also been documented outside the Sub-continent (Sanmarti *et al*., 2000; de la Hoz *et al*., 2008; Zanetti *et* al., 2008; Park *et* al., 2010; McMahon *et* al., 2011; Coppola *et al*., 2012).

5.1.4 HIV subset analyses

From the HIV subset analyses performed, only 1 out of the 20 (5.0%) HIV infected children under the age of 5 years was found to be positive for HBsAg, while no HBsAg was detected in the HIV uninfected population (0/89; Table 4.2). In contrast, a previous South African study reported HBsAg prevalence rates of 2.7% (2/73) and 0.4% (1/230) in HIV infected and uninfected children respectively (Simani *et al*., 2008). In terms of new HBV infections within HIV infected and uninfected populations, this same study by Simani *et al* found no IgM anti-HBc positive subjects from either population, as was the case in the current study. Thus the decline in the prevalence of new HBV infections as well as chronic carriage noted in this study also implies a reduction in new HBV/HIV co-infections and future chronic hepatitis B-related complications within HIV infected individuals, at least for this study population.

In terms of immunity to HBV infection, It was observed that immunity was significantly (p<0.001) higher in the HIV uninfected population (90.9%; 80/89) as compared to the HIV infected population (30.0%; 6/20) (Figure 4.11). This is in line with results reported

elsewhere, which indicated that HIV infected vaccinees generally have reduced seroprotection rates as compared to their HIV uninfected counterparts (Simani *et al*., 2008; Mphahlele and Mda, 2012). The difference in immunity rates observed for the HIV infected and uninfected populations in this study was however found to be wider than what has been previously reported, such as in the Simani *et al* (2008) study which found immunity rates of 85.7% and 78.1% in South African vaccinated cohorts (<2 years of age) of HIV uninfected and infected populations respectively (p=0.125). It should also be noted that susceptibility to HBV infection was very high (40%; 8/20, Table 4.2) within the HIV infected population of this study. Reports have shown however, that up to 70% of HIV infected, hepatitis B vaccinees do not respond optimally as a result of uncontrolled HIV replication or high HIV viral load and lower $CD4^+$ T cell count, which then increases the risk of susceptibility to HBV infection (van den Berg *et al*., 2009). Since records of HIV viral load and $CD4^+$ T cell count of study subjects were not considered for the purpose of this study, the cause of the high rate of susceptibility to HBV infection in the HIV infected population of this study could not be established. With such low rates of immunity compounded by the high rate of susceptibility to HBV infection in HIV infected children, as demonstrated by this study, a need arises for enforcing screening of anti-HBs titres and providing vaccine booster doses when required, in hepatitis B vaccinated infants and children with underlying HIV infection, since this population is regarded to be at a very high risk for HBV infection with a faster progression to chronic disease and death (Thio, 2003; van den Berg *et al*., 2009; Kim *et al*., 2009; Rani *et al*., 2009).

5.2 Long-term impact of hepatitis B vaccination

An evaluation of the long-term impact of hepatitis B vaccination within the framework of EPI-SA would be a measure of population immunity and chronic carriage of HBV infection in a national representative population, regardless of the vaccination status of study participants (Advanced Immunization Management, 2009; WHO, 2011). Thus the long-term indicator of the success of the national hepatitis B immunization programme would be an increase in immunity to HBV infection and a reduction in chronic carriage and related fatal sequelae as compared to when the hepatitis B vaccine had not been introduced into EPI-SA (Rani *et al*., 2009; WHO, 2011). Using a prospective approach, this study looked to measure the long-term impact of hepatitis B vaccination in a South African population, by comparing population immunity and chronic carriage of HBV infection between a post-vaccination population (aged 1-15 years) and a pre-vaccination population (aged 16-25 years). The

vaccination status of all study subjects was not considered during this study. Although the study population employed was not a true representation of the general South African population, it did however reflect a subset of the country's population and included subjects from five of the nine provinces in South Africa. This study also contributes novel data on the prevalence of HBV exposure (including immunity and chronic carriage) within a South African population over the age of 5 years.

5.2.1 Increased population immunity to HBV infection

In comparing immunity rates between the post- and pre-vaccination populations of the study, it was noted that immunity was significantly (p<0.001) increased in the post-vaccination population (56.7%) as compared to the pre-vaccination population (15.5%), with no significant difference between male and female subjects (Figure 4.5). This increase in immunity to HBV infection after national hepatitis B immunization is in line with what has been reported in studies conducted in other countries. Taiwan for example, which represents perhaps one of the best success stories of universal hepatitis B immunization, documented a 50.5% immunity rate almost 20 years into the hepatitis B immunization programme in that country (Ni and Chen, 2010). Similarly, in a national serosurvey conducted in China after 14 years of universal hepatitis B immunization, Liang and colleagues (2009) reported a 50.1% overall immunity rate, with a decline noted as participants' ages increased; from 71.2% in those aged 1-4 years to 55.5% and 57.5% in the 5-9 and 10-14 years age groups respectively. Among the different age strata in the post-vaccination population of this study, immunity rates also declined with increasing age from as high as 76.1% in those 1-5 years of age, reducing to 50.0% in those 6-10 years and 44.2% in those 11-15 years of age (Figure 4.4). Accordingly, geometric mean titres (GMT) of protective anti-HBs also waned with increasing age; from 110.2 mIU/mL in those 1-5 years of age, to 38.6 mIU/mL and 41.0 mIU/mL in 6-10 and 11-15 years age groups respectively. Waning of protective levels of anti-HBs over the course of time is a well documented phenomenon and does not necessarily imply a loss of immunity to HBV infection (Lu *et al*., 2004; Chavez *et al*., 2012; Poovorawan *et al*., 2010; Zhu *et al*., 2011). It could thus be inferred from the results obtained in this study that hepatitis B vaccine booster doses are not required for immunocompetent South African vaccinees, at least not within the first 15 years after primary vaccination. Delaying hepatitis B vaccine booster dose within this age group has also been confirmed elsewhere, such as in a study by Lu *et al*. (2004) which demonstrated that hepatitis B vaccine booster doses were not required for at least 15 years after neonatal hepatitis B vaccination in Taiwan subjects

(Ni and Chen, 2010). In addition to this, recent reports have shown that a full 3 dose regimen of the hepatitis B vaccine could provide long-term immunity for up to 20 years (Poovorawan *et al*., 2010).

5.2.2 Reduction in population HBV infection and chronic carriage

The incidence of acute HBV infection within the study population was determined by screening for IgM anti-HBc in those study subjects who had serological evidence of exposure or infection with HBV. Due to insufficient volumes of sera however, the presence of IgM anti-HBc could not be evaluated in all study subjects who fell into this category. From results obtained for those that could be tested, it was noted that none (0/35; 0.0%) of the study subjects within the post-vaccination population were positive for IgM anti-HBc as compared to 14.6% (14/96) of the pre-vaccination population who tested positive for IgM anti-HBc (Table 4.1). Thus the decline in the number of new HBV infections following introduction of universal hepatitis B immunization in the country is emphasized by this result.

In comparing the prevalence of chronic carriage between the post- and pre-vaccination populations of the study, a significant (p=0.008) reduction was observed within the post-vaccination population which had a total HBsAg prevalence of 1.5%, compared to the 4.0% found in the pre-vaccination population (Figure 4.8). Similar reductions in chronic carriage rates have been recorded in other regions of the world since the start of universal hepatitis B immunization (Davaalkham *et al*., 2007; Gidding *et al*., 2007; Park *et al*., 2010). China for example, revealed a reduction in chronic carriage in the country from 9.8% in 1995 to 7.2% in 2006 (Liang *et al*., 2009). In a national serosurvey conducted in Cambodia, Soeung *et al* (2009) reported a HBsAg prevalence of 3.5%; a vast decline from the 14% documented in some regions before universal hepatitis B immunization was introduced in that country in 2002. Within the African region, the majority of national serosurveys that have so far been conducted have focused on providing pre-immunization or baseline data, such as that published from South Africa (Vardas *et al*., 1999) and Uganda (Bwogi *et al*., 2009), while other field and laboratory studies have mainly documented the short-term impact of universal hepatitis B immunization in countries like The Gambia (Viviani *et al*., 1999), Egypt (Sherbini *et al*., 2006), as well as South Africa (Tsebe *et al*., 2001; Schoub *et al*., 2002).

It is worth mentioning that while there was no statistically significant difference (p=0.49) in chronic carriage rates between genders in the post-vaccination population, chronic carriage was significantly (*p*=0.03) higher in females (2.2%; 13/600) as compared to males (1.7%; 10/600) within the pre-vaccination population. All 13 HBsAg positive females within this population were also positive for HBV DNA (viral loads ranging between 3.7E+2 and 11.1E+36 IU/mL) while 6 out of the 13 were IgM anti-HBc positive, confirming the presence of both active and acute HBV infections. The concern for this group is the fact that they are at childbearing age (16-25 years) and as such carry a high risk (up to 90% due to high viremia) of vertically transmitting the HBV infection to their newborns, especially if these newborns were not to be administered timely (within at least 24 hours of birth) post-exposure prophylaxis (hepatitis B vaccine birth dose, with or without hepatitis B immuneglobulin) (Tran, 2009; Petrova and Kamburov, 2010; Shi *et al*., 2011). Previously, HBV chronic carriage rate among South African mothers had been reported at 3.2% while the HBV infectivity rate (HBeAg positivity) among pregnant women was shown to be <1% (Kew, 1996; Tsebe *et al*., 2001). Now although vertical transmission of HBV infection is not a major transmission route in South Africa, results from this study reaffirm the need for strengthening the screening of pregnant women for HBV infection in order to provide timely interventions when required. This also revives the debate over the need for introducing a hepatitis B vaccine birth dose into EPI-SA in order to prevent neonatal HBV infections.

Looking at HBV chronic carriage within the post-vaccination population, it was noted that chronic carriage rates increased with increasing age (0.5% for 1-5 years; 1.3% for 6-10 years and 2.5% for 11-15 years, Figure 4.7). In agreement with this finding, the national serosurvey conducted in China also reported an increase in HBV chronic carriage rates with increasing age (1.0% for 1-4 years, 1.4% for 5-9 years and 3.2% for 10-14 years) (Liang *et al*., 2009). It could be speculated that the reason for the low chronic carriage rate found in those under the age of 5 years may be due to the improved hepatitis B vaccination coverage in South Africa, which is currently estimated at 97% as compared to 88% in 2000 and 74% in 1997; the years in which study subjects between the ages of 11-15 years were born (Burnett *et al*., 2012; WHO; 2012b). To further explain the increase in chronic carriage rates among the older age groups; results from the subset analyses already proved that underlying HIV infection did not have a profound influence on HBV chronic carriage within the post-vaccination population, and thus focusing on the association made between waning immunity and increasing chronic carriage with increasing age (Figure 4.7), there is room for speculation about the need for hepatitis B vaccine booster dose later on in life within the

South African setting. This is because although hepatitis B vaccine booster doses are not recommended in immunocompetent individuals, the potential waning of immunity over time coupled with the increased risk of behavioural exposures in the older age strata (such as in those between 11-15 years who are within the sexually active age range) may warrant the need for reconsideration of the hepatitis B vaccine booster dose policy.

5.2.3 Reduction in population chronic hepatitis B sequelae

Monitoring the long-term impact of hepatitis B vaccination would be incomplete without an evaluation of the prevalence of population chronic hepatitis B sequelae such as liver cirrhosis, HCC and other extra-hepatic manifestations, that is if the national hepatitis B immunization programme has been in effect for long enough to measure these sequelae (Rani *et al*., 2009; WHO, 2011). For the purpose of this study however, the prevalence of chronic hepatitis B sequelae present in the study population was not evaluated. Results from other South African studies that did evaluate the impact of hepatitis B immunization on childhood cases of HCC and extra-hepatic manifestations like HBV-associated membranous nephropathy, have been documented previously (Bhimma *et al*., 2003; Moore *et al*., 2004; Moore *et al*., 2008). In light of the results obtained from this study, such as the increase in immunity rate even in the absence of vaccination records and the observed decline in new and chronic HBV infections within the post-vaccination population, this study does give some inclination as to the number of fatal chronic sequelae that could be averted in the future. The true long-term impact of hepatitis B vaccination on chronic hepatitis B sequelae in the country can only be established once a nationwide serosurvey is conducted.

5.3 Variations within the viral HBsAg

In the final objective, this study looked to investigate the presence of HBsAg variations by performing molecular characterization of HBV surface and overlapping polymerase genes in HBV DNA carriers within the study population. The HBsAg mutations are particularly relevant for the properties they confer to the resulting mutant strain, which may include immune, diagnostic and vaccine escape (Carman, 1997; Echevarría and Avellón, 2006; Yokosuka and Arai, 2006). The region of the HBsAg where most of these mutations occur is the Major hydrophilic region (MHR; aa110-aa165), which also bears the α antigenic determinant (aa124-aa147) (Carman, 1997). Escape mutants are reported to predominate over wild type strains within an infected individual due to selection pressure from the host

immune response or immunomodulators like hepatitis B immunoglobulin and the hepatitis B vaccine (Zuckerman and Zuckerman, 2003). For this reason, introduction of universal hepatitis B immunization has seen a frequency in the detection of escape mutants and HBsAg variants in general, in a number of countries like The Gambia (Fortuin *et al*., 1994; Whittle *et al*., 1995) and Taiwan (Hsu *et al*., 1999; Chang, 2010). The possibility of an uncontrolled increase in emergence of HBsAg mutants is however countered by the reduction in HBV chronic carriage in vaccinated populations as a result of the introduction of universal hepatitis B immunization (Chang, 2010).

The HBV S gene sequences from 9 post-vaccination and 28 pre-vaccination HBV DNA carriers were generated and analyzed in this study. Phylogenetic analyses revealed that the majority of HBV isolates circulating within the two populations were genotype A, with only 1 (from the pre-vaccination population) being genotype D (Figure 4.17). This is consistent with HBV epidemiology studies which reported the most predominant HBV genotype in South Africa to be the genotype A, followed by genotypes D and B (Kramvis *et al*., 2005; Kramvis and Kew, 2007). While 7 different types of amino acid variations were detected in the HBsAg of viral isolates from the post-vaccination population, a total of 18 different variation were observed for the pre-vaccination population (Figures 20 and 21). Thus HBsAg variations were more prevalent in the pre-vaccination population as compared to the post-vaccination population, consistent with what has been reported in a previous South African study (Hino *et al*., 2001).

In assessing amino acid variations within the HBV MHR (aa110-aa165) of the HBsAg a number of amino acid substitutions were detected within this region; K122R, N131T, T143S, A159P and E164D (Figure 4.22). Both K122R and N131T amino acid substitutions are considered to be polymorphisms which determine the *d*/*y* sub-serotype while the T143S has been described to reflect genotype variations (Soussan *et al*., 2001; Martin *et al*., 2010). The HBV isolate bearing the A159P substitution was found to be identical to HBV sequences published from neighbouring Zimbabwe by Gulube *et al* (2011). The final amino acid substitution, E164D is a lamivudine-selected mutation which was detected in a study subject from the pre-vaccination population. It is reported that this E164D mutation is linked with reduced binding to anti-HBs. However whether or not this has some influence on vaccine escape has not been confirmed (Torresi, 2002; Torresi *et al*., 2002). Altogether, results from this study are in agreement with previous reports which showed that the increase in the

prevalence of HBsAg variants as well as the detection of escape mutations including vaccine escape mutations, after the introduction of universal hepatitis B immunization, are occurrences yet to be observed in South Africa (Hino *et al*., 2001).

CHAPTER 6

6.1 CONCLUSIONS, LIMITATIONS AND RECOMENDATIONS

6.1 Conclusions

The work described in this study provides current data on population immunity and chronic carriage to HBV infection within the post-vaccination population of South Africa, almost 17 years after nationwide immunization against HBV infection.

The following conclusions could be drawn from the study:

- Introduction of the hepatitis B vaccine into EPI-SA has shown remarkable success in children under the age of 5 years; significantly increasing immunity (76.1%) to HBV infection while reducing the prevalence of new HBV infections (0.0%).
- Population immunity and chronic carriage to HBV has been greatly impacted since the introduction of universal hepatitis B immunization. In comparing the post-vaccination population with the pre-vaccination population, it was clear that there was a significant difference between the two groups. For example, immunity was higher (56.7% versus 15.5%) and chronic carriage reduced (1.5% versus 4.0%) in the post-vaccination population as compared to the pre-vaccination population.
- Within the HIV infected population, susceptibility to HBV infection remains a cause for concern, reaching as high as 57.6% (and 40.0% in <5 year olds) in the post-vaccination population as compared to 49.0% in the pre-vaccination population.
- Although amino acid variations within the viral HBsAg are present, vaccine escape-related mutants appear to be rare or even absent within the South African population.

6.2 Limitations of the study

The purposive sampling method used in this study led to sampling bias which would have been minimized had a larger sample size been used in the study. Due to financial and time constraints on this study however, a larger sample size could not be employed.

It should also be noted that the study population employed for this study was not quite representative of the general South African population as it comprised health facility-based

study subjects presenting for various ailments, and thus a high risk group. However with results from this study having strong compatibility with those from previous studies conducted in and outside South Africa, the major outcomes of this study should be afforded careful considered.

In terms of laboratory testing, a limitation to IgM anti-HBc screening was encountered as sera from some study subjects were insufficient. Screening for HBV DNA by real time PCR (qPCR) could not be performed in all HBsAg negative study samples (i.e. those positive for anti-HBs only and those negative for all primary serological markers) due to financial and time constraints on the project. Thus the prevalence of occult HBV infection within the entire study population was not established.

6.3 Recommendations

1. The main question emanating from this study is:

   "Has the time come for:

   - Inclusion of a hepatitis B vaccine birth dose into EPI-SA in view of the high prevalence of new (6.3%) and active (with viral loads raging from 3.7E+2 to 11.1E+36 IU/mL) HBV infections within females of childbearing age in the pre-vaccination population, and;
   - Reconsidering the booster dose policy for individuals with or without underlying HIV infection?"

2. This study recommends that future studies include an evaluation of the impact made by universal hepatitis B immunization on chronic hepatitis B sequelae (liver cirrhosis, HCC as well as extra-hepatic manifestations) in a South African population over the age of 5 years.

3. This study also advocates for a nationwide, hepatitis B serosurvey to be conducted in the country so as to better ascertain the long-term impact of hepatitis B vaccination.

CHAPTER 7

7. REFERENCES

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