Exploring the Impact of Human Immunodeficiency Virus on Hepatitis B Virus Diagnosis, Prevention and Control in Co-infected Adult South African Patients on Highly Active Antiretroviral Therapy

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EXPLORING THE IMPACT OF HUMAN IMMUNODEFICIENCY VIRUS ON HEPATITIS B VIRUS DIAGNOSIS, PREVENTION AND CONTROL IN CO-INFECTED ADULT SOUTH AFRICAN PATIENTS ON HIGHLY ACTIVE ANTIRETROVIRAL THERAPY

by

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SUPERVISOR: Professor MJ Mphahlele

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DECLARATION

I declare that the thesis hereby submitted to the University of Limpopo Medunsa Campus, for the degree of Doctor of Philosophy (Medical Virology) has not been submitted for any degree at this or any other university; that it is my own work in design and execution, and that all material contained herein has been dully acknowledged.

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Initials & Surname (Title) **Date**

[redacted]

DEDICATIONS

This study is dedicated to my late grandmother, Vho-Muswolwa Munyai, my family for their support throughout the years of studying, and finally to my fiancée, Shirley Livhuwani Muvhulawa, for overwhelming support.

"In the field of observation, chance favours only the prepared mind." – Louis Pasteur

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SUMMARY

Background and Objectives: South Africa is one of the countries highly affected by human immunodeficiency virus (HIV) and hepatitis B virus (HBV) infections. Some drugs (e.g. lamivudine) used as part of combination antiretroviral regimens for HIV treatment have dual activity against HBV and HIV. Despite high infection rate with both viruses, routine screening for HBV before initiation of treatment for HIV is not yet a standard practice. This study undertook to investigate: (1) the burden of HBV co-infection in HIV-positive patients enrolling for highly active antiretroviral therapy (HAART) at Dr George Mukhari hospital, (2) the impact of anti-HBV containing HAART regimens on HBV during the management of HBV/HIV co-infected patients, (3) the co-evolution of HBV and HIV drug-resistant strains, and (4) the correlation of HBV genotypes with response to anti-HBV containing HAART regimens.

Study Population and Methods: To investigate the burden of HBV/HIV co-infections, a cohort of 192 HIV patients who were candidates for ARV treatment at Dr George Mukhari hospital were studied by screening for HBV serological markers (HBsAg, anti-HBs and anti- HBc) (Elecsys 2010, Roche Diagnostics) and HBV DNA with an in-house nested PCR assay targeting HBV polymerase gene. Quantitation of HBV DNA positive samples was performed with Roche Cobas Taqman HBV test 48 assay. To investigate the impact of lamivudine-containing HAART regimens on HBV during the management of HBV/HIV co-infected patients, as well as the co-evolution of HBV and HIV drug-resistant strains, a total of 78 patients were studied. HBV virological response against lamivudine containing-HAART regimens [1a (lamivudine, stavudine and efaverenz); 1b (lamivudine, stavudine and neviripine)] was measured (Cobas Taqman HBV test 48, Roche diagnostics). HBV direct sequencing targeting HBV polymerase gene was performed on all baseline samples (n=78) and additional samples collected at various time points (n = 45). Direct sequencing was also performed on 30 HIV baseline samples targeting the HIV reverse transcriptase and protease genes (Spectru-Medix SCE 2410 Genetic Analysis System and ABI PRISM® 3100 Genetic Analyzer version 3.7). To explore the genetic diversity of HBV and HIV strains circulating in Pretoria and surrounding areas, as well as the correlation of HBV genotypes with response to lamivudine-containing-HAART regimens in co-infected patients, all baseline and follow-up HBV and HIV sequences were analysed, compared and correlated with treatment. Sequence alignments and phylogenetic studies for both HBV and

HIV were conducted with MAFFT, Mega 4 and neighbour joining phylogenetic trees generated with the PHYLIP programme.

Results: Three significant findings were observed in this study. *Firstly,* the majority of South African HIV patients enrolling for HAART were exposed to HBV infection and either had acute or chronic HBV infections. A total of 63.0% of patients were found to have one or more HBV markers, with 40.6% having detectable HBV DNA as an indication of replication. The study also detected 22.9% with positive HBsAg, and 23% of 77% HBsAg-negative patients having occult hepatitis B infection.

Secondly, HBV/HIV co-infected patients do benefit during the management of HIV infections with lamivudine-containing HAART regimens. A total of 68.4% of patients responded to HAART, with undetectable HBV DNA during 18 to 24 months of follow-up. A total of 91.3% of HIV patients also responded to HAART with an undetectable HIV viral load during 6 to 12 months of follow-up. However, a total of 18% of patients had persistent HBV DNA, yielding various HBV virological responses against lamivudine containing-HAART regimens. This proportion of patients poses a question regarding the management of HBV and HIV co-infections, as guidelines on the use of HAART with anti-HBV activity from developed countries, may not necessarily be followed in developing countries. The results further showed that baseline drug-resistance was more frequent with HIV than HBV in this cohort of patients. The following HIV primary drug resistant mutants were observed: nine major NRT's primary mutants, M41L (1/30), E44A (1/30), V75M (1/30), F77L (1/30), V118I (1/30), M184V (1/30), L210S (1/30), T215Y (1/30) and V90I (1/30), and five major NNRT's primary mutants were also detected, K103N (3/30), Y318CFSY (1/30), E138Q (1/30), P225H (1/30) and K238T. However, all follow-up samples had undetectable HIV viral load. In contrast to HIV, only one patient was detected with HBV mutant, M204I, at baseline. The mutant reversed to wild type during 6 months and other follow-up (12, 18 and 24 months).

Finally, this study indicated that the HBV genotype A is still the most prevalent genotype circulating in South Africa. Of the 78 HBV sequences, 77 were genotype A and 1 sequence was genotype G. This is the first report from Africa of the detection of HBV genotype G. HIV subtype C remains the predominant prevailing subtype in South Africa. HBV genotype or HIV subtype C was not observed to influence any treatment outcome following treatment with

lamivudine-containing HAART regimens. The study also indicated that patients on lamivudine-containing HAART regimens do benefit not only by suppressing HIV and HBV viral load, but also improving immunity (i.e. CD4 cells count increases).

Conclusion: Overall, the present study highlights the need for screening HBV before initiation of any HAART containing anti-HBV regimens in HBV/HIV co-infected patients. It necessitates the use of molecular assays for effective laboratory in diagnosis of occult HBV infections in HIV-positive patients, especially in developing countries where these assays are not widely available. While lamivudine-containing HAART regimens do benefit both HBV and HIV patients in co-infected individuals, however, whether HBV virological response is temporary or sustained is unknown at this stage. What is certain is that these patients require an effective monitoring programme as (1) a small percentage experience variable HBV virological responses (partial, reactivation, or no response), and (2) hepatitic flares are likely to develop if HAART is terminated (e.g. by patient), or the current HAART regimen is switched to another regimen without anti-HBV activity. HBV genotype A remains the dominant genotype in South Africa, but novel genotypes can be detected. HIV subtype C was found to be the prevalent subtype. HBV genotype or HIV subtype C were not seen to influence any treatment outcome following treatment with lamivudine-containing HAART regimens.

Recommendations: HIV patients should be screened for HBV before initiation of anti-HBV containing HAART regimens. The screening of HBV in HIV patients is also important since some drugs included as part of HAART (e.g. nevirapine) may cause hepatotoxicity and exacerbate HBV infections leading to increased morbidity and mortality due to liver complications. Immunization and immune boosters of HIV patients with low (< 10IU/L) or no immunity against HBV should be done as this could be beneficial, although these patients may not respond optimally, or their immunity may wane faster due to immunocompromised status. Monitoring of both HBV and HIV resistant strains should be conducted for timely detection for the occurrence of multiple resistant mutations, which could limit future therapeutic option for both viruses.

PUBLICATIONS AND PRESENTATIONS EMINATING FROM THESIS

PUBLICATIONS

A Lukhwareni, TE Ramulifho, KM Mphahlele, MJ Mphahlele. The burden of HBV in HIV positive patients enrolling for antiretroviral therapy in South Africa. Submitted to Journal of Medical Virology

A Lukhwareni, MP Gededzha, ME Nesengani, SG Selabe, MJ Mphahlele. Sensitivity of HBV DNA to lamivudine-containing antiretroviral regimens in HBV and HIV co-infected South African patients. Submitted to Journal of Clinical Virology

A Lukhwareni, MJ Mphahlele. Genetic diversity of HBV strains isolated from HIV co-infected South African patients. In preparation

A Lukhwareni, MJ Mphahlele. Genetic resistance profile and diversity of HIV strains from South African patients on antiretroviral therapy. In preparation

A Lukhwareni, MJ Mphahlele. Detection of a novel HBV genotype G in South African HIV patient on HAART. In preparation

CONFERENCE PROCEEDINGS

INTERNATIONAL

A Lukhwareni, KM Mphahlele, TE Ramulifho, MO Mzileni and MJ Mphahlele. Baseline study of hepatitis B virus (HBV) and HIV co-infections in patients receiving anti-retroviral drugs at Dr George Mukhari hospital. 27th African Health Sciences Congress, Durban, South Africa, 4th-8th December, 2006 (Oral presentation)

A Lukhwareni, MK Mphahlele, TE Ramulifho, MJ Mphahlele. 2006. Hepatitis B virus, hepatitis C virus and human immunodeficiency viruses co-infections in patients receiving antiretroviral drugs at Dr George Mukhari hospital, South Africa. Eighth International Congress on Drug Therapy in HIV infection, Glasgow, UK, 12th -16th November 2006 (Poster presentation)

NATIONAL

A Lukhwareni, KM Mphahlele, TE Ramulifho, ME Nesengani, MO Mzileni and MJ Mphahlele. Hepatitis B virus (HBV) and HIV co-infections in patients receiving anti-retroviral drugs at Dr George Mukhari hospital, Pretoria. 3rd South African AIDS Conference, Durban, South Africa, 5- 8 June 2007 (Poster presentation)

A Lukhwareni, KM Mphahlele, TE Ramulifho, ME Nesengani, MJ Mphahlele, SG Selabe and MO Mzileni. Hepatitis B virus and HIV co-infections at Dr George Mukhari hospital, Pretoria, South Africa: Implications on Diagnosis and Therapy. South Africa Medical Research Council Research Day, Cape Town, South Africa 18th October 2007 (Oral presentation)

LOCAL

A Lukhwareni, KM Mphahlele, MO Mzileni, MJ Mphahlele. HIV and HBV co-infections at Dr George Mukhari Hospital: Implications on ARV's. 3rd Academic day, 15th-16th August 2007, University of Limpopo (Poster presentation)

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ME Nesengani, **A Lukhwareni**, SG Selabe, MJ Mphahlele. Evaluating sensitivity of HBV DNA to lamivudine-containing antiretroviral regimens in HIV patients co-infected with HBV. 3rd Academic day, 15th-16th August 2007, University of Limpopo (Poster presentation)

A Lukhwareni, KM Mphahlele, TE Ramulifho, MJ Mphahlele, MO Mzileni. Baseline study of HBV and HIV co-infections in patients receiving ARV's at Dr George Mukhari hospital. 2nd Academic day, 16th-17th August 2006, University of Limpopo (Oral presentation)

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

aa.....	amino acid
ALT.....	alanine aminotransferase
AST.....	aspartate aminotransferase
ARV……	antiretroviral
AZT……	zidovudine
BCP……	basic core promoter
Bp……..	base pairs
cccDNA…….	covalently closed-circular deoxyribonucleic acid
CD4……	Cluster of differentiation
D4T……	Stavudine
DGMH……	Dr George Mukhari Hospital
DNA……..	deoxyribonucleic acid
ddNTPs…….	dideoxynucleotide triphosphate
ddATP……	dideoxyadenine triphosphate
ddCTP……..	dideoxycytosine triphosphate
ddGTP……..	dideoxyguanine triphosphate
ddTTP…….	dideoxythymine triphosphate
dNTPs……	deoxynucleotide triphosphate
dATP……..	deoxyadenine triphosphate
dCTP…….	deoxycytosine triphosphate
dGTP…….	deoxyguanine triphosphate
dTTP……..	deoxythymine triphosphate
HAART……..	highly active antiretroviral therapy
HBcAg……..	hepatitis B core antigen
HBeAg.....	hepatitis B e antigen
HBsAg.....	hepatitis B surface antigen
HBV.....	hepatitis B virus
HBP.....	hepatitis B polymerase
HBxAg.....	hepatitis B x antigen
HCC……..	hepatocellular carcinoma

HIV…….	human immunodeficiency virus
HIV –RT……..	HIV reverse transcriptase
NRT……	nucleoside reverse transcriptase
NNRT…….	non-nucleoside reverse transcriptase
NVP……	Nevirapine
PCR……...	polymerase chain reaction
S-gene……...	surface gene
IU/mL…….	International Units per millilitre
IU/L…….	International Units per Litre
WHO……...	World Health Organisation

NUCLEOTIDES

A……..	Adenine
C……..	Cytosine
T……..	Thymine
G……..	Guanosine
U……..	Uracil

AMINO ACID ABBREVIATIONS

A……..	Alanine
R……..	Arginine
N……..	Asparagine
D……..	Aspartic acid
C……..	Cysteine
E……..	Glutamic Acid
Q……..	Glutamine
G……..	Glycine
H……..	Histidine
I……	Isoleucine
L……...	Leucine
K……..	Lysine

M……..	Methionine
F……..	Phenylalanine
P……..	Proline
S……..	Serine
T……..	Threonine
W……	Tryptophan
Y……..	Tyrosine
V……..	Valine

CHAPTER ONE

1 EXPERIMENTAL PROPOSAL AND THESIS ORGANISATION

1.1 Experimental proposal

1.1.1 Study problem

The South African government has been introducing a large-scale supply of antiretroviral (ARV) drugs for the treatment of human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) in public hospitals since April 2004 (South Africa National Department of Health, 2003). Despite this development, research programmes to monitor the efficacy of these ARV drugs in HIV/AIDS patients co-infected with hepatitis B virus (HBV), do not exist. Both of these viruses are endemic and frequently detected as co-infections. Nevertheless, screening of HBV serological markers in HIV patients initiating highly active antiretroviral therapy (HAART) in sub-Saharan Africa is currently not yet a standard practice, due to financial constraints. HAART is defined as the use of several ARV drugs, typically comprising of three ARV drugs, usually two nucleoside analogues and either a protease inhibitor or a nonnucleoside reverse-transcriptase inhibitor, and are taken in combination. The ARV drugs target HIV at multiple stages of its lifecycle, minimising the development of resistant strains, and slowing the progression of the disease by lowering the viral load (Yeni *et al.,* 2002). Currently, it is not well established what proportion of HIV-positive patients harbours HBV infection in South Africa. Of the significant concern is that a number of ARV drugs for HIV have dual activity against HIV and HBV, and have not been extensively studied in co-infected individuals worldwide. This is despite reports of the detection of naturally and drug -induced occurring mutants which are associated with drug -resistant strains in naïve/treatment -experienced patients, both mono/co-infected HBV and HIV patients (Kobayashi *et al.,* 2001; Kirishima *et al.,* 2002; Heo *et al.,* 2004; Ohishi *et al.,* 2004; Selabe *et al.,* 2007). Therefore, baseline studies are needed for monitoring patients before and during HAART, especially with anti-HBV-containing HAART regimens.

Previous study from the HIV and Hepatitis Research Unit at Medunsa Campus has reported the prevalence of HBV co-infections to be approximately 40.1% in HIV-positive and 31.7% in HIV-negative women attending antenatal clinics (Burnett *et al.,* 2003). As a result, HBV and HIV co-infection studies are necessary as seen with the inclusion of HBV in the Guidelines for Therapy of HIV-associated opportunistic infections, in order to emphasise the importance of management of HIV co-infected patients. Although HAART has been shown to be successful in controlling HIV replication and significantly prolonging the survival of HIV-infected patients, the

impact of HAART in HBV/HIV co-infected patients, especially in developing countries is largely unknown. Furthermore, information on co-evolution of HBV and HIV drug-resistant associated strains is limited, or does not exist, and therefore data and information derived from this study will be useful for improving the prevention and treatment in the management of HBV and HIV co-infected patients in South Africa.

1.1.2 Literature review

Both HBV and HIV are endemic in sub-Saharan Africa, and South Africa has introduced HAART for HIV/AIDS patients in public hospitals. Studies have shown that the majority of these HIV/AIDS patients have been exposed to HBV or chronic carriers, since both viruses use the same mode of transmission. Consequently, a number of HIV-infected individuals have past exposure to, or are chronic carriers of, HBV (Saillour *et al.,* 1996; Burnett *et al.,* 2005). While HBV is now a vaccine-preventable disease in most parts of the world, the search for an HIV vaccine remains a challenge. However, the improved prognosis of HIV infection that occurred since the introduction of HAART has resulted in renewed emphasis being placed on co-morbidities associated with HIV-infection, and chronic viral hepatitis B and C are no exceptions. Antiviral therapies with activity against HBV and HIV have enabled targeted therapy in co-infected individuals; however, improved prognosis has been tempered by the development of antiviral resistance. HBV and HIV reverse transcriptase (RT) enzymes lack a 3' to 5'– exonuclease activity for proofreading, and therefore, have a high misincorporation rate. The error rate in HBV is similar to HIV, although the potential for variation in HBV is limited by the compact genome organisation (Locarnini, 1998). The high mutation rates have substantial implications for the development of drug-resistant mutations, once the selection pressure of antiviral therapy is introduced.

The published HBV RT antiviral-resistant mutations are located in similar or identical positions to HIV RT antiviral-resistant mutations (Bartholomeusz *et al.,* 2004). The well known studied classical mutant is the M204I/V for HBV RT or M184I/V for HIV RT. The M184I/V or M204I/V involves the replacement of methionine by isoleucine or valine at position 184 of the reverse transcriptase and is the main mutation that confers resistance to lamivudine (Boucher *et al.,* 1993). Other several key HIV RT point mutations associated with resistance to nucleoside analogues include: lysine (K) 65 arginine (R), K70R, leucine (L) 74 valine (V), glutamine (Q) 151 methionine (M), and threonine (T) 215 tyrosine/ phenylalanine (Y/F), and are all located in the region of the polymerase that interacts with the incoming nucleotide and within the conserved domains (Huang *et al.,* 1998). In HBV RT, resistance to lamivudine has been documented and

is primarily associated with changes at M204I/V in combination with L180M (Stuyver *et al.,* 2001). This has also been reported to occur in lamivudine-naïve patients, both co-infected with HIV and mono-infected (Selabe *et al.,* 2007).

Although the use of HBV and HIV antiviral therapy is not widely practiced in sub-Saharan Africa, the majority of HIV and HBV chronic carriers are drug-naïve. This is a concern, since the impact of HAART drugs on the natural history of HBV in the region is unknown. Thus, this study may provide contrasting information on the evolution of HBV and HIV drug-resistant strains. It is generally accepted that HIV co-infection exacerbates liver disease. Previous studies have reported HBV/HIV co-infected patients having a significant increased risk of dying from liver disease as a result of reactivation of "silent" chronic hepatitis B, or hepatotoxicity, and the risk was found to increase significantly after starting HAART, and the development of lamivudine-resistance (Liaw *et al.,* 1999; Hoffmann and Thio, 2007) and following withdrawal of lamivudine in individuals who have experienced immune reconstitution (Bessesen *et al.,* 1999). When HBV/HIV co-infected patients experience liver diseases due to HBV, HAART regimen will have to be switched to include drugs which are active against both HBV and HIV. This will necessitate monitoring of both HIV and HBV drug resistant strains (Hoff *et al.,* 2001; Manegold *et al.,* 2001). Finally, the introduction of HAART in South African public hospitals is very new, and research programmes to monitor the efficacy of the HAART, to characterise and detect the co-evolution of HIV and HBV drug-associated resistant strains, are currently not available.

1.1.3 Overall aim

The overall aim of this study was to explore the impact of HIV on HBV diagnosis, prevention and control in co-infected adult South African patients on HAART, by performing epidemiological studies on HBV, and molecular characterisation of HBV and HIV strains in co-infected patients. The cohort consisted of HIV-positive patients attending the ARV clinic at Dr George Mukhari Hospital (DGMH), Pretoria.

1.1.4 Broad objectives

- To investigate the burden of HBV co-infection in HIV-positive patients enrolling for HAART at DGMH in Pretoria
- To investigate the impact of anti-HBV-containing HAART regimens on HBV during the management of HBV/HIV co-infected patients, and the co-evolution of HBV and HIV drug-resistant strains

- To investigate the genetic diversity of HBV and HIV strains circulating in Pretoria and surrounding areas, and further explore the correlation of HBV genotypes with response to anti-HBV-containing HAART regimens

1.1.4.1 Investigating the burden of HBV co-infection in HIV-positive patients enrolling for HAART at DGMH in Pretoria

Rationale:

Co-infections of HBV and HIV are common given the shared routes of transmission (sexual, parenteral and mother to child). It has been reported that up to 80% of HIV-infected patients have serological markers of present or past HBV infection (Scharschmidt *et al.,* 1992; Sinicco *et al.,* 1997). Approximately 10% of HIV-infected patients are co-infected with HBV. However, most of the data is generated from developed countries. There is limited data on the burden of HBV and HIV co-infections in South Africa. Therefore, the study was to investigate the burden of HBV co-infections in HIV-positive patients before initiation of HAART by screening baseline bleeds. Serological and molecular assays were used to screen all patients for HBV serological markers. This part of the research was crucial to establish a study cohort for follow-up during the duration of the study.

1.1.4.2 Investigating the impact of anti-HBV-containing HAART regimens on HBV during the management of HBV/HIV co-infected patients, and the co-evolution of HBV and HIV drug-resistant strains

Rationale:

A number of ARV drugs have dual activity against HBV and HIV, and one of them, lamivudine, is included in comprehensive treatment guidelines for HIV in South Africa. Therefore, in co-infected patients, care is needed to monitor both HBV and HIV during treatment, as both viruses are sensitive to lamivudine. Furthermore, care is needed to treat both viruses optimally, without encouraging emergence of resistant strains. In patients co-infected with both HIV and HBV, long-term treatment of HIV with lamivudine-containing HAART regimens could result in mutations in the YMDD motifs of both HBV and HIV. Many studies have focused on development of HIV-resistant strains during HAART, including lamivudine-resistant HIV strains (Horban *et al.,* 2002; Derdelinckx *et al.,* 2004; Turner *et al.,* 2004), and, as such, limited data is available for lamivudine-resistant HBV strains in HBV and HIV co-infected patients. This study was to investigate the co-evolution of HBV- and HIV lamivudine associated -resistant strains in co-infected patients on HAART-containing lamivudine. Thus, it is important to understand HBV/HIV co-infection, as South Africa has a high prevalence of chronic hepatitis B and is experiencing expanding antiretroviral programmes.

1.1.4.3 Investigating the genetic diversity of HBV and HIV strains circulating in Pretoria and exploring the correlation of HBV genotypes with response to anti-HBV-containing HAART regimens.

Rationale:

Although HBV is hyperendemic in Africa, very little is known about the relationship of different genotypes with disease progression or response to anti-HBV therapy. Few studies have reported the recombinant genotypes being detected in Africa, with limited studies reporting a trend in the distribution of the genotypes in Africa (reviewed by Kramvis and Kew, 2007). However, the most significant part is the correlation of HBV genotypes with response to anti-HBV drugs being given to HIV patients as part of ARV treatment, as there are currently no studies from Africa. Therefore, this study seeks to investigate HBV and HIV diversity in Pretoria and surrounding areas, and the correlation of HBV genotypes with response to anti-HBV drugs as part of the expanding ARV programmes for treatment of HIV patients in South Africa. This is also significant, as there are no studies on the impact of lamivudine-containing HAART regimens on different HBV genotypes in HBV/HIV co-infected patients in sub-Saharan Africa. Analysis of the study will also include the potential impact of HBV genotypes on prognosis of HIV patients on HAART.

1.2 Thesis organisation

The thesis consists of nine chapters. **Chapter One** is "Experimental Proposal and Thesis Organisation", **Chapter Two** is "General Literature Review" and **Chapter Three** is "Overview of the study Plan". **Chapters Four to Six** are organised around the three broad objectives of this study, with each chapter containing its own independent background introduction, experimental methods, results, discussion and conclusions. In brief, **Chapter Four** is "Investigation of the burden of HBV co-infection in HIV-positive patients"; **Chapter Five** is "Investigation of the impact of anti-HBV-containing HAART on HBV during the management of HBV/HIV co-infected patients and co-evolution of HBV and HIV drug-resistant strains". **Chapter Six** is "Investigation of the genetic diversity of HBV and HIV strains circulating in Pretoria and surrounding areas, and further exploring the correlation of HBV genotypes with response to anti-HBV-containing HAART regimens". Where there was an overlap in experimental methods or procedures, this was referred to the previous chapter(s) and section(s). Finally, **Chapter Seven** combines the discussion, conclusions and recommendations, while **Chapter Eight** and **Nine** contains references and appendix respectively.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 Introduction

In addition to the growing pandemic of HIV, there is an enormous burden of infection with chronic viral hepatitis, leading to very high rates of co-infections with HBV, hepatitis C virus (HCV) and HIV. There is overwhelming evidence that HIV impacts very negatively on the progression of HBV-and HCV-related chronic liver diseases (Alberti *et al.,* 2005). Since the introduction of HAART, death from AIDS-related opportunistic infections has declined, but hepatitis B-related liver diseases have become the leading cause of morbidity and mortality worldwide (Lewden *et al.,* 2005; Palella *et al.,* 2006). Due to an increase in the roll- out of HAART-containing anti-HBV activity in developing countries, drug-resistant strains against HBV and HIV have become a major concern (Kobayashi *et al.,* 2001; Kirishima *et al.,* 2002; Heo *et al.,* 2004; Ohishi *et al.,* 2004; Selabe *et al.,* 2007; Hofmann and Thio, 2007). Antiviral drug resistance is defined by the presence of virus mutations that can reduce drug susceptibility, compared with the susceptibility of wild-type viruses. This can either be mediated by changes in the molecular target of therapy or in other virus proteins that indirectly interfere with a drug's activity. Development of resistance to multiple antiviral agents may result in limited alternatives to treatment regimens, with long-term virological success of subsequent therapies being short-lived. This is in-view of HIV, the etiological agent of AIDS responsible for the pandemic worldwide (especial sub-Saharan Africa), and HBV, the etiological agent responsible for hepatocellular carcinoma and cirrhosis (Williams, 2006; Hoffmann and Thio, 2007; Benhamou, 2007). Co-infections with HBV and HIV are particularly common, with up to 80% of HIV-infected individuals having been exposed to HBV, and up to 10% have chronic HBV due to their similar modes of transmission (reviewed in Burnett *et al.,* 2005; Hofmann and Thio, 2007). While HBV has a successful and efficacious vaccine for prevention in over 80% of countries worldwide, management of chronic hepatitis B infections in HIV co-infected individuals has become a challenge. This is because of current ARV regimens that include several drugs, such as lamivudine, adefovir, tenovir and entecavir, that have dual activity against both HBV and HIV, and may ultimately results in development of drug-resistant HIV and HBV variants (Heo *et al.,* 2004; Ohishi *et al.,* 2004; Selabe *et al.,* 2007). The HBV is a DNA virus; however, its replication is through an RNA-replicative intermediate requiring an active viral reverse transcriptase/polymerase enzyme. The reverse transcriptase of HBV lacks a conventional proofreading function which is found in other higher-order polymerases (McMahon *et al.,* 2007;

Hoffmann and Thio, 2007; Benhamou, 2007; Zoulim *et al.,* 2007; Yuan and Lee, 2007). Therefore, HBV exhibits a mutation rate more than 10-fold higher than other DNA viruses and more closely resembles the replication characteristics of RNA viruses like HIV. As a result, HIV and HBV share a number of characteristics that affect selection and use of antiviral therapy and drug resistance, and lessons learned from aspects of HIV resistance may provide insight on how to address similar challenges found when addressing potential HBV resistance (Benhamou, 2006 and 2007; Jain *et al.,* 2007).

In contrast to HBV, HIV vaccine development remains an enormous problem; hence, ARV drugs are the only therapeutic tools that are currently used to treat HIV/AIDS patients. The ultimate challenge is to use the understanding of the HIV lifecycle to continue to develop anti-HIV drugs that can suppress and are effective against the current HIV drug-resistant strains. Unfortunately, HIV infections cannot be cured with ARV drugs, so that drug therapy, once initiated, must be continued for the life of the patient (Finzi et *al.,* 1999; Sarafianos *et al.,* 2009). With the current significant development of ARV drugs that are able to suppress both HBV and HIV replication, the evolution and development of drug -associated resistant strains has become a major public health concern. This literature review will focus on HBV and HIV-1 and their disease manifestations.

2.2 Hepatitis B virus

Viral hepatitis B remains a major public health problem and the most common cause of liver disease world-wide. In 1963, Blumberg and colleagues, in a search for polymorphic serum protein, discovered a previously unknown antigen in the blood of an Australian aborigine, which was named Australia antigen. Soon afterwards it was recognised that the appearance of this antigen was related to type B hepatitis (Blumberg *et al.,* 1965), and eventually Dane *et al.,* (1970), discovered virus-like particles that carried antigen on their surface, in the serum of hepatitis B patients using immune and electron microscopic method. These particles were later named HBV.

2.2.1 Classification

HBV is a prototype virus of the *Hepadnaviridae* family and genus Orthohepadnavirus. It is a preferentially hepatotropic agent, and was originally called serum hepatitis. Hepatotropy implies entry into and, in a number of cases, survival of the virus in the hepatocytes. The consequence is necroinflammatory hepatic disease of varying duration and severity (Francois *et al.,* 2001). Other members of *Hepadnaviridae* related to HBV are the woodchuck hepatitis virus, which was isolated from eastern woodchucks (Summer *et al.,* 1978), ground squirrel hepatitis virus

obtained from Beechy ground squirrels (Testus *et al.,* 1996), tree squirrel hepatitis and duck hepatitis B virus (DHBV) isolated from Pekin ducks (Mason *et al.,* 1980). They all share unique genomic and structural features including similar virion size and ultra structure, with an envelope surrounding a spherical nucleocapsid that contains a similar viral DNA genome in terms of size, structure and organisation. HBV has unusual features, similar to retroviruses. It replicates through an RNA intermediate and can integrate into the host genome. Its unique method of replication involves reverse transcription of a greater than genome length RNA, using a viral encoded protein primer. Finally, hepadnaviruses have moderate narrow host range, which is usually limited to each species (Ganem and Schneider, 2001; Hollinger and Liang, 2001).

2.2.2 Structure of HBV particles

During the course of infection, human serum contains multiple types of HBV particles. Under the electron microscope, three types are observed: (i) Complete, infectious virions of 42nm in diameter, called Dane particles (Figure 2.1) (Dane *et al.,* 1970); and (ii) incomplete (lack HBcAg, polymerase, and genome) non-infectious viral components of two forms (spherical moieties with size of 22nm and filamentous particles of diameter 22nm) (Figure 2.1).

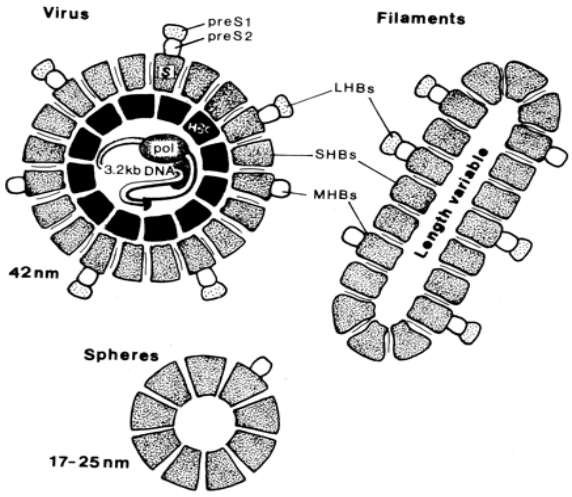

Figure 2.1: Structure of HBV particles (adapted form Mahoney, 1999).

High levels of these non-infectious particles are found in excess, as compared to Dane particles, during infection, and induce a significant immune response from the majority who are infected.

2.2.3 Genomic organisation of HBV

The genome of HBV is a covalently closed-circular DNA (cccDNA) virus of an approximately 3200 base pairs. It is an enveloped virus with a genome uniquely organised in a partly double-stranded pattern (Gerlich and Robinson, 1980; Ganem and Varmus, 1987). The genome encodes four overlapping open reading frames (ORFs), overlapping each other and covering the entire genome (Figure 2.2). The ORFs include: core (C) [pre-core/core], surface (S) [(pre-S1/S2/S)], polymerase (P) and HBX-encoding (X) regions (Lau and Wright, 1993). The viral polymerase is covalently attached to the 5' end of the minus strand which is approximately 3200 bases in length. The minus strand is the longest, and contains ORF that encode for viral proteins and the cis-elements responsible for regulation of HBV gene expression and replication. A plus strand of variable length maintains the circular structure of the cohesive hybridisation that straddles the 5' and 3' ends of the minus strands (Gerlich and Robinson, 1980).

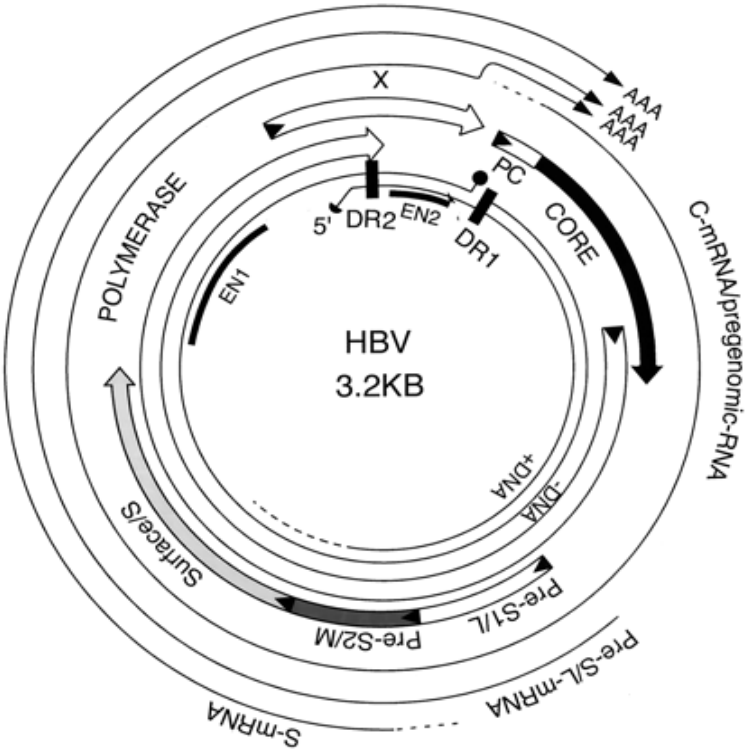

Figure 2.2: Schematic genomic organisation of HBV (Seeger and Mason, 2000).

The four protein-coding regions are shown between the inner and outer circles, and they include the pre-core/core gene, the polymerase gene, and the X gene. The envelope genes pre-S1 (Large), pre-S2 (Middle), and small surface (S or HBsAg) overlap with the polymerase ORF. Direct repeats, DR1 and DR2, as well as the positions of the two enhancers, ENI and ENII, are shown.

2.2.3.1 The Surface gene

The *S* ORF encodes for the viral surface envelope proteins, the HBsAg, and can be structurally and functionally divided into the pre-S1, pre-S2, and S regions. Each begins with its own translation start codon (ATG), but all end with a common translation stop codon (TAA). The three resulting proteins are LHBsAg, MHBsAg and SHBsAg or HBsAg proteins (Figure 2.3). All three proteins are required for the formation of the HBV envelope (Lau and Wright, 1993).

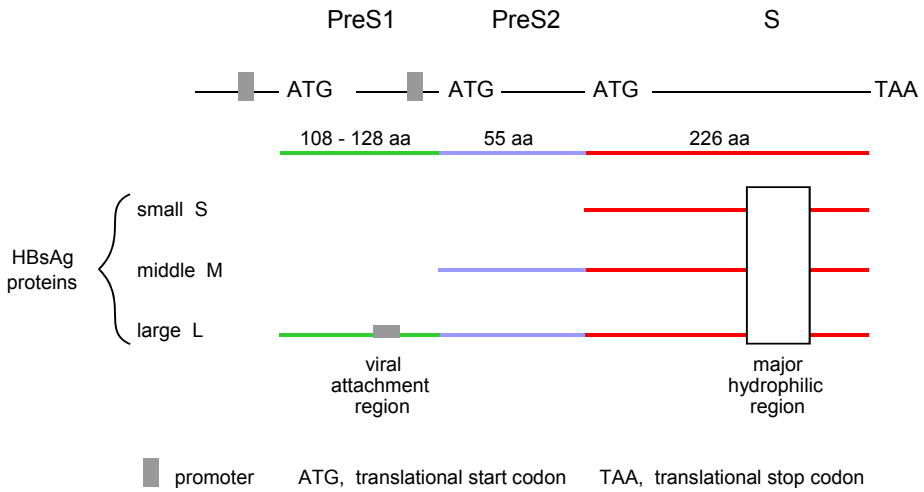

Figure 2.3: Schematic organisation of the HBV surface gene (Carman, 1996)

The HBsAg is coded for by the S-gene, and is the most important gene as it contains neutralising epitope. It is composed of 226 amino acids (Figure 2.3). HBsAg is of crucial importance, because it contains major neutralising epitope and is therefore used in commercial hepatitis B vaccines. The presence of antibodies to HBsAg (anti-HBs) alone is sufficient to protect from HBV infection (Carman, 1996).

2.2.3.2 The pre-core/core gene

The C- ORF encodes either the viral nucleocapsid HBcAg or hepatitis B e antigen (HBeAg), depending on whether translation is initiated from the core or precore regions, respectively. The pre-core/core has two translation start codons (AUG); translation from the first leads to the preprotein that, after loss of the signal sequence and the carboxyterminal nucleophillic sequence, leads to HBeAg. HBeAg is secreted into the serum, and its function remains largely undefined, although it has been implicated as an immune tolerogen, whose function is to promote persistent infection (Milich and Liang, 2003). Translation from the second AUG leads to core protein, which is the building block of core particles and, therefore, virions. Both HBeAg and HBcAg translation end at a common translation stop signal (UAG). Both the HBcAg and the

HBeAg are products of the pre-core/core gene, and they share about 160 amino acids. Besides HBcAg and HBeAg being structurally related and sharing some epitopes, they are not immunologically identical. Due to proteolytic processing, HBeAg assumes a different three-dimensional conformation. This conformation allows HBeAg to shift the immune response against the virus in a humoral direction (Carman *et al.,* 1989; Carman, 1996; Milich *et al.,* 1998).

2.2.3.3 The Polymerase gene

The polymerase gene (Pol) is the largest open reading frame and encodes for the multi-functional polymerase protein. Its protein is 90kD in size and has RNA- and DNA-dependent polymerase activities, which are responsible for priming and reverse transcription of the pre-genomic HBV RNA (Lau and Wright, 1993). The polymerase gene overlaps all six other genes including the core gene that encodes for HBcAg and the precore gene that encodes for the HBeAg, the three envelope genes PreS1, PreS2, and HBsAg, that encodes for the large, middle, and small envelope proteins, respectively, LHBs, MHBs, and HBsAg. It also overlaps the X gene that encodes for the multifunctional X protein (Figure 2.2). The polymerase gene contains four functional regions: (i) terminal protein used in protein priming of HBV DNA synthesis, (ii) the spacer region, (iii) the reverse transcriptase (RT) that has RNA- and DNA-dependent DNA polymerase activities, and (iv) the ribonucleaseH (RNase H) that cleaves the RNA in the RNA-DNA hybrids during reverse transcription (Chang, 1990; Radziwill *et al.,* 1990; Lanford *et al.,* 1999) (Figure 2.4).

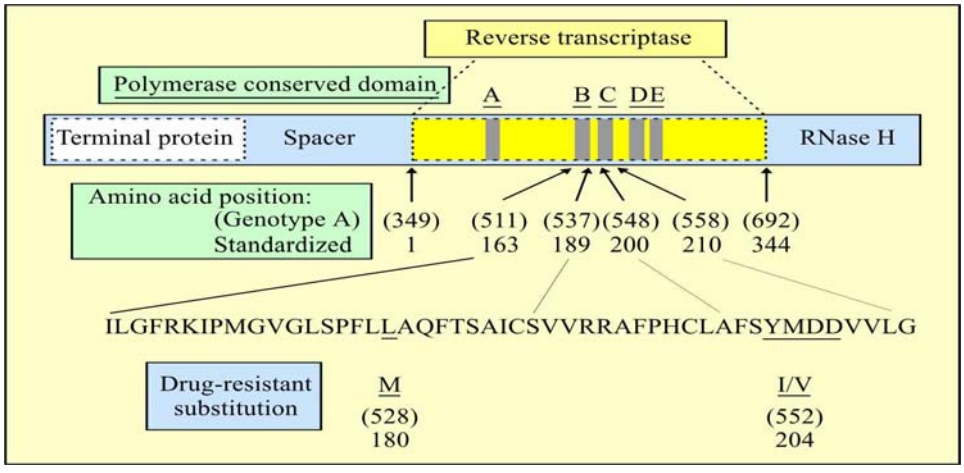

Figure 2.4: Schematic domains of HBV polymerase gene (adapted from Kamiya, 2003). Reverse transcriptase is situated in the middle (Domains A-E).

The hepatitis B polymerase also requires the presence of the metal ions and the presence of the stem-loop for polymerase/reverse transcription activity to occur (Tavis *et al.,* 1998; Urban *et*

al., 1998). HBV has a complex genomic replication strategy, which uses protein priming for the synthesis of the minus and plus strands via a number of polymerase strand transfers to generate the partially double-stranded genomic HBV DNA. There are potentially three sites of action for a nucleoside analog in the HBV replication process. The first is the reverse transcription of the HBV pregenomic (pg) RNA into minus strand DNA (Mack *et al.,* 1988). The second is the formation of the incomplete positive strand DNA. The third is the conversion of the partially double-stranded DNA (either derived from the intracellular conversion pathway or the initial entrance of the virus into the cell) to the fully complete double-stranded covalently closed circular form (cccDNA). This final conversion is likely to be mediated by cellular enzymes and is therefore not an ideal target for nucleo(ti)side analogs.

2.2.3.4 The HBx gene

The X gene is the smallest of the four ORFs of the HBV genome (Figure 2.2). It codes for 154-amino acid polypeptide with a molecular weight of 17kD (Lau and Wright, 1993). The X gene was the last of the HBV genes to be characterised and is thought to be an early gene. The biological functions of the HBxAg have not been fully characterised. The main function of the X protein is transcriptional activation and it has been shown to transactivate a number of cellular and viral promoters (Kekule' *et al.,* 1993; Lau and Wright, 1993; Henkler and Koshy, 1996; Arbuthnot *et al.,* 2000).

2.2.4 Epidemiology of HBV

2.2.4.1 Transmission of HBV

Transmission of HBV occurs in various ways, but with the main routes being parenteral and sexual. Infection may occur via microlesions of mucous membranes, allowing the virus to pass into the bloodstream. This is the basis for the frequent transmission of HBV via sexual contact. Direct inoculations – for instance, in the medical profession via contaminated needles ("needle stick" injury) – also account for the majority of occupational-acquired infections. HBV can be transmitted from a carrier mother to her neonate. The frequency of HBV infection and patterns of HBV transmission vary markedly in different parts of the world. In areas of high endemicity such as Africa and Asia, most infections occur at birth or during early childhood, with variable mechanisms of transmission, i.e. horizontally (close contact) and vertically (maternal-neonatal transmission), compared to low endemic areas (North America and Western Europe) (Davis *et al.,* 1989; Robson and Kirsch, 1991; Kew, 1996; Kiire, 1996; Mahoney, 1999).

A South African study has found that 8.1% of 0-6 month old babies and 8.9% of 7-12 months old babies are HBsAg-positive, suggesting an increase in early infancy transmission of HBV (Vardas *et al.,* 1999).

2.2.4.2 Prevalence of HBV

HBV infection is a major global public health problem, warranting high priority efforts in prevention and control. Over two billion of the world's population have been exposed to HBV and an estimated 400 million are chronically infected, with a rate of 10 million new carriers each year. Approximately 17% of the carriers will die from the consequences of their infection, with an overall annual mortality from HBV infection of about 1 million (Mphahlele *et al.,* 2002; Alexander *et al.,* 2006).

There are approximately 50 million chronic carriers of HBV in Africa, with 25% mortality risk. In sub-Saharan Africa the carrier rates are high, ranging from 9-90% (Kiire, 1996). South Africa is a country with intermediate HBV endemicity and pockets of high endemicity. Most of the infections occur early in childhood by as yet unidentified horizontal routes. Of the approximately 400 million HBV carriers in the world, about 3 million reside in South Africa. Deaths from chronic liver disease are estimated at 14 000-18 000 per annum (Mphahlele *et al.,* 2002).

2.2.4.3 HBV genotypes and subtypes

HBV has been classified into eight genotypes (A to H), based on an intergroup divergence of 8% or more in the nucleotide sequences. The genotypes have been associated with different geographical areas: genotype A with Europe and sub-Saharan Africa, genotypes B and C with east Asia, genotype D with the Mediterranean and Middle East regions, genotype E with Western Africa, and genotype F with the Americans. Genotype G has been observed in France and the United States (Stuyver *et al.,* 2000). Previous studies reported genotype A to be the most predominant genotype in South Africa, while genotype E is predominant in West Africa (Magnius and Norder, 1995; Bowyer *et al.,* 1997; Kramvis *et al.,* 1997; Kidd-Ljunggren *et al.,* 2002).

The distributions of genotypes over different regions can be explained by migration that occurs over a period of time. They may also reflect the origin of the immigrants and other patterns of migration. An example of this pattern is seen with South Africa, where different genotypes are found, genotypes A and D, which correlate with migration from North-western Europe (UK and Netherlands), Southern Europe and India. Recombination between genotypes has been reported to occur in South Africa (Owiredu *et al.,* 2001). It is unclear how these events of

recombination have arisen in HBV. Genotype H has been recently discovered (Kramvis and Kew, 2007).

Other than genotypes, HBV has been subdivided into nine different serological types. This is based on using sub-specific antibodies against HBsAg, reflecting the genetic variability of HBV. Of the defined determinants, one is common to all subtypes and is called the "a determinant". Two pairs of mutually exclusive sub-determinants (d or y, and w or r) of the major envelope protein are also commonly found. They have been widely used to differentiate HBV strains in clinical, virological and epidemiological studies (Norder *et al.,* 1993). These serotypes have been shown to be geographically distributed. For example, adr is confined to the East, ayw2 predominates in the Mediterranean region and adw2 is found in the Pacific region. Serotype adw2 predominates in Northern Europe and sub-Saharan Africa, but its prevalence decreases from East Africa towards Central and West Africa and is accompanied by a corresponding increase in the unique African serotype, ayw4 (Magnius and Norder, 1995).

2.2.5 The lifecycle of the HBV

2.2.5.1 Entry and uncoating

The HBV lifecycle follows the model formulated for the overall replicative cycle of hepadnavirus using DHBV. The initial phase of HBV infection involves the attachment of mature virions to host cell membranes, likely involving the pre-S domain of the surface protein (Figure 2.5). However, early events of the viral life cycle, including entry, uncoating, and delivery of the viral genome into the nucleus, are not well understood (Klingmuller and Schaller, 1993). This is, in part, due to the absence of cell lines that are susceptible to HBV. Attempts to define the receptor for HBV have yielded a plethora of candidates such as apolipoprotein H (Mehdi *et al.,* 1994). Despite these findings, the proteins and mechanisms of HBV entry into cells have yet to be well characterised. The DHBV system has suggested that HBV genome uncoating occurs at the nuclear membrane (Qiao *et al.,* 1999).

2.2.5.2 Replication of HBV genome

Following HBV entry into the hepatocytes, circular, partially double-stranded DNA is released from the viral nucleocapsid and is converted to covalently-closed circular DNA within the hepatocyte nucleus. The cccDNA serves as the template for transcription of functional genomic and subgenomic mRNAs. The smaller, subgenomic transcripts are 2.4, 2.1, and 0.9 kb and they serve as mRNA for the expression of the surface proteins and HBX protein. The larger genomic transcript is longer than one genome in length, 3.5 kb, and it serves as mRNA for the synthesis of HBeAg, core and polymerase proteins (Figure 2.5).

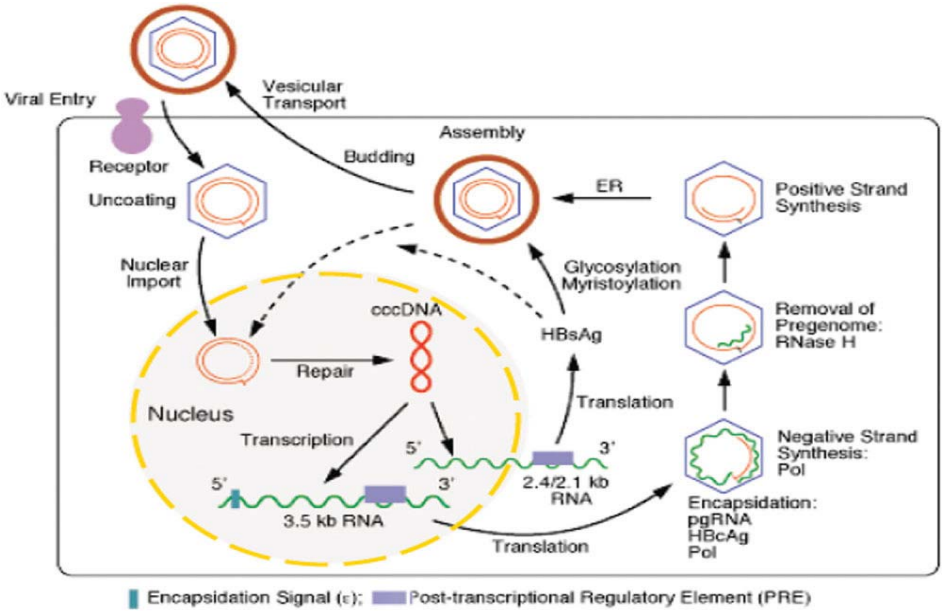

Figure 2.5: Schematic diagram of HBV life cycle (Ghany and Liang, 2007)

The 3.5-kb genomic transcripts consist of two species with different 5' ends: the pregenomic and the precore RNAs. Pregenomic RNA (pgRNA) serves as the template for HBV reverse transcription and the messenger RNA for core and polymerase; the precore direct RNA directs the translation of the precore gene product. The viral mRNAs are transported to the cytoplasm, at which translation of viral proteins, nucleocapsid assembly, and viral replication occurs. Replication occurs within a nucleocapsid that consists of the core protein, the pregenomic RNA, and the polymerase (Ganem and Varmus 1987, Seeger and Mason, 2000). It is believed that binding of the polymerase protein to the stem loop results in pgRNA encapsidation (Pollack *et al.,* 1994). The 5'- and 3'-ends of this greater than genome length transcript contain a terminal repeat that includes DR1 and the stem loop RNA packaging signal (ε). The polymerase interacts with the 5'-end of packaging signal to prime reverse transcription (Wang and Seeger, 1993; Tavis *et al.,* 1994). Upon binding of the polymerase to the stem-loop, the polymerase begins to reverse transcribe the pgRNA template for three to four bases. The polymerase protein is actually covalently attached onto the growing (-) DNA strand. The polymerase serves as the primer when initiating reverse transcription. The polymerase protein uses a bulge in the stem-loop as its template for initiating reverse transcription (Wang and Seeger, 1993; Tavis *et al.,* 1994). The complex of pgRNA with HBV polymerase associates with core proteins to form viral capsids. Following protein priming, the subsequent steps of HBV reverse transcription are completed within the viral capsid. This process, involving template switches, DNA synthesis

and RNase degradation, eventually results in the formation of the characteristic circular, partially double-stranded hepadnavirus DNA (Nassal and Schaller, 1993; Nassal and Schaller, 1996).

2.2.5.3 Assembly and budding

Once partially double-stranded DNA has been produced, nucleocapsids can undergo a maturation event that facilitates their acquisition of an outer envelope via budding into endoplasmic reticulum (Figure 2.5). The assembled nucleocapsid is enveloped by a lipoprotein membrane formed by cell-derived lipid bilayer and virus surface proteins, and the virion is exported through a secretory pathway. A proportion of the nucleocapsid particles also migrate into the nucleus to maintain the pool of cccDNA (Nassal and Schaller, 1993).

2.2.6 Disease profile

The clinical presentations of HBV infection show a very broad spectrum, ranging from asymptomatic patients without any detectable evidence of liver disease ("healthy carrier") to severely ill patients with jaundice, edema, ascites, upper gastrointestinal bleeding and other signs and symptoms. Occasionally, HBV infections can be fulminant or chronic, progressing to chronic liver disease with substantial morbidity and mortality resulting from the complications of liver cirrhosis and HCC (Robinson, 1994).

2.2.6.1 Acute infection

The consequences of acute HBV infection are highly variable, and this can be either symptomatic or asymptomatic. The incubation period ranges from 6 weeks to 6 months, and the development of clinical manifestations are highly age-dependent. Exposure against HBV is marked by a well–defined immunological response which results in the resolution of the infection and protective immunity, i.e., anti-HBs. The first serologic marker of HBV infection to appear is HBsAg. The HBsAg usually persists in serum throughout the period of clinical illness, and is commonly used to diagnose acute HBV infection. The disappearance of HBsAg and the appearance of anti-HBsAg (anti-HBs) mark the resolution of acute infection. The anti-HBc generally appears at approximately the same time as HBsAg. HBc IgM develops initially and is eventually replaced by HBc IgG (Di Bisceglie, 1988) (Figure 2.6).

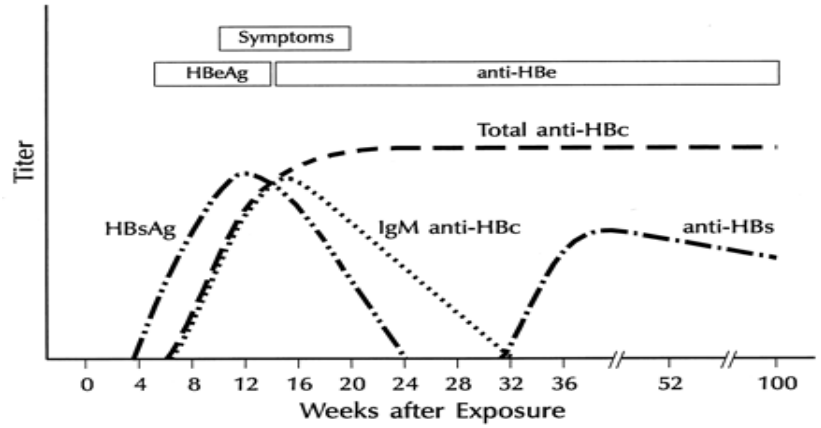

Figure 2.6: Characteristics of acute hepatitis B with recovery (Mahoney, 1999)

Newborns generally do not develop any clinical signs or symptoms, and infection produces typical illness in only 5 to 15% of children 1-5 years of age (Hyams, 1995). Older children and adults are symptomatic in 33 to 50% of infections, with symptomatic infections varying in severity from mild to fulminant forms. Clinical signs and symptoms of acute HBV infection include fever, anorexia, nausea, malaise, vomiting, jaundice, dark urine, clay-coloured or pale stools, abdominal pain and, occasionally, arthralgias, and arthritis. Fulminant hepatitis occurs in about 1 to 2% of persons with reported acute disease and has a case-fatality ratio of 63 to 93% (Mahoney, 1999).

2.2.6.2 Chronic infection

During early HBV infection, the HBeAg, HBsAg, and HBV DNA are usually present in high titres, and there are mild to moderate elevations in serum aminotransferase levels. This is followed by either the disease activity resolving, either with persistence of high levels of HBeAg and HBV DNA (the “immune tolerance phase”) or with loss of HBeAg and fall of HBV DNA to low or undetectable levels (“inactive carrier state”) (Mahoney, 1999). Chronic infection has been defined as the persistence of HBsAg in the serum of an individual for at least 6 months, or longer, or the presence of HBsAg and the absence of anti-HBc immunoglobulin M (IgM) (Figure 2.7).

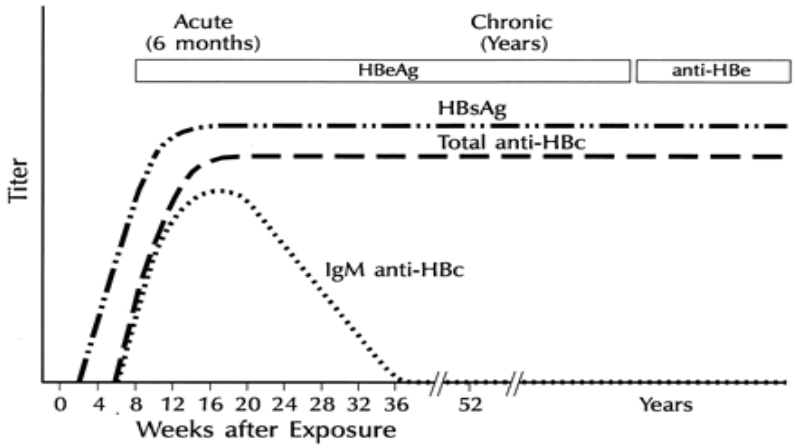

Figure 2.7: Characteristics of progression to chronic hepatitis B infection (Mahoney, 1999).

This results when the immune system does not mount an efficient response, due to immaturity, immune deficiency, or genetic factors. Chronic infection begins when the immune response that normally clears the infection fails to take place or is too weak to be effective. Despite the fact that most adulthood HBV infections are transient, approximately 1 to 5 % of people infected as adults, and more than 90% of those infected as neonates, fail to mount a sufficient immune response to clear the virus, and develop a lifelong chronic infection. The risk of developing chronic infection varies inversely with age (Hyams, 1995). Immunocompromised individuals are almost always chronic, following exposure to HBV. Patients infected as adults often have the most severe and progressive liver disease. Up to 90% of children infected between 1 and 5 years of age develop chronic infection, compared to 6 to 10% of acutely infected older children and adults (Mahoney, 1999). Individuals infected and becomes hepatitis B chronic carriers at adult stage have poor prognosis. About 10 to 25% will die of either liver cancer or cirrhosis. Smaller numbers, particularly those infected as adults, will die or require liver transplantation, due to rapidly progressing liver disease, leading to hepatic failure (Di Bisceglie, 1988).

2.2.7 Pathogenesis

It is well known that the outcomes of HBV infection depend on dynamic interaction and balance of viral replication rate and host immune response. The cellular and humoral immune responses to HBV infection are complex. Both humoral and cellular immune responses are involved in virus elimination. Infection with HBV is marked by the development of antibodies directed against each of the individual viral antigens: anti-HBs, anti-HBc and anti-HBe. An adequate multispecific anti-HBV T cell response against HBV proteins results in clearance of

HBV infection, whereas, a defective immune response causes persistence of HBV infection (Chang and Chisari, 1999).

Resolution of acute infection is associated with a vigorous polyclonal helper (Th) and cytotoxic T lymphocyte (CTL) response to multiple viral antigens in the infected livers (Ganem and Price, 2004). Moreover, although destruction of virally infected hepatocytes is evident during the resolution of acute infection, it has been demonstrated that the noncytolytical reduction of viral gene products in infected cells by cytokines, such as interferon gamma and tumour necrosis factor, released from activated T lymphocytes, is likely to play an important role in terminating the infection (Guidotti *et al.,* 1999).

Most studies have suggested that HBV is not directly cytopathic to infected hepatocytes and that the cellular response to several viral proteins correlate with the severity of clinical disease and viral clearance (Chisari and Ferrari, 1995; Isogawa *et al.*, 2005; Chang and Lewin, 2006). It is believed that the antibody response to viral envelope antigens contributes to clearance of the virus, and that cytotoxic T cells mediate viral clearance by killing infected cells. In addition, it has been shown that cytotoxic T lymphocytes inhibit HBV gene expression through the secretion of antiviral cytokines, and that the expression of these cytokines may be the principal mechanism of viral clearance during HBV infection (Chisari, 1997). It is hypothesised that chronic infection is related to a weak T-cell response to viral antigens, while neonatal immune tolerance to viral antigens appears to play an important role in viral persistence among persons infected at birth. The basis of a poor T-cell response in adults is not well understood (Mahoney, 1999).

2.2.8 Laboratory diagnosis

The diagnosis of HBV infection is based on a collection of clinical, biochemical, histological, and serologic findings. HBV serological assays have become a critical and primary assay in distinguishing between clinical symptoms due to HBV and other forms of viral hepatitis. A number of HBV antigens and their respective antibodies can be detected in serum after infection, and proper interpretation of the results is essential for the correct diagnosis of the various clinical forms of HBV infection (Table 2.1).

Table 2.1: HBV Serological markers and their significance

Serological Markers	*Definitions*	*Significance*
HBsAg	Hepatitis B surface antigen	Acute or chronic hepatitis B infection.
HBeAg	Viral product secreted in blood, identical to HBcAg in part	Marker of increased infectivity when present
Anti-HBs	Antibody to HBsAg	Immunity due to past infection and vaccination
Anti-HBc	Antibody to HBcAg (IgG and IgM)	Infection at some undefined time, acute, chronic or past infection
Anti-HBc-IgM	IgM antibody to HBcAg	High titres are proof of acute or recent hepatitis B infection
Anti-HBe	Total antibody to HBeAg	Replaces HBeAg; may indicate decreased infectivity
HBV DNA	Viral DNA	Direct evidence of replication and hence infectivity

There are different assays for HBV diagnosis, and these include: (i) serological, (ii) molecular and (iii) biochemical assays.

2.2.8.1 Serological Markers

There are different sensitive immunoassays available for diagnosis of HBV infections. This includes assays such as radioimmunoassay (RIA) and enzyme-linked immunosorbert assay (ELISA), which are used to identify HBV antigens or antibodies (Hollinger, 1996). Routine HBV markers used for diagnosis, and their significance, are listed in Table 2.1. The HBsAg in serum is an indirect marker for the presence of the virus or it's DNA in the liver, and can be detected in both acute and chronic infections. Undetected HBsAg does not rule out a new or chronic infection, since approximately 5% of the infections fail to develop HBsAg. During a typical course, anti-HBs appear shortly after disappearance of HBsAg. The rise of anti-HBs is associated with the elimination of HBsAg and signifies viral clearance from the liver, attesting to the end of the infectivity of the patient and corroborating immunity to HBV. Anti-HBs is the only antibody found using routine methods following effective immunisation against HBV. Antibodies to the HBcAg (anti-HBc, IgM and IgG) are detected at disease onset. Presence of anti-HBc (IgG) signals current or past contact with HBV. Testing for IgM antibodies to HBcAg (anti-HBc IgM) helps in staging the disease. The anti-HBc IgM antibodies are present in high titres at disease onset, and usually drop within weeks to months, below the sensitivity of routine assays during the normal course of hepatitis B infection. Antibody to HBeAg (anti-HBe) usually appears shortly after clearance of HBeAg, often at the peak of clinical illness. Thus, loss of HBeAg and appearance of anti-HBe is a favourable serological marker during acute hepatitis B, indicating the initiation of recovery (Wolfgang, 1999). HBeAg correlates with the presence of high levels of HBV replication and infectivity (Liang and Ghany, 2002).

2.2.8.2 Molecular assays

The DNA of the virus is the only marker allowing direct detection of HBV in the blood. HBV DNA in serum is associated with the presence of an infectious virus. Therefore, detection of HBV DNA is direct evidence of the presence of an infectious virus in blood. Methods for detecting HBV DNA are either based on direct nucleic acid hybridisation, or on nucleic acid amplification as in the polymerase chain reaction (PCR) or ligase chain reaction (LCR). Direct hybridisation based methods have a detection limit of about 0.3 pg HBV DNA/ml, allowing the detection of approximately 10^5 virion particles per ml. Despite their relatively high sensitivity, direct hybridisation methods do not rule out infectivity with certainty. Amplification-based methods, such as PCR or LCR, allow the detection of a very small number of DNA molecules with a 1,000 to 10,000-fold increased sensitivity, compared to direct hybridisation methods (Wolfgang, 1999). However, PCR assays are prone to false-positive results. Branched-chain DNA assay have also been shown to be useful for the detection of HBV DNA (Chen *et al.,* 2000). The limitation of molecular assays is that they are not routinely used for screening of HBV infections by clinical laboratories, but are for research purposes only and are also expensive for clinical laboratories.

2.2.8.3 Other tests

There are other supplementary tests that can be performed, and these include biochemical testing of the functioning of the liver and histological evidence of liver damage. Liver function tests include bilirubin, AST, ALT, alkaline phosphatase, gamma-glutamyl transpeptidase, albumin and globulin (Hollinger, 1996). However, ALT and AST are the two most important indicators of hepatocellular damage in viral hepatitis, and may fluctuate or may remain consistently elevated in chronic active HBV. Other liver function tests may include bilirubin, alkaline phosphatase and gammaglutamyl transpeptidase albumin. Globulin plays an important role in assessing the synthetic functioning of the liver (Hollinger, 1996).

2.2.9 Prevention and control

Acute HBV is a notifiable disease in the South Africa. Several approaches are being applied to control and prevent HBV transmission, and reduce the incidence of hepatitis B worldwide. These include the screening of blood donors for HBV infection markers, vaccination of those at risk, administration of hepatitis B immunoglobulin (HBIG) (often in conjunction with HBV vaccines), universal precautions and education of health care workers and high-risk groups concerning routes of transmission (Hollinger, 1996).

An effective and safe vaccine against HBV has been available for more than 20 years and many countries have included it in the Expanded Programmes on Immunization (EPI). The EPI

Global Advisory Group (1991), the World Health Assembly (1992) and WHO (1994) recommended the integration of hepatitis B vaccine into national immunisation programmes by 1995 in countries with high endemicity, and in all other countries of the world by 1997. The main aim of vaccination is to prevent chronic HBV infection, chronic liver disease, cirrhosis, HCC and deaths from these complications, and acute symptomatic HBV infection, and hopefully to eradicate HBV in the future. South Africa introduced the hepatitis B vaccine into its EPI in April 1995 (Aspinall and Kocks, 1998; Mphahlele *et al.,* 2002; Tsebe *et al.,* 2002).

The recommended series of three intramuscular doses of hepatitis B vaccine induce a protective antibody response in >90% of healthy adults younger than 40 years. After age 40, the cumulative age-specific decline in immunogenicity drops below 90%, and by age 60 years, only 70% of vaccinees develop protective levels of anti-HBs. Booster doses studies of adults have demonstrated that >90% of vaccinees have such immune memory when challenged with hepatitis B vaccine, indicating that the immune system would be able to respond rapidly to HBV exposure (Mahoney, 1999). It is expected that the vaccine will reduce the burden of chronic carriage by half in the coming decades.

2.2.10 HBV Treatment

Different nucleoside analogues have been introduced in the management of chronic HBV infection. The primary goal in treating chronic HBV infection is to reduce and further halt liver inflammation, lower the viral load to undetectable levels, and ideally, eradicate the virus and produce protective antibodies (anti-HBs). Most of the nucleoside analogues act by inhibition of HBV polymerase activity, resulting in the decrease of viral replication. There are currently five drugs with approval by the US Food and Drug (FDA) Administration for the treatment of chronic hepatitis B. These include: interferon alfa-2b (1992), lamivudine (1998), adefovir dipivoxil (2002), entecavir (March 2005), peginterferon alfa-2a (May 2005), with Tenofovir and emtricitabine still in clinical trials (Yuan and Lee, 2007). They are administered orally, and most of them have been shown to have an excellent tolerance and safety profile. Standard interferon alfa-2b has been replaced by peginterferon alfa-2a because of its improved pharmacokinetics of the pegylated form of interferon and the greater convenience.

Besides having anti-HBV activity, lamivudine, emtricitabine, adefovir, entacavir and tenofovir have also been shown to be effective against HIV; hence a caution when prescribed in HBV/HIV co-infected patients (Jain *et al.,* 2007). Adefovir was first developed as an anti-HIV drug and is now registered at a lower dosage for treatment of hepatitis B (Herrero-Martinez, 2001; Lok, 2002; Zoulim *et al.,* 2007; Yuan and Lee; 2007).

IFN-α is recommended when pre-treatment HBV DNA levels are less than 200 pg/ml, aminotransferase levels are greater than 100 U/L, and acute liver necroinflammation is present (Herrero-Martinez, 2001). Interferon–α–2b has been shown to have a response rate of 30-40% within 3 to 4 months (Lok, 2002). Lamivudine is a nucleoside analogue, which has been shown to clear HBV DNA within one year, particularly in 16-18% of HBeAg positive carriers (Lok, 2002), and is able to bring complete remission in 65-70% of HBeAg negative chronic carriers (Papatheodoridis *et al.,* 2002). It has also been shown to be equally efficacious in children (Jonas *et al.,* 2002). Prolonged effective antiviral therapy is required for eradication of chronic HBV infection, but long-term treatment with lamivudine has been found to be associated with progressively increasing rates of viral resistance (Papatheodoridis *et al.,* 2002).

2.3 Human Immunodeficiency Virus (HIV)

The human immunodeficiency virus type 1 (HIV-1) is an etiological agent responsible for causing AIDS, which is a slow, progressive and degenerative disease of the human immune system. The HIV has complex pathogenesis which is characterised by the interplay of both viral and host factors. Since it was first isolated in 1983 (Gallo and Montagnier, 2003), the HIV/AIDS epidemic has continued to exceed all expectations in the severity and scale of its impact. An intense global research effort into understanding the individual steps of the viral replication cycle and its dynamics during an infection has inspired researchers in the development of a wide spectrum of antiviral strategies (Nielsen *et al.*, 2005).

2.3.1 Classification

Human Immunodeficiency viruses belong to a class of *Retroviridae* family, and a member of the genus, Lentivirus. They also belong to a subgroup of retroviruses known as lentiviruses or "slow" viruses. This is so-called because the course of infection with these viruses is characterised by a long interval between initial infection and the onset of serious symptoms. Two species of this retrovirus infect human: HIV-1 and HIV-2 (Chiu *et al.,* 1985). They are closely related to the Simian Immunodeficiency Virus. HIV-1 is more virulent than HIV-2, and it is responsible for the majority of global HIV infections. HIV-2 is less virulent, less transmissible and geographically limited to West Africa (Reeves and Doms, 2002). Other lentiviruses which infect non-human species are: the feline immunodeficiency virus which infects cats, and the simian immunodeficiency virus which infect monkeys and other non-human primates. They are characteristically responsible for long-duration illnesses associated with a long period of incubation. Both of these viruses' primarily target similar cells, mainly the immune system cells, often causing immune deficiency and AIDS-like symptoms (Lévy, 1993). This study focuses mainly on HIV-1, which is responsible for the majority of global HIV infections, with sub - Saharan Africa being the worst affected.

2.3.2 Morphology of HIV

The structure of mature HIV virions has a spherical morphology of 100–120 nm in diameter. It consists of a lipid bilayer membrane that surrounds a dense truncated cone-shaped nucleocapsid (core) which consists of two copies of positive single-stranded genomic RNA molecules. The genomic RNA molecules include the HIV polymerase which consist of protease (PR), reverse transcriptase (RT), and integrase (IN) (Figure 2.8). Other genomic RNA molecules include accessory genes: *Vpu, Vif, Vpr and Nef*, and some cellular factors (Hirsch and Curran, 1990; Montagnier and Clavel, 1994). Each viral particle contains 72 glycoprotein

complexes which are integrated into this lipid bilayer. The matrix protein p17 is anchored to the inside of the viral lipoprotein membrane. The p24 core antigen contains two copies of HIV RNA. The HIV RNA is part of a protein-nucleic acid complex, which is composed of the nucleoprotein p7 and the RT p66 (Rubbert and Ostrowski, 2003). The viral particle contains all the enzymatic equipment that is necessary for replication: RT (p66/p51), an IN (p32) and PR (p10) (Rubbert & Ostrowski, 2003) (Figure 2.8).

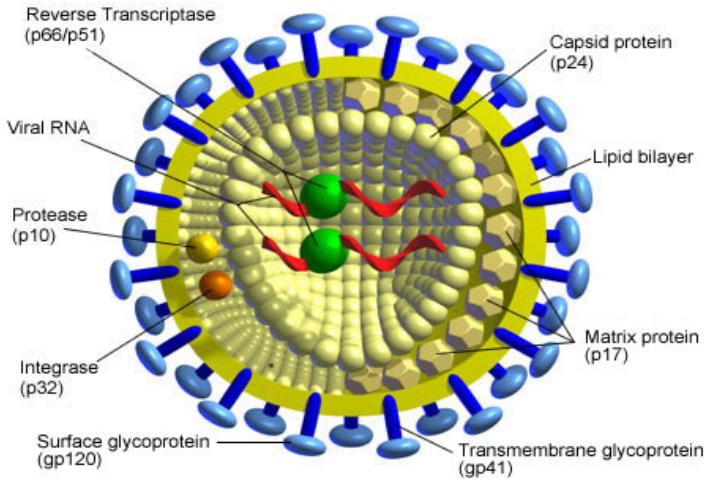

Figure 2.8: Schematic representation of HIV virion (Rubbert and Ostrowski, 2005)

2.3.3 Genomic organisation of HIV

The HIV genome consists of two identical 9.2 kb single-stranded positive sense RNA molecules within the virion. The RNA genome consists of nine genes, three structural genes; (i.e. group specific antigen (*gag*), polymerase (*pol*) and envelope for *env*), and six regulatory genes (i.e. *tat, rev, nef, vif, vpr, and vpu*) (Figure 2.9). Like other retroviruses, HIV depends mainly on three genes for replication: *gag, pol and env* (Sierra *et al*., 2005). These three genes (gag, pol and env) are common to all retroviruses. The "classical" structural scheme of HIV genome is: 5'LTR-gag-pol-env-LTR 3', flanked by LTR ("long terminal repeat"). The LTR's regions represent the two end parts of the HIV genome that are connected to the cellular DNA of the host cell after integration. Their regions do not encode for any viral proteins. Each LTR contains an U3, R and U5 region (Starcich *et al*., 1985). The U3 contains binding sites for cellular transcription factors (Huang and Jeang, 1993). The R-region contains the trans-activation response element (TAR) implicated in Tat-mediated trans-activation (Richter *et al*., 2002).

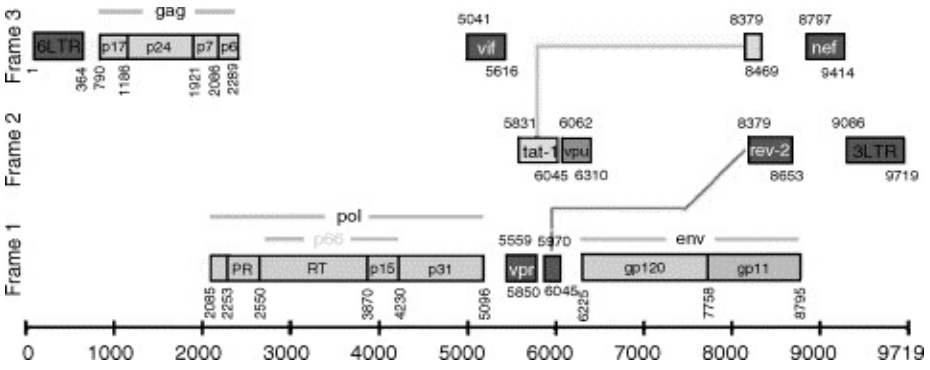

Figure 2.9: Organisation of the proviral HIV-1 genome (strain HXB2) (Sierra *et al.,* 2005).

Numbering positions are according to HXB2CG. Four main regions can be distinguished in the HIV genome: (1) LTR: regulatory regions located at both edges of the RNA molecules, flanking the coding region. (2) The *gag-pol gene* encodes two polyprotein precursors. The *gag* polyprotein includes the proteins of the nucleocapsid, and the *gag-pol* polyprotein comprises some structural proteins and three viral enzymes: PR, RT and IN. (3) The *env* gene encodes the gp160 polypeptide precursor containing the exterior gp120 and the transmembrane gp41. (4) HIV accessory genes: *tat, rev, nef, vif, vpr and vpu* (for HIV-1) or *vpx* (for HIV-2).

The persistent form of the HIV genome is proviral double-stranded DNA within infected cells. Infectious virions contain two copies of a single-stranded RNA genome, each of which contains the genetic information necessary to encode all viral proteins (Sierra *et al.,* 2005). Three genes; *gag, pol and env*, carriers required information for structural proteins for new virus particles, while the six regulatory genes, *tat, rev, nef, vif, vpr, and vpu,* are necessary to produce proteins that control the ability of HIV to infect a cell, and produce new copies of the virus, or cause disease. Each of these six regulatory genes also codes for accessory proteins, *tat, rev, nef, vif, vpr, and vpu*. They enhance virus production and are essential for virus replication. The accessory protein *Vp*u is unique to HIV-1, while *Vpx* is unique to HIV-2 (Figure 2.9) (Kirchhoff *et al.,* 1995; Collins *et al.,* 1998; Sierra *et al.,* 2005).

2.3.3.1 The gag gene

The *gag* gene (group specific antigen) encodes a polyprotein precursor (Gag) whose name, p55, is based on its molecular weight (55-kDa). The major structural components of HIV are synthesized as a 55-kDa polyprotein, Gag (Demirov and Freed, 2004). P55 is cleaved by the viral protease to the mature gag proteins, matrix protein (also known as MA or p17), capsid protein (CA or p24), nucleocapsid protein (NC or p7) and p6, p2 and p1. Both *pol* and *gag* are encoded by the same mRNA, but translated into different proteins through initiation in alternative reading frames. Frame shifting during the translation of the viral gag-pol messenger RNA is essential for the production of *pol* gene products (PR, RT and IN). Although all nine

viral genes are essential for assembly of an infectious particle, the *gag* gene alone can direct the synthesis, transport to the plasma membrane, and assembly of the structural precursor polyprotein Gag (Sierra *et al.,* 2005).

2.3.3.2 The envelope gene

The envelope gene codes for the envelope glycoprotein gp160, which is a precursor to two glycoproteins: gp41 and gp120. The gp120 protein is embedded in and extends exterior to the viral lipid membrane, and is primarily responsible for host cell receptor binding and host cell tropism, the CD4 and co-receptors (CCR5 and CXCR4). Due to its physical location in the virion, the gp120 has been observed to contain a number of recognition sites for various adaptive immune responses, including neutralising antibodies; this includes the receptor (Goudsmit *et al.,* 1988), helper T lymphocytes (Fenoglio *et al.,* 2000) and cytotoxic T lymphocytes (Walker *et al.,* 1986; Tsubota *et al.,* 1989). Gp120 is organised into five conserved regions (C1-C5) and five highly variable domains (V1-V5) (Freed and Martin, 1995). Therefore, two potentially important positive selective forces acting on the env gene are changes in optimal host cell receptor affinity and evasion of host immune responses. The gp120 portion of env has been broadly categorised into five hypervariable regions (V1 to V5), with conserved regions interspersed (Modrow *et al.,* 1987).

2.3.3.3 The Polymerase gene (Pol)

The *pol* gene encodes for the enzymes that are initially synthesised as part of a large polyprotein precursor, p160 (*gag-pol*). These enzymes are results of synthesis from a rare frame shifting event during p55 translation (Jacks *et al.,* 1988). The individual pol-encoded enzymes include: RT, PR and IN, and are cleaved from p160 by the viral PR (Figure 2.10).

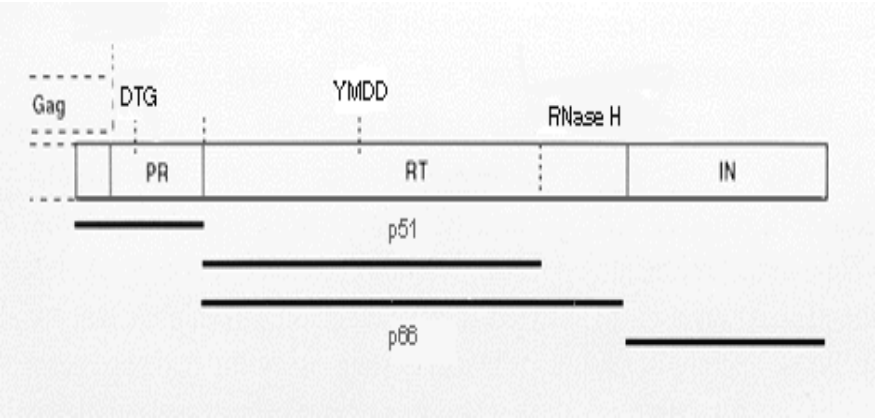

Figure 2.10: Schematic diagram of HIV polymerase showing PR, RT and IN (http://www.bioafrica.net/proteomics/POLprot.html#references/12/5/2008)

2.3.3.3.1 Reverse transcriptase gene (p66/p51)

The RT is essential for the life cycle of HIV, and is responsible for RNA-dependent DNA polymerisation and DNA-dependent DNA polymerisation. It converts the single-stranded genomic RNA into the double-stranded DNA, which is subsequently integrated into the host chromosome and passed on to all progeny cells. RT has two enzymatic activities: a polymerase that can copy either RNA or DNA, and an RNase H that degrades the RNA strand of RNA–DNA intermediates formed during viral DNA synthesis. The enzyme is a heterodimer of 1000 amino acids' residues consisting of two subunits, p66 and p51. The p51 subunit is composed of the first 440 amino acids, while the p66 subunit is composed of all 560 amino acids of the polymerase gene. Although the p51 and p66 subunits share 440 amino acids, their relative arrangements are significantly different. The p66 subunit contains the DNA-binding groove and the active site; the p51 subunit displays no enzymatic activity and functions as a scaffold for the enzymatically active p66 subunit. The general shape of the polymerase domain of the p66 subunit can be likened to a human hand with subdomains referred to as 'fingers', 'palm', and 'thumb'. The remainder of the p66 subunit contains an RNaseH subdomain and a connection subdomain (Kulpa *et al.,* 1997; Shafer, 2002; Tsai *et al.,* 2006).

Most RT inhibitor resistance mutations are in the 5' polymerase coding regions, particularly in the "fingers" and "palm" subdomains. Structural information for RT has been found mostly from X-ray crystallographic studies of unliganded RT (Rodgers *et al.,* 1995), RT bound to an NNRTI (Kohlstaedt *et al.,* 1992), RT bound to double-stranded DNA (Jacobo-Molina *et al.,* 1993), RT bound to double-stranded DNA and the incoming dNTP (ternary complex) (Huang *et al.,* 1998), and an RT bound to double-stranded DNA containing an AZT-terminated DNA primer pre- and post-translocation (Sarafianos *et al.,* 2004). There have been fewer structural determinations of mutant RT enzymes than of mutant protease enzymes (Sarafianos *et al.,* 1999; Sarafianos *et al.,* 2004).

2.3.3.3.2 Protease gene

The HIV-1 protease enzyme is responsible for the post-translational processing of the viral *gag* and *gag-pol* polyproteins, and releasing the structural proteins and enzymes necessary for the assembly of infectious viral particles. The enzyme is an aspartic protease composed of two non-covalently associated, structurally identical monomers of 99 amino acids in length. The active site of the enzyme is formed at the dimer interface and contains two catalytic aspartic acid residues, each of which is contributed by the subunits to form two aspartic acid-threonine-glycine motifs (residues 25 to 27). It has a binding cleft that specifically recognizes and cleaves at least 9 different sequences on viral precursor polyproteins to produce the matrix, capsid,

nucleocapsid, and p6 proteins from the *gag* polyprotein and the PR, RT, and IN proteins from the *gag-pol* polyprotein (Erickson and Burt, 1996). The protease of HIV is a symmetrically assembled homodimer with a central, symmetric, substrate-binding cavity. The active site contains well -defined subsites in which inhibitor or substrate side chains participate in tight binding interactions. Two identical flexible flap regions project over the active site, and also participate in the binding of inhibitors and substrates. Protease inhibitor (PI) resistance mutations have been described in the substrate cleft of the enzyme that impairs the binding between the inhibitor and the mutant PR (Velazquez-Campoy *et al.,* 2003; King *et al.,* 2004; Kagan *et al.,* 2005). The resistance reduces the binding affinity between the inhibitor and the mutant protease enzyme. Other mutations either compensate for the decreased kinetics of enzymes with active site mutations, or cause resistance by altering enzyme catalysis, dimer stability, inhibitor binding kinetics, or active site reshaping through long-range structural perturbations (Erickson *et al*., 1999). For the PR gene to develop resistance, multiple mutations are required in the enzyme flap and other parts of the molecule, and this activity of PI has been referred to as a "genetic barrier" to drug resistance (Condra *et al.,* 1996).

2.3.3.3.3 Integrase (IN)

The HIV-1 IN is a 32 kDa protein composed of 288 amino acids encoded by the 3' end of the HIV *pol* gene. The enzyme is composed of three functional domains: (i) the N-terminal region, which encompasses amino acids 1–50, and is responsible for coordinating zinc binding, (ii) the catalytic core, which encompasses amino acids 51–212, and containing catalytic triad, and (iii) a C-terminal region, which encompasses amino acids 213–288, and is responsible in host DNA binding (Van Maele and Debyser, 2005; Rhee *et al.,* 2008). The IN being a multidomain enzyme, is required for the integration of viral DNA into the host genome. The enzyme is responsible in catalysing the cleavage of the conserved 3' dinucleotide CA (3' processing) and the ligation of the viral 3'-OH ends to the 5'-DNA of host chromosomal DNA (strand transfer) (Rhee *et al.,* 2008). Because of its unique character of the involvement in the integration in the host chromosome, the IN has also become an ideal target for drug design (Hickman *et al.,* 1994; Debyser *et al.,* 2002; Van Maele and Debyser, 2005).

2.3.4 Epidemiology of HIV

2.3.4.1 Transmission of HIV

Since the beginning of the pandemic, three main transmission routes of HIV have been identified:

2.3.4.1.1 Sexual route

The majority of HIV infections are acquired through unprotected sexual relations. Sexual transmission occurs when there is contact between sexual secretions of one partner with the rectal, genital or oral mucosal membranes of another (Royce *et al.,* 1997).

2.3.4.1.2 Blood or blood product route

This transmission route is particularly important for intravenous drug users, haemophiliacs and recipients of blood transfusions (though most transfusions are checked for HIV) and blood products. It is also of concern for persons receiving medical care in regions where there is prevalent substandard hygiene in the use of injection equipment (e.g. reused needles in Third World settings). Health care workers (nurses, laboratory workers, doctors, etc.) are also directly concerned, although more rarely. Also concerned by this route are people who give and receive tattoos, piercing and scarification procedures (Schreiber *et al.,* 1996).

2.3.4.1.3 Mother-to-child transmission (MTCT)

The transmission of the virus from the mother to the child can occur *in utero* during the last weeks of pregnancy and at childbirth. In the absence of treatment, the transmission rate between the mother and child is 25%. However, where treatment is available, combined with the availability of caesarean section, this has been reduced to 1%. Breast feeding also presents a risk of infection for the baby (Coovadia, 2004). The use of physical barriers, such as the latex condom, is widely advocated to reduce the sexual transmission of HIV. Current research is clarifying the relationship between male circumcision and HIV in differing social and cultural contexts. UNAIDS believes that it is premature to recommend male circumcision services as part of HIV prevention programmes, even though male circumcision may lead to a reduction of infection risk in heterosexual men by up to 60% (32%−76%; 95% CI) (Auvert *et al.,*2005). Moreover, South African medical experts are concerned that the repeated use of unsterilised blades in the ritual circumcision of adolescent boys may be spreading HIV (Siegfried *et al.,* 2005; WHO, 2005; Williams *et al.,* 2006).

2.3.4.2 Prevalence of HIV

By 2007, 33.2 million people (30.6–36.1 million), (representing around 1.2% of the global population) were reported to be currently living with HIV/AIDS, with 24.7 million residing in sub-Saharan Africa (UNAIDS, 2007). In 2006, over 4.3 million (3.6–6.6 million) new infections occurred, and of these, more than 90% were in sub-Saharan Africa and South East Asia (UNAIDS 2006). South Africa harbours nearly 5.5 million (i.e. around 18%) people living with HIV/AIDS; the 2006 annual national antenatal survey estimated HIV prevalence of 26.5% among South African pregnant women (UNAIDS, 2006; Department of Health, South Africa, 2006).

The most affected continent at the present time is Africa, with an estimated 25 million persons currently infected with HIV, and more than 3 million with advanced disease in need of treatment. HIV infections have dramatically increased over the past decade, despite the knowledge of the mechanisms of transmission. In Southern Africa, the infection rates were less than 1% of the population in 1990, when large-scale surveillance programmes were initiated, whereas by 2004, infection rates in antenatal screening surveys were well in excess of 30% of persons in the same areas, with epidemics expanding in Asia every year (Piot *et al.,* 2004; Pettifor *et al.,* 2005).

2.3.4.3 HIV genotypes and subtypes

The HIV-1 has been classified into three different groups: a "major" group (or group M) which represents the majority of globally prevalent HIV-1 strains; an "outlier" group (or group O); and a "non-M/non-O) group (or group N) (Simon *et al.,* 1998). High HIV diversity is driven by a high mutation rate and an extensive recombination and evolution. Based on the genetic sequence analyses of the envelope gene of the virus, the group M HIV strains are further classified into nine subtypes and a variety of recombinant forms (Spira *et al.,* 2003). The implications of HIV diversity are important, especially in vaccine development. Another aspect of diversity was the report of individuals being co-infected, or superinfections (i.e. becoming infected with two strains at one time or subsequent infection with a second strain, respectively), that may impact on the progression of the disease (Altfeld *et al.,* 2002; Ramos *et al.,* 2002). Distribution of HIV subtypes vary according to geographic regions, with clade C virus being the most prevalent worldwide, and responsible for the HIV epidemic – especially in the sub -Saharan Africa. Clade B is the most prevalent in the developed Western world. The difference between clades can be up to 35%, and even within a similar clade variation can be 20% among isolates (Korber *et al.,* 2001; Hemelaar *et al.,* 2004).

2.3.5 The life cycle of the HIV

The HIV life cycle consists of six steps: binding/fusion, reverse transcription, integration, transcription, translation, and viral assembly and maturation (Figure 2.11).

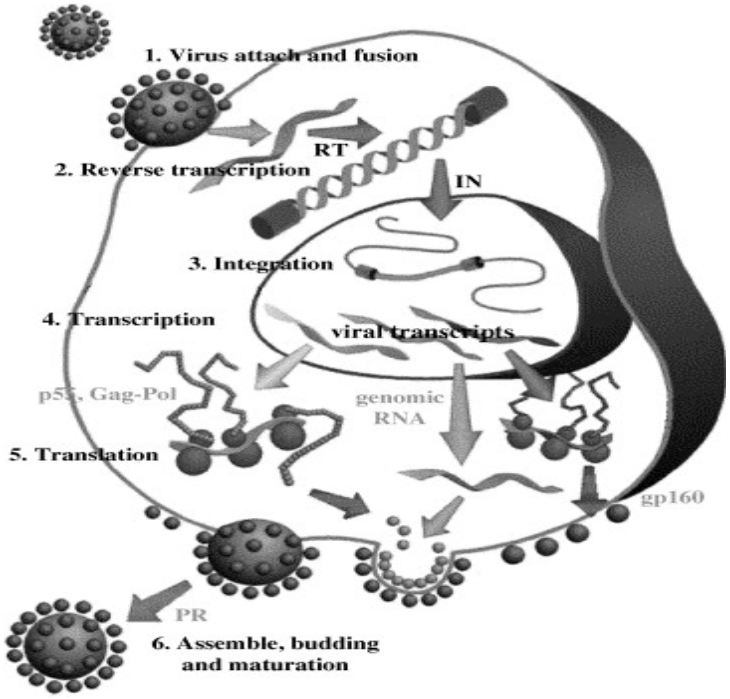

Figure 2.11: Summarised life cycle of HIV (adapted from Sierre *et al.,* 2005)

The main steps are sequentially numbered from 1 to 6: (1) HIV attachment and binding to CD4, appropriate co-receptor, resulting in fusion of the viral envelope and the cellular membrane and the release of viral nucleocapsid into the cytoplasm; (2) HIV RNA reverse transcription by the RT; (3) Integration of double-stranded proviral DNA into cellular DNA by the IN; (4) The transcription of proviral DNA by the cellular RNA polymerase II; (5) The mRNAs are translated by the cellular polysomes; and (6) Viral proteins and genomic RNA are transported to the cellular membrane and assemble. Immature virions are released. Polypeptide precursors are processed by the viral PR to produce mature viral particles (reviewed by Sierre *et al.,* 2005).

2.3.5.1 Entry of HIV into cells

Infection typically begins when an HIV particle, which contains two copies of the HIV RNA, encounters a cell with a surface molecule called cluster designation 4 (CD4). Cells carrying this molecule are known as CD4+ cells (Moore *et al.,* 1993; Weiss, 1993). One or more of the virus's gp120 molecules binds tightly to CD4 molecule(s) on the cell's surface. The binding of HIV gp120 to CD4 results in a conformational change in the gp120 molecule allowing it to bind to a second molecule on the cell surface known as a co-receptor, CCR5 or CXCR4 vivo (Berger *et al.,* 1999; Morris *et al.,* 2001; Clapham and McKnight, 2001). The HIV envelope and the cell

membrane then fuse, leading to entry of the virus into the cell. The gp41 of the envelope is critical to the fusion process. In the early stage of HIV disease, most people harbour viruses that use, in addition to CD4, a receptor called CCR5 to enter their target cells. With disease progression, the spectrum of co-receptor usage expands in approximately 50% of patients to include other receptors, notably a molecule called CXCR4. The evolution of HIV-1 co-receptor use during disease progression has been documented in both adults and children (Scarlatti *et al.,* 1997; Bjorndal *et al.,* 1997). The evolution usually involves change from CCR5 use (R5 phenotype) to CXCR4 use alone (X4) or in combination with CCR5 (R5X4) and/or other minor co-receptors (multitropic viruses). The virus that uses CCR5 is called R5 HIV and the virus that uses CXCR4 is called X4 HIV (Bjorndal *et al.,* 1997; Nabatov *et al.,* 2004). Although CD4+ T cells appear to be the main targets of HIV, other immune system cells with and without CD4 molecules on their surfaces are infected as well. Among these are long-lived cells called monocytes and macrophages, which apparently can harbour large quantities of the virus without being killed, thus acting as reservoirs of HIV. CD4+ T cells also serve as important reservoirs of HIV; a small proportion of these cells harbour HIV in a stable, inactive form. Normal immune processes may activate these cells, resulting in the production of new HIV virions. Cell-to-cell spread of HIV also can occur through the CD4-mediated fusion of an infected cell with an uninfected cell (Dalgleish *et al.,* 1984; Klatzmann *et al.,* 1984; Littman, 1998).

2.3.5.2 Reverse transcription and proviral integration

By definition, retroviruses possess the ability to convert their single-stranded RNA (ssRNA) genomes into dsDNA during the early stages of the infection process (Figure 2.11). The RT enzyme is responsible for catalysing the reverse transcription process together with the RNase H enzyme. The RT possesses three essential activities important for replication of the HIV: RNA dependent DNA polymerase (i.e. reverse transcriptase), RNase H activity (i.e. cleaves the genomic RNA in RNA/ DNA hybrids during DNA synthesis), and DNA-dependent DNA polymerase activity (i.e. for synthesis of the second strand of the proviral DNA) (Coffin, 1996; Joshi and Lamothe, 2000). HIV integration encompasses a series of molecular events that follows the completion of reverse transcription in the cytoplasm of the infected cell, and ends with the initiation of the transcription from the proviral DNA. The IN protein carries out the initial DNA breaking and joining reactions responsible for the attachment of HIV cDNA to host DNA, i.e. 3'-processing and strand transfer. Prior to integration, two nucleotides are removed from each 3'-end in the linear cDNA precursor (terminal cleavage). This reaction may be important to the virus by preparing a defined substrate for subsequent reaction steps because RT often adds

mono template bases to the 3'-ends of unintegrated cDNA. The recessed 3'-ends are then joined to protruding 5'-ends of breaks made in the target DNA (strand transfer). The remaining DNA strands are then attached, probably by the action of host DNA repair enzymes, to complete the formation of an integrated provirus (Garcia and Gaynor, 1994; Sechi *et al.,* 2004). Thus, retrovirus integration requires at least two viral components, the retroviral enzyme integrase, and cis-acting sequences at the retroviral DNA termini U3 and U5 ends of the long terminal repeats. Because of the HIV feature of integrating into the host chromosome, IN has also become an ideal target for drug design (Debyser *et al.,* 2002).

After fusion, the viral core enters the cytoplasm and the viral RNA is copied into double-stranded cDNA. This process is mediated by the viral RT, in a complex consisting of RT, the viral genome, and a cellular $tRNA_3^{lys}$. The latter acts as primer and initiates negative strand DNA synthesis by binding to the primer binding site region, located immediately 3' to the U5 region (Coffin, 1996; Joshi and Lamothe, 2000).

The integrated viral DNA (the provirus) serves as the template for the synthesis of viral RNA and is maintained as part of the host cell genome for the lifetime of the infected cell. Integration of viral DNA is catalysed by IN, a 32-kd protein generated by PR-mediated cleavage of the C-terminal portion of the HIV *gag-pol* polyprotein. The steps in the integration process were originally elucidated in studies using *murine leukemia virus* (Fujiwara and MizuuchiK, 1988; Brown *et al.,* 1989). The pre-integration complex (Farnet and Haseltine, 1991) docks to the nuclear membrane directed by *Vpr* gene (Popov *et al.,* 1998) and enters the nucleus through the nuclear pore (Le Rouzic and Benichou, 2005; Haffar and Bukrinsky, 2005).

The newly made HIV DNA moves to the cell's nucleus, where it is spliced into the host's DNA with the help of HIV IN enzyme. Prior to integration, the viral DNA can be found in the nucleus in three forms: linear, 1-LTR or 2-LTR circles (Wu, 2004). The HIV DNA integrated into cells DNA is called a provirus.

2.3.5.3 Transcription

Once the proviral DNA is integrated, to produce new viruses, RNA copies must be made that can be read by the host cell's transcription factors (Figure 2.11). These copies are called mRNA, and production of mRNA is called transcription, a process that involves the host cell's own enzymes. Viral genes, in concert with the cellular machinery, control this process; the *tat* gene, for example, encodes a protein that accelerates transcription. Genomic RNA is also transcribed for later incorporation in the budding virion (Harrich *et al.,* 1996; Jordan *et al.,* 2001; Harrich and Hooker, 2002).

2.3.5.4 Translation

After HIV mRNA is processed in the cell's nucleus, it is transported to the cytoplasm. HIV proteins are critical to this process; for example, a protein encoded by the *rev* gene allows mRNA encoding HIV structural proteins to be transferred from the nucleus to the cytoplasm. Without the *rev* protein, structural proteins are not made. In the cytoplasm, the virus co-opts the cell's protein-making machinery including structures called ribosomes to make long chains of viral proteins and enzymes, using HIV mRNA as a template (Figure 2.11).

2.3.5.5 Assembly and budding

This is the last step of HIV replication, wherein newly synthesised HIV core proteins, enzymes, and genomic RNA assembled inside the cell. This is followed by the formation of an immature viral particle which buds off from the cell, acquiring an envelope that includes both cellular and HIV proteins from the cell membrane. During this part of the viral life cycle, the core of the virus is immature and the virus is not yet infectious. The long chains of proteins and enzymes that make up the immature viral core are now cut into smaller pieces by a viral enzyme called protease. This step results in infectious viral particles (Gelderblom, 1991; Sierre *et al.,* 2005) (Figure 2.11).

2.3.6 Disease profile

The WHO Disease Staging System for HIV Infection and Disease was first produced in 1990 and updated in September 2005 (WHO, 2005). The clinical staging is used once HIV infection has been confirmed (serological and/or virological evidence of HIV infection). It is an approach for use in resource-limited settings and is widely used in Africa and Asia. Most HIV/AIDS - related conditions are opportunistic infections that can be easily treated in healthy people. The staging system is different for adults, adolescents and children. The following stages are applicable for all people.

Stage I: HIV disease is asymptomatic and not categorised as AIDS.

Stage II: includes minor mucocutaneous manifestations and recurrent upper respiratory tract infections.

Stage III: includes unexplained chronic diarrhoea for longer than a month, severe bacterial infections and pulmonary tuberculosis.

Stage IV: includes toxoplasmosis of the brain, candidiasis of the oesophagus, trachea, bronchi or lungs, and Kaposi's sarcoma; these diseases are used as indicators of AIDS.

2.3.7 Pathogenesis

During HIV infection, CD4+ cells are the primary targeted cells for the establishment of primary or acute infection. The infection with HIV results in a progressive destruction of immune cells and impairment of immune functions by reduction of the CD4 T-lymphocytes subset in peripheral blood and lymphoid organs. The HIV infects a large number of CD4+ cells and replicates rapidly (Ho *et al.,* 1995). During the acute or primary phase of infection, the blood contains many viral particles that spread throughout the body, seeding various organs, particularly the lymphoid organs. Two to 4 weeks after exposure to the virus, up to 70% of HIV-infected people suffer flu-like illness and a rash, an illness termed acute HIV infection syndrome (Levy, 2006). The virus enters target cells via cell surface molecules, including CD4 and chemokines co-receptors (CXCR4, CCR5) (Berger *et al.,* 1999). CD4_ cells, also plays a central role in the immune response, signalling other cells such as the cytotoxic T cell (CD8+ T cells) and the B cells to neutralise replicating HIV (Fahey *et al.,*1990). The CD4+ T cell count may rebound somewhat, and even approach its original level and then remain free of HIV-related symptoms for years, despite continuous replication of HIV in the lymphoid organs that had been seeded during the acute phase of infection, – a stage referred to as the asymptomatic stage (Piatak *et al.,* 1993). During this asymptomatic stage, infection is accompanied by persistent viral replication in lymph nodes and a rapid turnover of plasma virions and CD4+ T lymphocytes, referred to "clinical latency". Upon transmission to a new host, HIV targets CCR5+ CD4+ effector memory T cells, resulting in acute, massive depletion of these cells from mucosal effector sites. Normally, a healthy person has a CD4-count of 800 to 1200 CD4 - T cells per cubic millimeter (mm^3) of blood. As CD4-T cells are destroyed by HIV and as these cells decrease in number, holes develop in the immune repertoire. However, as the CD4-count falls <200 cells/mm^3, the patient becomes particularly vulnerable to the serious opportunistic infections and cancers that typify AIDS, the end stage of HIV disease. AIDS is defined as a CD4-count of <200 cells/mm^3, or the presence of a serious infection, such as *Pneumocystis carinii pneumonia*, toxoplasmosis, cytomegalovirus infections of the eye or intestine, as well as debilitating weight loss, diarrhoea, HIV dementia and cancers, Kaposi's sarcoma and lymphomas (CDC, 1993).

2.3.8 Laboratory diagnosis

Diagnosis assays to detect HIV have improved since the discovery of the virus. The available assays include those for screening blood, diagnosing infection, and monitoring disease progression. Hence, they can be further classified as follows:

a) Screening assays which are designed to detect all infected individuals, detecting either antigen, antibody or both

b) Viral load assays to detect viral nucleic acids to monitor HIV infected patients and

c) Resistance assays to test for resistance to antiretroviral drugs.

2.3.8.1 Combination assays 4th generation

The fourth-generation immunoassay is called "combination assays" due to its ability to simultaneously detect HIV antibodies and HIV-1 p24 antigen. Fourth-generation immunoassays have targeted reduction of the seronegative window period to achieve a continued decrease in the residual risk of transfusion-transmitted HIV infection. This is because antibodies to the HIV are absent in the very early phase (window period) of HIV infection. The combination assays were developed to shorten the diagnostic window period. The diagnostic window period is a gap in which no signs of infection can be detected in an infected individual. The window period between the presence of HIV RNA in plasma and antibody seroconversion varies between 10.2 and 27.4 days, depending on the route of infection. HIV infection was detected between 9.4 and 17.4 days earlier by p24 Ag testing than with third-generation assays (Kleinman *et al.,* 1988). For safe diagnosis, the diagnostic window period should be as short as possible. The development of combination assays fourth-generation assays has permitted the simultaneous detection of HIV antigen and antibody and detected HIV infection on an average of 4.4 days of infections. Because of its simultaneous detection of HIV p24 antigen and HIV antibodies in a single assay, it has given more confidence for screening assays (Weber *et al.,* 1998).

2.3.8.2 Molecular assays

There are various commercial and "in house" assays available for quantitative HIV nucleic acid testing. Most of these assays are based on different technologies, which include: PCR, branched DNA (b-DNA), nucleic acid sequence-based amplification (NASBA), ligase chain reaction (LCR), or quantitative detection of reverse transcriptase activity. The quantitative detection of HIV RNA in plasma has become an important clinical tool, both as a prognostic and therapeutic marker, by being used to monitor and estimate HIV infectiousness (Berger *et al.,* 2001). Currently, most of the quantitative assays (i.e. molecular assays) have sensitivity that can detect as little as approximately 50 copies/ml. An undetectable viral load does not mean the patient is cured of HIV. It should also be noted that no viral load test should be used as a diagnostic tool.

2.3.9 Prevention and control

Currently, the use of latex condoms, sexual education on abstinence and faithfulness are widely advocated to reduce new infections. Male circumcision has also come into focus as another mechanism to reduce transmission of HIV. The UNAIDS believes that it is premature to recommend male circumcision services as part of HIV prevention programmes, even though male circumcision may lead to a reduction of infection risk in heterosexual men by up to 60%. Moreover, South African medical experts have also warned in the repeated use of unsterilised blades in the ritual circumcision of adolescent boys may be spreading HIV (Siegfried *et al.,* 2005; WHO, 2005; Williams *et al.,* 2006).

Mother -to -child transmission has been reduced by the introduction of the dual therapy treatment with nevirapine (NVP) and zidovudine (AZT). When combined with the availability of caesarean section, HIV transmission has been reduced to 1%. Breast-feeding also presents a risk of infection for the baby (Coovadia, 2004).

2.3.10 Treatment of HIV

Common classes of antiretroviral drugs include: fusion/entry inhibitors, nucleoside, nucleotide (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI), integrase inhibitors and protease inhibitors (PI). Because of the HIV polymerase being essential for viral replication, it has been one of the most popular antiviral and newer drugs' design targets.

2.3.10.1 Reverse Transcriptase inhibitors

The discovery of HIV reverse transcriptase inhibitors was the first critical part of treating and managing HIV -infected individuals advancing to AIDS (Panel Antiretroviral Guidelines for Adult and Adolescents, 2008). Reverse transcriptase inhibitors were the first anti-HIV drugs, and are still a critical part of treating patients who have HIV infection. Their mode of action is by inhibiting the transcription from viral RNA to DNA. They are divided into two classes, based on their structure and how they inhibit HIV reverse transcriptase enzyme: Nucleoside transcriptase inhibitors and Non-nucleoside reverse transcriptase.

2.3.10.1.1 Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs)

NRTIs have been advocated by WHO, (2004) as an integral part of HAART in the ARV roll-out programmes as HIV first line therapy, especially in developing countries. The WHO, (2004); has advised the developing countries to include NRTIs as an integral part of HIV management due to low cost. Their mode of action is based on arresting the synthesis of viral DNA by reverse transcriptase, with their effectiveness being achieved during phosphorylation by cellular

kinases, once they are converted to triphosphates. Nucleotide analogues only require two instead of three phosphorylation steps, and NRTIs compete with naturally occurring deoxynucleotide triphosphates. They are incorporated by reverse transcriptase into the nascent chain of viral DNA. Because these drugs lack a 3' hydroxyl group, no additional nucleotides can be attached to them, and the synthesis of viral DNA is arrested. This results in chain termination, due to the fact that the analogues lack a 3' hydroxyl group and no further nucleotides can be attached (Gupta and Pillay, 2007). Development of resistance to this class of drugs has been reported to occur by two main mechanisms:

(i) *Impairment of the incorporation of the analogue* into DNA. Examples of this mechanism include mutations such as the M184V, Q151M, L74V and K65R. They reduce the affinity of the RT active site for NRTIs.

(ii) *Removal of the analogue from the prematurely terminated DNA chain*. Classical example of this mutations are collectively called thymidine analogue (TAMs). These are selected by failing regimens that contain thymidine analogues such as AZT or stavudine (d4T).

Interestingly, the presence of other mutations can affect this resistance mechanism. For example, M184V slows the *in vitro* emergence of TAMs and actually reduces their primer unblocking action (Phillips *et al.,* 2005), while the K65R mutation is antagonistic to TAMs on the same genome (Parikh *et al.,* 2006).

2.3.10.1.2 Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

NNRTIs form a very important class of drugs for treatment of HIV infection in the developing world, with durable potency when combined with a dual NRTI backbone as well as low cost resulting from generic manufacturing. Currently licensed NNRTIs (NVP and efavirenz (EFV)) are also part of standard first-line therapy in developed and developing countries (WHO, 2004). NNRTIs have a high affinity for a hydrophobic pocket located in proximity to the substrate binding site, and binding results in arrest of DNA synthesis (Esnouf *et al.,* 1997). HIV- 2 has been shown to be naturally resistant to NNRTIs, owing to the absence of tyrosine at position 188 of RT, which prevents stable binding (Witrouw *et al.,* 1999). A major limitation to the use of NNRTIs, however, is the low genetic barrier to resistance for this class. A single point mutation in the pocket can prevent NNRTI binding and confer resistance. Major NNRTI-associated mutations leading to high-level resistance include K103N, Y181C, Y188C/L/H, V106A/M, G180A/S and A98G (Shih *et al.,* 1991; Boyer *et al.,* 1993; Witrouw *et al.,* 1999; Hsoiu *et al.,* 2001; Hirsch *et al.,* 2003).

Another important role for NNRTI is the HIV prevention of mother-to-child transmission (PMTCT) program. Hence the concern for a possibility of the PMTCT program being compromised by the rapid of resistance emergence to NNRTIs from monotherapy (Havlir *et al.,* 1996; Jackson *et al.*, 2000). This is evidenced by the suggestion that the use of single-dose NVP can result in detectable NVP resistance and can potentially compromise the efficacy of future NVP-containing HAART. However, the incidence of NNRTI resistance has been reduced by co-administering a short course of AZT/3TC as part of PMTCT (Mackie *et al.,* 2004; McIntyre *et al.,* 2005).

Current research has shown a promising next-generation of NNRTI such as etravirine (TMC-125). This next-generation NNRTI has shown to retain a good activity of up to 24 weeks in experienced patients with two or less major NNRTI mutations (Vingerhoets *et al.,* 2006).

2.3.10.2 Protease inhibitors (PIs)

Due to lack of funding, PIs are limited available in developing countries. This has resulted in developing countries adopting the WHO public health ART strategy of using the class as second line therapy (WHO, 2004). HIV protease is responsible in cleaving large polyprotein precursors. These include the *gag-pol* that encodes the viral enzymes PR, RT and IN. Cleavage by protease enzyme results in formation of mature viral particles, while the dysfunction of the enzyme renders them non-infectious. Most PIs have been designed based on knowledge of natural substrates and their structure which is a homodimeric structure in the substrate-binding cleft and active site (Erickson-Viitanen *et al.,* 1989; Kumar *et al.,* 1996).

Resistance to protease inhibitors is the result of amino acid substitutions that emerge either inside the substrate-binding domain of the enzyme or at distant sites (Kaplan *et al.,* 1994). For resistance associated with PIs to develop, it requires multiple mutations to accumulate. Some of these 'secondary mutations' are actually polymorphisms found in wild-type viruses that alone do not appreciably reduce susceptibility, but along with so-called "primary mutations" can produce higher levels of resistance. Primary mutations include D30N, M46I/L, G48V, I50V/L, V82A/F/T, I84V and L90M. Secondary mutations include L10F/I/R/V, K20I/M/R, L24I, V32I, L33I/F, M36 I/L/V, I54L/M/V, L63P A71V/T and V77I (Hicks *et al.,* 2006; Baxter *et al.,* 2006; Lazzarin *et al.,* 2006; Johnson *et al.,* 2006).

The arrival of the new formulation of combined lopinavir/ritonavir that does not require refrigeration is set to make this the boosted PI of choice in tropical countries, as long as cost

permits. However, developing countries are reserving PIs as second-line agents (RSA HIV treatment Guideline, 2003; Benhamou, 2007).

2.3.10.3 Fusion/Entry inhibitors

HIV enters the target cells through an intricate sequence of interactions between the HIV envelope glycoprotein (gp) complex (gp120–gp41) and specific cell-surface receptors, the CD4 receptor and the chemokines co-receptors, CCR5 or CXCR4 (Kilby and Eron, 2003). Fusion inhibitors are relatively new class of compounds and are also referred to as viral entry inhibitors. Enfurvitide (T-20), a 36-amino acid peptide, is the only licensed fusion inhibitor that mimics HR2 and binds to HR1, thereby preventing the conformational change and thus membrane fusion. Loss of efficacy is generally accompanied by the appearance of mutations at the T-20 binding site, which is the HR1 region of gp41. In particular, mutations at positions 36–45 emerge, most frequently with substitutions at positions 36, 38, 40, 42, 43 and 45 (e.g. G36D/E/S, 38A/M/E, Q40H/K/P/R/T, N42T/D/S, N43D/K or L45M/L). This relatively low genetic barrier to resistance suggests that enfurvitide should be used only in combination with at least one other active drug (Lu *et al.,* 2006).

2.3.11 HIV treatment in developing countries

The World Health Organization (WHO) and the Joint United Nations Programme on HIV/AIDS (UNAIDS) launched the '3 by 5 Initiative' in December 2003 as a simplified and strategic plan to increase access to HAART regimens to 3 million people by the end of 2005 (WHO, 2005). The WHO plan recommended regimens consisting of lamivudine (3TC) with stavudine (d4T) or AZT, and either NVP or EFV (WHO, 2004). As a result of the availability of a simple fixed dose combination, the d4T/3TC/NVP regimen has become widely used. However, limited data is available regarding the use of this regimen in resource-limited settings.

The HIV treatment in developed countries is routinely accompanied by regular monitoring of virological, immunological and opportunistic infections (i.e. viral load, CD4, viral hepatitis, ALT and resistance testing). The main aim of regular monitoring is to optimise the virological response to subsequent therapies (Kantor *et al.,* 2004). By contrast, implementation of intensive and expensive virological monitoring is currently not feasible in resource-limited settings. Instead, developing countries such as South Africa are using the WHO public health approach to ART delivery, which aims to maximise survival gains from three drug classes. This approach includes standard first-line therapy consisting of two NRTIs plus a NNRTI or three NRTI simplification regimens (to reduce drug interactions and NNRTI-associated toxicity), and

second-line therapy (boosted PI) with at least one NRTI, with treatment switch guided by clinical disease progression. One major consequence of this strategy is the likely emergence of high-level resistance during first-line therapy, since most people will stay on a virologically failing regimen for longer periods. Any such resistance emerging on first-line therapy may compromise the efficacy of second-line therapy (Gupta and Pillay, 2007).

2.4 HBV and HIV co-infections

Up to 90% of patients with HIV have serological markers of past or active HBV infection (Treitinger *et al.,* 2000; Rogers *et al.,* 2000; Lodenyo *et al.,* 2000; Saillour *et al.,* 1996; Shao *et al.,* 1993). This is due to similarities in epidemiology and mode of transmission of HBV and HIV infection, respectively. While HBV does not appear to influence the rate of HIV progression in co-infected patients, HIV affects HBV viral replication and clearance, leading to higher serum HBV DNA levels, lower rate of serum HBeAg and serum DNA decreased liver injury (and hence significantly lower ALT levels), and an increased loss anti-HBs (Horvath and Raffanti, 1994; Mastroianni *et al.,* 1998; Sinicco *et al.,* 1997; Taylor *et al.,* 1988; Waite *et al.,* 1988). Additionally, HIV immunosuppression may be associated with reactivation of HBV infection in persons who have lost detectable HBsAg, or HBeAg, or developing AIDS (Horvath and Raffanti, 1994; Mastroianni *et al*.,1998; Waite *et al*., 1988), or re-infection in patients who have lost protective anti-HBs or are progressing to AIDS (Horvath and Raffanti, 1994).

Frequent detection of HBV DNA was also reported in HIV co-infected patients with “occult” hepatitis B, in comparison with HIV-positive patients with “seropositive” hepatitis B, implying that in HIV and HBV co-infected patients, HIV results in poor detection of serological markers of HBV infection, despite active replication of the virus (Mphahlele *et al.,* 2006).

2.4.1 Comparisons of HIV and HBV

Both viruses share similar modes of transmission, hence, co-infection is very common (Burnett *et al.,* 2005). While HBV is a DNA virus; its replication is through an RNA-replicative intermediate requiring an active viral reverse transcriptase/polymerase enzyme, an unusual feature similar to retroviruses like HIV reverse transcriptase. The reverse transcriptase enzyme lacks a conventional proofreading function, 3’ to 5’–exonuclease activity for proof-reading, and therefore, has a high misincorporation rate. Because of this lack of proof-reading activity, the HBV has a similar error rate to HIV (Ganem and Schneider, 2001; Hollinger and Liang 2001). The potential for variation in HBV is limited by the compact genome organization (Locarnini, 1998). HBV exhibits a mutation rate more than 10-fold higher than other DNA viruses, and more closely resembles the replication characteristics of RNA viruses like HIV (Ganem and

Schneider, 2001; Jain *et al.,* 2007). This leads to a high mutation rate and constant production of new viral variants, even in the absence of antiviral treatment. The rate at which nucleotide substitutions develop varies at different stages of infection. The natural evolutionary rate for the HBV genome in chronic hepatitis B is approximately 1.4 - 3.2 x 10^{-5} substitutions per site per year, which is approximately the same as retroviruses (10^{-5}) but 10^{4} times higher than DNA genomes (Okamoto *et al.,* 1987; Girones and Miller,1989; Mimms, 1995; Ganem and Price, 2004). The high mutations lead to complex mixtures of genetic variants, also known as "quasispecies," which result from this high-level, low-fidelity replication, and circulate in various reservoirs specific to each virus. Under the selective pressure of antiviral therapy that does not profoundly suppress viral replication; drug-resistant strains are selected for as the dominant species, with the potential loss of virological suppression. Both viruses have rapid development of drug-resistant viral variants during suboptimal therapy, and it's because of both the high rate of replication and turnover of virus (HIV produces ~ 10 billion new viral particles per day while HSV produces at least 10 times that number in each infected individual) (Perelson *et al.,* 1996), and the high error rate of the HIV reverse transcriptase and HBV polymerase enzymes. As a result of similar polymerase enzyme, HBV and HIV share a number of antiviral drugs, and hence the development of similar antiviral resistance patterns during antiviral therapy (Iser and Sasadeusez, 2008).

The HBV polymerase is a multifunctional protein that contains a number of regions designated domains A–G, and is homologous to other RNA -dependent polymerases. Within these domains, four functional regions are observed: a priming region, a spacer region of unknown function, a catalytic region that functions as a RNA-dependent RNA polymerase/DNA polymerase, and a carboxyl terminal region that has ribonuclease H activity. The reverse transcriptase is situated in the middle of domains A-E (Seeger and Mason, 2000; Kamiya *et al.,* 2003). Although the crystal structure of HBV polymerase is unknown, much of its structure has been deduced from HIV RT based on their homology (Kohlstaedt *et al.,* 1992; Huang *et al.,* 1993; Jacobo-Molina *et al,* 1993). Alignment of HBV and HIV viral genome sequences, and comparison with the polymerase gene, showed significant homology and predicted the presence of an RNase H domain at the carboxyterminus of the polymerase, and a reverse transcriptase domain including the much conserved catalytic YMDD motif (Bartholomeusz *et al., 2004*). Additionally, the HBV polymerase has an amino-terminal extension, which is unique among all known reverse transcriptases and includes a spacer domain dispensable for enzymatic activity and the terminal protein domain harbouring the primer for reverse

transcription (Bartenschlager and Schaller, 1988; Xiong and Eickbush, 1990; Zoulim *et al.,* 1994).

In HBV, the antiviral resistance mutations also provide valuable information on the structure of the HBV polymerase. The published HBV RT antiviral-resistant mutations are located in similar or identical positions to HIV RT antiviral-resistant mutations when the amino acid sequences are aligned (Delaney *et al.,* 2001). The interactions between lamivudine triphosphate and HBV polymerase aided in the prediction of three dimensional structure and homologies with the HIV RT. This also showed that the mutations M204V and M204I are found in the C domain of the viral polymerase. These mutations were also implicated in preventing the correct interaction of the HBV RT with lamivudine triphosphate by steric hindrance (Lee *et al.,* 2001). Regardless of their amino acid sequences and differences in domain structure, both polymerases have been observed to appear to have a common right-handed configuration with a thumb, a palm, and a fingers domain (Steitz; 1999; Doublie *et al,* 1999). The palm domain appears to be the active site and catalyses the phosphoryl transfer reaction; the fingers domain facilitates interactions with the incoming dNTPs as well as the template base to which it is paired; and the thumb domain may play a role in positioning the duplex DNA, processivity, and translocation. Nucleic Acids and dNTPs bind at a site that is located in the palm subdomain adjacent to the 3' terminus of the primer strand (Tantillo *et al,* 1994). An interesting property of the HBV polymerase seems to be its preference for nucleotides with the L-configuration, in contrast to other polymerases that prefer nucleotides with the D-configuration (Davis *et al,* 1996). The catalytic region can be subdivided into 7 domains: A–G (Kohlstaedt *et al,* 1992). Domain A of HIV RT is in close proximity to the 2 aspartic acid residues in domain C and forms part of the dNTP binding pocket (Kohlstaedt *et al.,* 1992; Jacobo-Molina *et al.,* 1993). Residues in this domain are involved in the coordination of the incoming triphosphate moiety of the dNTP and the magnesium ions. Domain B for HBV RT forms an α-helix with a loop region and is involved with positioning of the primer-template strand to the catalytic region. Domain C contains a sequence of 4 amino acids, YMDD, which is highly conserved among viral polymerases/reverse transcriptases that binds 2 magnesium ions and represents the active site of the enzyme (Beese *et al.,* 1991; Jacobo-Molina *et al.,* 1993; Poch *et al.,* 1998).

2.4.2 Clinical aspects of HIV and HBV co-infection

There is overwhelming evidence that HIV co-infection impacts very negatively in the modification of the natural history of HBV infections (Gatanaga *et al*., 1997; Bodsworth et al., 1991; Hadler *et al*., 1991; Gilson *et al*., 1997; Colin *et al*., 1999; Mphahlele *et al*., 2006).

However, there hasn't been any convincing evidence that showed HBV to impact the course of HIV disease (Horvath and Raffanti, 1994; Colin *et al*., 1999; Chung and Kinm, 2001). More recently, a retrospective study on the effect of HBV on the course of HIV infection in 458 HIV-positive patients revealed that HIV viral loads were lower over time in HBsAg -positive patients, and there was no effect on the progression of HIV to AIDS or death. The finding leads to speculation that the transmission of HIV from co-infected patients may be decreased due to lower levels of replication, but there is no evidence to support this. The data was even adjusted for confounding factors (use of HAART, baseline viral loads, CD4 cell counts, age, race, and risk factors for transmission of HIV), but did not demonstrate any effect on progression of HIV to AIDS (Benhamou, 2007). Other studies have also suggested HBV protein (HBx) being responsible in superinducing ongoing HIV replication and HIV long-term repeated transcription by synergising with tat-protein and T-cell activation signals. These findings indicate that HBx could promote faster progression to AIDS in HBV/HIV-co-infected individuals. Studies from the pre-HAART era did not demonstrate a significant impact of HBV carriage on HIV disease progression (Law *et al.,* 2004; Konopnicki *et al.,* 2005).

While HBV does not seem to influence HIV disease progression, there is overwhelming evidence that HIV impacts very negatively on the HBV natural infections. This includes: (i) Increase in progression to HBV chronic carriage; (ii) Reduced persistence of anti-HBs and anti-HBc; (iii) Increased HBV infectivity (iv) Increased transmission of HBV; and (v) Impact on liver disease and (vi) occult hepatitis B infections diagnosis (Gatanaga *et al*., 1997; Bodsworth et al., 1991; Hadler *et al*., 1991; Gilson *et al*., 1997; Colin *et al*., 1999; Mphahlele *et al*., 2006). Studies have shown that HIV/AIDS individuals co-infected with HBV are less likely to clear acute HBV infection spontaneously, resulting to chronic infection. The prevalence of chronicity has also been shown to be up to 25% (Gatanaga *et al*., 1997) compared with 3–5% in HIV seronegative, homosexual men. Higher risk of liver-related death has been reported, as compared to those mono-infected with either virus (Richards *et al*., 1983; Hyams, 1995; Thio *et al*., 2002).

HIV infection leads to impairment of the immune system, resulting in HBV reactivation despite seroconversion to anti-HBs antibody, due to lower CD4 count (Bodsworth *et al.,* 1991). The HBV DNA levels have been shown to be substantially higher than in HIV-seronegative individuals, and rates of seroconversion from HBeAg to anti-HBe are lower (Bodsworth *et al.,* 1991; Hadler *et al.,* 1991; Gilson *et al.,* 1997; Colin *et al.,* 1999).

2.4.3 Management of HBV and HIV co-infected patients

While therapy for HIV infections have well-developed treatment guidelines, HBV/HIV co-infected patients treatment guidelines have been recently developed and they still show conflicting data regarding HBV treatment, e.g. time for initiation and choice of HBV treatment. The principles of treatment of HBV/HIV co-infections are similar to that in HBV mono-infected patients, based on viral load, biochemical abnormalities and severity of liver pathology (Albert *et al*., 2005; Keeffe *et al*., 2007; WHO, 2006), hence, the difficulties in deciding when to initiate therapy, the choice of therapy, and which virus to initiate therapy for, in patients co-infected with both HBV and HIV. The goals of treatment and treatment plan for HBV/HIV co-infections should therefore reflect the needs of individual patients and also depend on the clinical status for both HIV and HBV, and whether they will be treated concurrently. At least three options in treatment of HBV/HIV co-infected patients are possible:

a. Patients who require anti-HIV therapy

b. Patients who require anti-HBV therapy, and

c. Patients who require treatment for both infections.

2.4.3.1 Patients who require anti-HIV therapy

There is conflicting data regarding recommendations about the choice of regimen during initiation of HAART in HBV/HIV co-infected patients who requires anti-HIV therapy only (Benhamou, 2006; Benhamou, 2007). The important consideration for this criterion of patients is to reserve drugs with double activity against both viruses. Patients with persistent controlled HBV replication (serum HBV DNA $<10^4$ copies/mL) may not need drugs with dual activity. However, they should be monitored for ALT and serum HBV DNA every 3 or 4 months. Some patients with HBV/HIV co-infection may have a high serum HBV DNA ($>10^{4–5}$ copies/mL) and no, or mild, liver disease, and they recommended as for patients with indications for both viruses in order to prevent hepatitis of immune reconstitution (Benhamou, 2006).

Current HIV treatment guidelines recommend that in the absence of an AIDS-defining illness, ART should be started in patients with blood CD4 cell counts in the range 200–350 cells per μL (South Africa National Department of Health, 2003; Gazzard, 2008). The decision to initiate HAART is also based on several considerations and other clinical evaluation. The main consideration is the need for HAART, and if it is needed in drug-naive patients presenting with low CD4 cell counts. Other factors include if the patient is co-infected with other opportunistic infections such as HBV and *Mycobacterium tuberculosis*. The inclusion of anti-HIV drugs with further anti-HBV activity (such as lamivudine, emtricitabine or tenofovir) seems to be the best choice for patients co-infected with HBV. If anti-HBV therapy does not begin at the same time

as HAART, delay its introduction until HIV replication is controlled or there is evidence of liver disease. Specifically, monitor HBV DNA for the anti-HBV treatment thresholds (Benhamou, 2006; Benhamou, 2007).

2.4.3.1.1 Initiation of HIV HAART regimen

The most common initial regimens consist of two nucleoside analogs, combined with either a (possibly boosted) PI, an NNRTI or a third nucleoside analog. The Panel on Clinical Practices for Treatment of HIV Infection, convened by the Department of Health and Human Services, USA, produced a consensus guideline for the initial regimen (Table 2.2). However, these guidelines are not always followed, especially by the developing countries, due to financial constraints (Table 2.3). The WHO also recommended an initial HAART regimen for developing countries with limited resources, such as South Africa (WHO, 2003).

Table 2.2: Antiretroviral regimens recommended for treatment of HIV infection in ARV's naïve patients from Department of Health and Human Services USA (Source: www.aidsinfo.nih.gov)

Preferred Regimens	*Regimens*
NNRTI - based	Efavirenz + (Lamivudine or ematricibine) + (Zidovudine or Tenofovir)
PI - based	Lopinavir/ritonavir (coformulation) + (lamivudine or Emtricitabine) + zidovudine
Alternative regimens	
NNRTI- based	Efavirenz + (lamivudine or emtricitabine) + (abacavir or didanosine or stavudine)
	Nevirapine + (lamivudine or emtricitabine) + (zidovudine or stavudine or didanosine or abacavir or tenefovir)

Table 2.3: South African recommended adult ART regimens (adopted from South African ARV guidelines and routine monitoring, 2003)

Regimen	*Drugs*	*Monitoring tests*	*Frequency*
1a	stavudine / Lamivudine / Efavirenz	■ CD4 cells ■ Viral Load ■ ALT	■ Staging, 6-monthly ■ Baseline, 6-monthly ■ Symptomatic
1b	stavudine / Lamivudine / Nevirapine	■ CD4 cells ■ Viral Load ■ ALT	■ Staging, 6-monthly ■ Baseline, 6-monthly ■ Baseline, week 2, 4 and 8, thereafter 6-monthly
2	zidovudine / didanosine / lopinavir / ritonavir	■ CD4 cells ■ FBC ■ Fasting cholesterol and triglyceride ■ Fasting glucose	■ Staging,6-monthly ■ Baseline, then monthly for 3 months, then 6 monthly (with CD4 and viral load) ■ Baseline,6 months and thereafter every 12months ■ Baseline and 12 months

Key: FBC = Full blood count; ALT = Alanine transaminase

2.4.3.1.2 Treatment monitoring of HIV disease progression

Plasma HIV RNA levels are also measured immediately before, and again at 2–8 weeks after, initiation of antiretroviral therapy. This measurement allows the clinician to evaluate initial therapy effectiveness, because for the majority of patients, adherence to a regimen of potent antiretroviral agents should result in a substantial decrease (~1.0 log10) in viral load by 2–8 weeks. Patients' viral load mostly continues to decline during the following weeks and, for the majority of patients, should decrease below detectable levels by 16–24 weeks. Rates of viral load decline below the limit of detection are affected by the baseline CD4 T cell count, the initial viral load, potency of the regimen, adherence to the regimen and previous exposure to antiretroviral agents. These differences must be considered when monitoring the effect of therapy. After the patient is initiated on therapy, HIV RNA testing should be repeated every 3–4 months to evaluate the continuing effectiveness of therapy. With optimal therapy, viral levels in plasma at 24 weeks should be below the limit of detection (South Africa National Department of Health, 2003; Kantor *et al.,* 2004; WHO, 2005).

2.4.3.1.3 Virological and immunological treatment success

Virological treatment success is usually understood as a viral load decrease to below the level of detection of 50 copies/ml. On HAART, viral load declines in two phases: an initial, very rapid decrease in the first weeks followed by a slower phase, in which plasma viraemia is reduced

only slowly. A decrease to below the level of detection should be reached by 3-4 months; in cases of very high baseline viral load this may take 4 or 5 months. A viral load above the level of detection after 6 months of treatment is generally seen as failure. The same is true if a rebound in viral load is confirmed by a second determination after a short interval. In such cases, improvements in therapy (e.g. compliance, change in the regimen) should be considered. Promptly immunological treatment success is generally defined as an increase in the CD4 cell count. As with the decrease in viral load, the increase in CD4 count occurs in two phases. After a first, usually rapid increase over the first three to four months, further increases are considerably less pronounced (South Africa National Department of Health, 2003; WHO, 2005; Hoffmann *et al.,* 2007).

2.4.3.1.4 Change of HAART regimens

Antiretroviral therapy can be changed, due to a number of circumstances which may be beneficial to the patient. The changes can be due to drug failure, poor tolerance of the regimen, toxicity and inability to adhere to the regimen (South Africa National Department of Health, 2003). Drug failure may be associated with resistance; hence, resistance testing is an important tool for identifying the correct drug combination and the failing regimen. Patients' adherence to treatment should also be assessed prior to making any changes, especially if there is no evidence of drug-associated resistance strains (South Africa National Department of Health, 2003; Kantor *et al.,* 2004; Hoffmann *et al.,* 2007).

2.4.3.2 Patients who require anti-HBV therapy

The aim of antiviral treatment for chronic hepatitis B is to reduce serum HBV DNA to low or undetectable levels and prevent progression to cirrhosis, liver failure and HCC. HBsAg seroconversion is the ultimate, but elusive, goal of chronic hepatitis B therapy. A more achievable objective is to halt the progression of chronic hepatitis B-associated liver disease (Hoofnagle *et al.,* 2003; Liaw *et al.,* 2004). In view of the suppressive, rather than curative, nature of most chronic hepatitis B therapy, treatment is usually prolonged and may need to be continued indefinitely to maintain benefit through persistent HBV suppression. Treatment is most beneficial and efficacious for those in the immunoactive phase. Patient characteristics that favour treatment success are low HBV DNA levels, HBeAg positivity, or evidence of hepatic inflammation noted either on liver biopsy or by liver enzyme elevations (Soriano *et al.,* 2005). Patients with chronic HBV infection and normal ALT levels, who are immune tolerant or are inactive carriers, are not candidates for therapy. By contrast, patients with elevated ALT levels,

high HBV DNA levels and necroinflammation on liver biopsy, are candidates for treatment of chronic hepatitis B (Lok and McMahon, 2004; Keeffe and Marcellin, 2007).

Therapeutic management of HBV in the co-infected patient, for whom HAART is not required, is problematic. The goals of HBV treatment in the HBV/HIV co-infected patient not only include HBV suppression, but also the prevention of HBV and HIV reverse-transcriptase resistance mutations associated with drug resistance. The HBV treatment guidelines for HBV in HIV co-infections recommend the use of HBV mono-infections treatment guidelines. However, patients who do not require HAART for HIV therapy should not receive therapy for HBV infection that also has activity against HIV (i.e. lamivudine, tenefovir, emtricitabine); early HIV resistance is likely, with consequent limitations of HIV therapeutic options. The HBV-DNA threshold associated with progressive HBV-related liver disease is unknown, but HBV treatment guidelines in HIV-negative individuals have recommended that treatment must be considered for HBeAg-positive patients with detectable serum HBV-DNA levels above 10^5 copies/ml, whereas lower HBV DNA thresholds should be considered for HBeAg-negative patients and those with decompensated cirrhosis (thresholds of 10^4 and 10^3 copies/ml, respectively) (Keeffe *et al*., 2004). Under these circumstances it is reasonable to propose in HBeAg positive patients: pegylated interferon alpha 2a, adefovir or entecavir. Newer drugs that have no anti-HIV activity (telbivudine, clevudine) may also be useful in this situation. The duration and dose for pegylated interferon remains unknown during treatment for HBV/HIV co-infected patients. However, the objective should be HBeAg seroconversion. In HBeAg negative patients there is currently not enough data to propose pegylated interferon, because the chance to achieve HBsAg seroconversion and to maintain HBV DNA suppression off therapy is low. More data is needed to ensure that adefovir and entecavir do not select resistance mutations in HIV patients (Benhamou, 2006; Benhamou, 2007; Hirsch, 2007; McMahon *et al,* 2007). Some experts have cautioned against the use of adefovir alone at a dose of 10 mg, since there is a theoretical risk of HIV resistance, but resistance has not been shown *in vivo*. Entecavir has not been evaluated in patients with HIV and HBV co-infection who are not receiving effective treatment for HIV at the same time. There is one reported case of resistance to HIV in a patient who was receiving entecavir without antiretroviral therapy (Hirsch, 2007; McMahon *et al,* 2007).

2.4.3.2.1 Drugs with anti-HBV activity only

2.4.3.2.1.1 Adefovir dipivoxil

Adefovir dipivoxil is a guanosine analogue with a flexible acyclic linker instead of the unnatural L-nucleoside ring of lamivudine. Development of resistance to adefovir is slower compared with lamivudine, with the reported rate being 2% at 2 years and 29% at 5 years (Yang *et al.,* 2002; Hadziyannis *et al.,* 2006). The US FDA approved adefovir dipivoxil in 2002 for the treatment of chronic hepatitis B on the basis of the results of pivotal trials showing favourable safety and efficacy over 1 year of treatment with 10 mg daily vs placebo in patients with HBeAg-positive and HBeAg-negative disease (Hadziyannis, 2007). Several studies have confirmed the long-term safety and efficacy of adefovir over 4-5 years of treatment. Hadziyannis and colleagues (Hadziyannis *et al.,* 2005) investigated the virological, biochemical, and histological efficacy of adefovir over 4 to 5 years of therapy, determined safety and tolerability, and assessed the rate of HBV antiviral resistance. The results showed increasing improvement in hepatic fibrosis, with 55% in the 4-year cohort and 71% in the 5-year cohort demonstrating a ≥ 1-point improvement in the Ishak fibrosis score. The majority of patients normalised ALT levels and suppressed serum HBV DNA < 1000 copies/mL, and 5% lost HBsAg. There were only a few renal adverse events, with 3% having increased serum creatinine ≥ 0.5 mg/dL (maximum increase of 0.8 mg/dL, and maximum value of 1.5 mg/dL).

2.4.3.2.1.2 Entecavir

The US FDA approved entecavir in March 2005 for the treatment of chronic HBV infection on the basis of the results of pivotal trials showing favourable safety and superior efficacy of 0.5 mg daily, compared with lamivudine 100 mg/daily over 1 year of treatment in patients with HBeAg-positive and HBeAg-negative chronic hepatitis B (Opio *et al.,* 2005; Lai *et al.,* 2006). Entacavir has been shown to interfere at three different steps of HBV replication: priming, synthesis of minus strand by reverse transcription and synthesis of plus strand. It has been shown to have modest activity against herpes simplex virus and minimal activity against HIV, and therefore requires caution when treating HBV/HIV co-infected patients. Treatment with entecavir vs lamivudine resulted in a significantly greater rate of histological improvement, reduction in serum HBV DNA, HBV DNA undetectability <300 copies/mL, and ALT normalisation in both patient populations. Although entecavir is the most potent licensed oral agent in terms of effect on serum HBV DNA, there was no difference in HBeAg loss (22% vs 20%) or seroconversion (21% vs 18%) between entecavir and lamivudine after 1 year of therapy in patients with HBeAg-positive chronic hepatitis B. In both of the entecavir studies of nucleoside-naive patients, no

evidence of entecavir resistance was noted over 48 weeks of treatment (Gish *et al.,* 2005; Rivkin, 2007).

The emergence of resistance has been observed only in a proportion of patients pre-treated with lamivudine, with no resistance observed in nucleoside-naive patients treated with entecavir. It is known that lamivudine-resistance substitutions (M204V/I with/without L180M) reduce entecavir susceptibility 8-fold, and virological rebound due to entecavir resistance requires pre-existing lamivudine substitutions plus additional changes at the reverse transcriptase residues T184, S202, and/or M250 that can be selected by lamivudine (Colonno *et al.,* 2005).

2.4.3.3 Patients who require treatment for both infections

The interactions of HIV, HBV, and HAART complicate disease course and management of patients with HBV and HIV co-infections. The treatment end points for HBV infection in HIV infected individuals are the suppression of viral replication (the absence of HBV DNA or HBeAg in serum) and improvement in liver disease. However, more research is needed to define the optimal strategy for the management of HBV infection in patients with HIV infection. Until recently, guidelines for anti-HBV therapy have been recommended by the first European Consensus Conference on the Treatment of HBV in HIV co-infected patients (Alberti *et al.,* 2005; Thio and Locarnini, 2007). Five different parameters are generally used to assess HBV-related liver damage and to monitor treatment response: serum HBV-DNA, HBeAg, HBsAg, ALT and liver histology. The optimal time to initiate anti-HBV treatment in HIV co-infected patients is not well established. However, based on available evidence, the consensus conference advised using the HBV DNA criteria applied to HBV mono-infected patients. The initiation of treatment for HBV depends on HBeAg serostatus and serum HBV DNA. In HBeAg-positive patients, a serum HBV DNA level >20,000 IU/mL (10^5copies/ml), justifies consideration of anti-HBV therapy. In HBeAg-negative patients, the cut-off is a serum HBV DNA level >2000 IU/mL (Alberti *et al.,* 2005), and also ALT levels should be considered. If the results of liver function tests are out of proportion to HBV DNA levels, it is prudent to investigate other causes of liver disease (Brook *et al.,* 2007). If the patient meets the HBV DNA thresholds for considering anti-HBV therapy, the next step depends on whether they require anti-HIV therapy. The indication for initiation of HAART in HIV infection is based on clinical assessment, CD4 cell count and viral load. These three important factors determine whether therapy should be started, or if it can still be delayed. For HIV patients who do not meet therapy criteria, it is recommended that continued monitoring HIV viral load, CD4 count –and also opportunistic infections, is performed. HIV patients with CD4 cell counts below 200/350 /µl, or WHO Stage IV

disease, irrespective of CD4 count, should be treated with ARV. However, in patients with CD4 counts of less than 350cells/ µl and co-infected with HBV, the use of agents with dual anti-HIV and anti-HBV activity should be considered and will likely decrease the emergence of HBV resistance (Mauss, 2006; Thio and Locarnini, 2007).

The significance of ALT elevation after HAART initiation in HIV/HBV co-infected patients is also complex. It can be associated to 5 different situations: (1) HAART-related hepatotoxicity; (2) HBV resistance to antiretroviral with dual activity included in the HAART regimen; (3) discontinuation of a HAART regimen, including anti-HBV drugs, in a noncompliant patient; (4) super delta virus or HCV infection or other causes; and (5), more frequently, ALT flares in association with the control of HBV replication (Zoulim, 2006). In this latter situation, ALT returns to normal levels in 4 to 12 weeks if anti-HBV drug therapy is maintained and liver lesions improve (Benhamou *et al.,* 2001).

The treatment of both HBV and HIV infections should be coordinated. Similarly, when changing or initiating new antiretroviral regimens, it is important to continue administering agents with anti-HBV activity, since there is a risk of the immune reconstitution syndrome during recovery of CD4 cell. This syndrome may be difficult to distinguish from hepatotoxicity (Liaw *et al.,* 2004; Schmutz *et al.,* 2006).

In summary, early introduction of HAART -containing anti-HBV activity has been shown to be beneficial in co-infected patients who meet the criteria for ART. However, a low CD4 cell counts and high HBV viral loads at initiation of HAART have been associated with an increased risk for immune reconstitution hepatitis, and therefore require regular monitoring (Benhamou, 2007).

2.4.3.3.1 HBV and HIV drugs with dual activity

Availability of agents with dual activity against HIV and HBV facilitates management of co-infected patients. It also mandates careful monitoring of treatment effects on both infections to detect and avoid triggering resistance or relapse (Lok and McMahon, 2004; Keeffe *et al.,* 2006). Lamivudine is the most extensively studied analogue for the treatment of HBV and HIV.

2.4.3.3.1.1 Lamivudine

Lamivudine was the first FDA-approved nucleoside analogue for treatment of chronic HBV infection, and has been widely utilised. It was first approved for the treatment of HIV in the mid-1990s, and approved for treatment of chronic HBV infection in 1998. As a result, lamivudine has been the most affordable drug of choice for treatment of HIV and HBV, even in developing countries (Hoffmann and Thio, 2007). Development of resistance strains against lamivudine

during treatment of HBV has been the most extensively studied drug update (Allen *et al.,* 1998; Leung *et al.,* 2001; Liaw *et al.,* 2004).

2.4.3.3.1.2 Resistance to lamivudine

Lamivudine-resistance mutations occur within domains A, B, and C of the HBV polymerase. The catalytic YMDD motif resides in domain C and is commonly mutated in lamivudine-associated resistance HBV strains (Allen *et al.,* 1998; Leung *et al.,* 2001; Liaw *et al.,* 2004; Selabe *et al.,* 2007). Whereas several patterns of lamivudine resistance exist, nearly all patterns include the mutations M204V/I/S in the YMDD motif of domain C. These are two primary mutations associated with antiviral resistance to lamivudine, M204V and M204I. The M204I mutation is usually associated with the compensatory L180M mutation (Selabe *et al.,* 2007). Other rarer mutations are found at L80V/I, I169T, V173L, A181T, T184S, and Q215S. Lamivudine binds at a pocket in the surface of the polymerase (palm region) formed in part by residue 204. The primary lamivudine mutations M204V/I are predicted to reduce the accessible surface area between the polymerase and lamivudine because the methyl group on the β-branched side chain of valine/isoleucine impinges on the sulphur atom in the unnatural L-oxathiolane ring of lamivudine. These structural alterations have 2 main effects: (1) steric hindrance to decrease binding of lamivudine to the viral polymerase, and (2) reduced catalytic activity to incorporate lamivudine triphosphate into replicating viral DNA (Allen *et al.,* 1998; Lee and Chu, 2001; Das *et al.,* 2001; Lok *et al.,* 2003; Yuan and Lee, 2007).

Hepatitis B virus resistance strains associated with lamivudine have been reported to increase with longer duration of lamivudine treatment, reaching 65% to 70% of all patients by year 5 of therapy (Lok *et al*., 2003). The incidence of resistance is also related to the magnitude of HBV DNA suppression. Studies have reported that only 8% of patients with HBV DNA suppression to ≤200 copies/mL after 24 weeks of therapy developed resistance, compared with 13% of patients with viral load between 200 and 10^3 copies/mL, and increased to 64% for those who continued to have >10^4 copies/mL after 24 weeks of treatment (Yuen *et al.,* 2001).

The most important concern is that common lamivudine-resistance mutations such as M204V and M204I can demonstrate cross-resistance primarily to other nucleoside analogues. This is also seen rarely with other mutations such as A181T and Q215S mutations that have cross-resistance to the acyclic phosphonate nucleotide analogues adefovir and tenofovir, in lamivudine treatment. Thus, even in patients naive towards adefovir and tenofovir, lamivudine

treatment can result in resistance mutations that are cross-resistant to these nucleotide analogues (Locarnini, 2008).

Studies have found that patients who develop lamivudine resistance have lower rates of HBeAg seroconversion than patients without resistance, in spite of ongoing lamivudine therapy (Guan *et al.,* 2001; Lai *et al.,* 2003). This was confirmed by Lai *et al.,* (2003), who followed patients for 4-5 years of continued lamivudine treatment, with 75% to 80% of patients achieving HBeAg seroconversion. A total of 38% of patients who had the YMDD mutation had a significantly lower rate of HBeAg seroconversion.

Also observed, was that patients with lamivudine-resistance HBV strains appeared to have an increase in hepatitis flares with longer duration of resistance and an increase in liver disease–related morbidity and hepatic decompensation after 4 years of continued lamivudine treatment. Hepatic fibrosis tends to increase with duration of lamivudine resistance; however, HBeAg seroconversion can still occur in the presence of resistance, but at a reduced rate compared with that observed in patients who have no resistance (Liaw *et al*., 1999). They also found that patients with lamivudine resistance mutations experienced a significantly higher rate of hepatitis flares during the year following the emergence of resistance, compared with patients without lamivudine resistance ($P < .005$) (Lok *et al*., 2003). This was observed with the study by Dienstag *et al*., (2003); approximately 27% of patients with lamivudine resistance greater than 2 years had progressed to bridging fibrosis. By contrast, 83% of patients without resistance had reduction of bridging fibrosis in the same observation period (Dienstag *et al*., 2003).

2.4.3.3.1.3 Lamivudine mutants before therapy

YMDD mutants have also been detected in some asymptomatic HBV carriers and patients with chronic hepatitis B who have never received lamivudine treatment (Kobayashi *et al*., 2001; Kirishima *et al*., 2002; Heo *et al*., 2004; Ohishi *et al*., 2004; Selabe *et al*., 2007). The first study reported YMDD mutants before lamivudine therapy in Japanese asymptomatic hepatitis B chronic carriers was published by Kobayashi *et al*., (2001). The patients with YMDD mutants were all positive for anti-HBe antibody. Subsequently, these other studies reported that YMDD mutants can emerge before therapy (Kirishima *et al*, 2002; Heo *et al*, 2004; Ohishi *et al*, 2004). However, YMDD mutants in pre-treatment sera were found not to be frequent, being detected in only one of the 20 (5%) patients with chronic hepatitis B (Heo *et al*, 2004). Likewise, YMDD mutants were detected in pre-treatment serum of only one of the 62 (1.6%) anti-HBe patients before lamivudine therapy (Ohishi *et al*, 2004). Viruses, especially RNA viruses and

retroviruses, exist in the hosts as quasispecies. HBV, despite being a DNA virus, replicates via an RNA intermediate and shows high-mutation frequency (Orito *et al*, 1989), partly because of the lack of proof-reading enzymes that assure fidelity of DNA replication. Using a two-stage PCR with peptide nucleic acid (PNA) clamping, Ohishi *et al* (2004) identified rare polymerase variants of hepatitis B virus. Many new variants such as tyrosine-arginine-aspartate-aspartate (YRDD) and tyrosine-methionine-aspartate-asparagine (YMDN), which had stop codons in the overlapping surface gene, were detected in therapy-naïve chronic hepatitis B patients (Ohishi *et al*, 2004).

2.4.3.3.2 Management of HBV/HIV co-infected patients in developing countries

Of the seven agents used for treating chronic hepatitis B in the developing countries, only lamivudine is widely available through most of Africa and Asia. It is being used as part of current ARV regimens (WHO, 2004). Recently, Hoffmann and Thio, (2007), reported the recommendations for the management of HBV and HIV co-infections during HAART use in the regions with limited resources. First, screening of HBsAg and liver enzymes should be performed before starting HAART. Second, routine monitoring of liver enzymes should ideally occur once or twice during the first 3 months of HAART and when CD4 or HIV RNA is assayed. The presence of HBsAg and repeatedly elevated liver enzymes suggest active disease with necroinflammatory activity and the need for anti-HBV therapy. Detection of HBV DNA is also helpful, but this assay is unlikely to be available in resource-limited settings. The presence of HBeAg adds further weight to starting anti-HBV therapy, but this assay also might not be available to many treatment programmes. In high-income countries, HBV-specific agents, such as adefovir dipivoxil and interferon α, are available for use for HBV suppression in patients who have not reached immunological criteria for HAART. These agents are not available in most low- and middle-income countries, leading to the need to consider using lamivudine or tenofovir disoproxil fumarate-containing HAART for management of both chronic hepatitis B and HIV.

While the WHO, (2004), has advocated for an increased distribution of HAART in poor resource countries, there is a need for further research on HAART in HBV/HIV co-infections, initiation time for management of chronic hepatitis B in co-infected individuals before WHO immunological criteria for HAART are met. Another important aspects include long-term efficacy of combination regimens in patients with HBV/HIV co-infection, especially with the current tremendously expansion in the use of HAART in developing countries (Liaw *et al.,* 2004; Schmutz *et al.,* 2006; Hoffmann and Thio, 2007)

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CHAPTER THREE

3 OVERVIEW OF THE STUDY PLAN AND RECRUITMENT OF STUDY POPULATION

3.1 Study area

Patients selected for the study were recruited from Tshepang HIV clinic, located within Dr George Mukhari hospital (DGMH) (formerly Ga-Rankuwa hospital), which is an academic hospital for the University of Limpopo Medunsa Campus. The hospital is located 31 km north-west of Pretoria city centre. The hospital draws its patients mainly from 4 provinces in South Africa: Gauteng, North West, Limpopo and Mpumalanga, and other neighbouring African countries. In addition, there are a number of urban, semi-urban, and rural developments around the DGMH /Medunsa complex. These include Akasia (urban), Ga-Rankuwa, Soshanguve, Mabopane (urban and semi-urban) and many other rural sites. The study targeted patients mainly from all surrounding DGMH areas, Ga-Rankuwa, Soshanguve, Mabopane and Pretoria as a whole.

3.2 Ethical clearances

Ethical clearance was obtained from the Research and Ethics Committee of the University of Limpopo, Medunsa campus, South Africa (Project number: MP 07/2005) and also from DGMH. The study was also permitted by the Department of Internal Medicine, Medunsa campus, as they are responsible for the management of DGMH Tshepang HIV out-patient clinic.

3.3 Introduction of HAART at DGMH

This is one of the national hospitals which have been selected for providing an ARV roll out programme for HIV/AIDS patients when South Africa introduced the national comprehensive HIV and AIDS care management programme to all HIV patients, as well as the HIV PMTCT programme to consenting antenatal women. The treatment programme started in July 2004. The significant part of the programme is to offer HAART to eligible HIV-infected individuals. A significant number of HIV-positive individuals surrounding the hospital areas attend the HIV/AIDS management, care and treatment facility at Tshepang HIV outpatient clinic.

3.4 Establishing and maintaining the study cohort

The study population was initially envisaged to comprise at least 300 adult HIV/AIDS patients receiving HAART at DGMH, and of these, at least 40% (i.e. at least 120 patients) were expected to have been exposed to HBV, based on our previous studies (Burnett *et al.,* 2003).

Of the 120 patients with exposure to HBV, at least 26% (i.e. 78 patients) were chronic carriers of HBV. It was then planned that the 78 patients would form the core experimental group of this study, and would be followed up to study the impact of HIV treatment on HBV as well as other HBV and HIV viral characteristics as indicated in the study objectives. Almost all patients on ARV therapy were requested to participate in the study, as the population of patients on HAART was small at the beginning of the treatment programme. The other reason for recruiting all patients was to minimise the number of patients to be lost during the follow-up period. Ultimately, a total of 192 HIV patients were recruited from over 1000 patients screened for ARV enrolment at DGMH Tshepang clinic. The 192 HIV patients were selected, based on the evidence of either past or current markers of HBV infection, following screening for HBV serological markers, and therefore there was no need to continue recruiting.

3.5 Study population and selection criteria

The study population comprised 192 samples from HIV/AIDS adult patients attending Tshepang HIV outpatient clinic at DGMH (Table 3.1). These patients were selected for this study on the basis that their baseline bleeds and their records for their CD4+ levels, ALT, and HIV viral load were obtained (Table 3.1). The gender and ages of selected patients were indicated. The baseline records for their CD4+ levels, ALT and HIV viral loads were done by different National Health Laboratories Services, (NHLS) at DGM hospital, i.e. Haematology, Chemical Pathology and Virology NHLS laboratories.

Table 3.1: Demographic features of the study population (n = 192)

Characteristics	**Epidemiology data**
Age (yrs)	
Mean	37.131
Range	14 - 68
Gender	
Females	131 (68.2%)
Males	61 (31.8%)
CD4 cell (cells/µL)	
Mean	115.99
Range	2 - 1069
HIV viral load (copies/ml)	
Mean	187409
Range	25 - 1000 000
Alanine Transaminase (ALT) (IU/L)	
Mean	31.781
Range	8 - 304

3.6 Collection of samples

Blood samples were collected between September 2004 and March 2007. To avoid unnecessary bleeding of study participants, the researcher in the Department of Virology collaborated with other departments, such as Haematology and Chemical Pathology, to follow up leftover blood samples of patients from the Tshepang HIV clinic.

3.7 Storage of samples

The blood specimens were kept at 4^{o}C for 1-3 hours to allow for clotting, and then centrifuged. The plasma fraction was removed and stored at -70^{o}C until further tests were performed.

CHAPTER FOUR

4 INVESTIGATING THE BURDEN OF HBV CO-INFECTION IN HIV-POSITIVE PATIENTS ENROLLING FOR HAART AT DR GEORGE MUKHARI HOSPITAL, PRETORIA

4.1 Background

4.1.1 Global burden of HIV and HBV infections

By 2007, 33.2 million people (30.6–36.1 million), (representing around 1.2% of the global population) were reported to be currently living with HIV/AIDS, with 24.7 million of these residing in sub-Saharan Africa (UNAIDS, 2007). In 2006, over 4.3 million (3.6–6.6 million) new infections occurred, and of these, more than 90% were in sub-Saharan Africa and South East Asia (UNAIDS, 2006). South Africa harbours nearly 5.5 million (i.e. around 18%) people living with HIV/AIDS. The 2006 annual national antenatal survey estimated HIV prevalence of 26.5% among South African pregnant women (UNAIDS, 2006; South Africa Department of Health, 2006).

In contrast to HIV infections, the proportion of people living with chronic hepatitis B viral infection worldwide is at least 6 times higher than that of those infected with HIV. About 400 million people worldwide are estimated to be chronic carriers of HBV, representing over 5% of the global population, and 620 000 people die annually from complications of chronic hepatitis B infections. In the setting of HIV co-infections, however, the mortality rate from chronic hepatitis B infections increased beyond that of each viral infection alone (Martin-Carbonero *et al.,* 2001; Thio *et al.,* 2002; Salmon-Ceron *et al.,* 2005; Goldstein *et al.,* 2005).

About 45% of the global population reside in areas of high endemicity (≥ 8% HBV carriage), which includes Asia (with 75% of the world's HBV carriers), sub-Saharan Africa (with the second largest population of carriers) and the Western Pacific. In these endemic areas, the lifetime risk of HBV infection is estimated at greater than 60%, with early childhood infections being the most common. It is estimated that 50 million inhabitants of sub-Saharan Africa are chronic hepatitis B carriers, and of these, 12.5 million will eventually die from liver disease, which translates to between 230 000 and 250 000 deaths from HBV infection per year (Kew, 1996; Burnett *et al.,* 2005; Mphahlele, 2006).

4.1.2 The burden of HBV in HIV-positive patients in sub-Saharan Africa

HIV and HBV infections share major risk factors, and both are endemic in sub-Saharan Africa, warranting high priority efforts in prevention and control (Buve *et al.,* 2002; UNAIDS, 2007; Mphahlele *et al.,* 2002; Burnett *et al.,* 2005). Previous studies from sub-Saharan Africa and developed countries have reported that a number of HIV-infected individuals have either past exposure to, or are, chronic carriers of HBV, due to similar modes of transmission (Scharschmidt *et al,* 1992; Tswana and Moyo, 1992; Kashala *et al.,* 1994; Horvath and Raffanti, 1994; Basaras *et al.,* 1999; Lodenyo *et al.,* 2000; Nacro *et al.,* 2001; Rogers *et al.,* 2000; Herrero-Martínez, 2001).

While hepatitis B is now a vaccine-preventable disease, and the vaccine is almost universally available to all the world's children (Kane and Brooks, 2002), the search for an HIV vaccine remains a challenge. There is limited data on the interaction of HIV and HBV in co-infected persons, and the impact HIV co-infection may have on HBV prevention and control, from sub-Saharan Africa. These remains so, despite overwhelming evidence from around the world that HIV co-infection can impact negatively on the transmission, natural history, prevention and control, and treatment of HBV (reviewed by Burnett *et al.,* 2005; Hoffmann and Thio, 2007).

4.1.3 Impact of HIV in HBV/HIV co-infected patients in sub-Saharan Africa

There is overwhelming evidence that HIV has a significant impact on HBV infection (Cooley and Sasadeusz, 2003; Burnett *et al.,* 2005; Mphahlele *et al.,* 2006; Mphahlele, 2006; Hoffmann and Thio, 2007). The impact of co-infection is especially apparent in regions with widespread use of HAART, since competing risks of mortality from opportunistic infections are diminished. In areas with HAART, liver failure has emerged as a major cause of death in HIV-infected individuals. However, studies from sub-Saharan Africa are limited (Bica *et al.,* 2001; Martin-Carbonero *et al.,* 2001; Thio *et al.,* 2002; Salmon-Ceron *et al.,* 2005; Hoffmann and Thio, 2007; Hoffmann *et al.,* 2007).

The impact of HIV co-infection on HBV prevention and control includes the possibility of: increased prevalence of HBV in HIV-infected persons; increased HBV infectivity and transmission; rapid progression to active chronic hepatitis B and accelerated need for HBV therapy; limitation in the choice of drugs; the need to perform HBV DNA testing in HBsAg negative sera, due to frequent detection of occult hepatitis B in HIV co-infected persons; and finally, the need for administering additional hepatitis B vaccine doses and ascertaining levels of protective anti-HBs following hepatitis B vaccination. Of these factors, increased prevalence, infectivity and transmission of HBV, as well as factors associated with rapid progression of HBV in HIV co-infected patients, have been linked with HIV co-infections (reviewed by Burnett *et al.,*

2005; Hoffmann and Thio, 2007). Suffice it to mention that the impaired immune response brought about by HIV co-infection results in an increased pool of active chronic HBV carriers, due to re-infections, reactivations and new infections. Thus, HIV co-infection may result in an accelerated need for therapy, due to increased morbidity and mortality from chronic hepatitis B infection. More important are the implications of co-infections on chronicity, laboratory diagnosis, implications for therapy and limitation in the choice of therapy, and implications for immunisation (Feld *et al.,* 2005; Burnett *et al.,* 2005; Mphahlele *et al.,* 2006; Mphahlele, 2006; Hoffmann and Thio, 2007). This chapter investigates the burden and diagnostic implications of HBV/HIV co-infections in South African patients being enrolled for the HAART programme.

4.2 Study problem, hypothesis, purpose and objectives

4.2.1 Study problem

Formal investigations into the burden of HBV in HIV-positive patients on ARV therapy do not exist in South Africa; hence, testing of HBV serological markers is not yet part of standard care for HIV patients on ARV in most treatment centres. This study is important, since one of the drugs (lamivudine) included as part of HAART regimens, has a dual activity against HIV and HBV. However, screening for HBV is not standard practice in South Africa, because of financial constraints. For example, lamivudine is widely approved for the South African operational plan for the ARV roll out programme, it is included as part of HAART in 2 of 3 adult regimens and 3 of 5 paediatric regimens (Operational Plan for Comprehensive HIV and AIDS Care, Management and Treatment for South Africa. Pretoria, South Africa, 19 November 2003), and yet there are no studies on the burden of HBV infection on HIV-positive patients in receiving lamivudine-containing HAART regimens in South Africa. HBV infection in individuals with HIV requires detailed assessment and specific attention. Patients must initially be identified as HBV-infected, and in order to achieve this, all HIV-infected patients should be screened prior to initiation of ARV therapy. It is recommended that both HBsAg and anti-HBc assays should be performed in individuals with advanced immunodeficiency, as this can result in a decreased sensitivity of HBV serological assays. This study investigated the burden and diagnostic implications of HBV/HIV co-infections in patients who were candidates for HAART at DGMH, Tshepang HIV outpatient clinic.

4.2.2 Hypothesis

Because of a high background prevalence of HBV in sub-Saharan Africa, patients at an advanced stage of HIV infection may experience HBV re-infection (due to waning immunity) or

HBV reactivation (due to host immunosuppression status), leading to an increased prevalence of active HBV infection in such HIV patients. In addition, HIV may modify the natural course of HBV infection in co-infected patients, or lead to an atypical HBV serological profile, and this will negatively impact on the outcome of laboratory diagnosis of HBV infections.

4.2.3 Purpose

To investigate the burden of HBV in HIV-positive patients who were candidates for HAART at DGMH.

4.2.4 Specific objectives

This study screened for HBV infection markers from baseline bleeds of HIV patients before receiving HAART, and determined the extent of HBV/HIV co-infections. The specific objectives were:

a. To investigate overall baseline exposure or chronic carriage to HBV in HIV-positive patients by screening HBV serological markers (i.e. HBsAg, anti-HBs, anti-HBc)
b. To investigate the extent of "atypical HBV serological profiles" such as occult HBV infections, in HIV-positive patients
c. To investigate the proportion of HIV patients with active HBV infections (i.e. HBV DNA positive).

4.3 Study population and methods

4.3.1 Study population and selection criteria

The study population comprised 192 samples from HIV/AIDS adult patients who were candidates for HAART at DGMH. These patients were selected for this study on the basis that their baseline bleeds were obtained and their CD4+cells levels, ALT and HIV viral load records were available (see Table 3.1 in Chapter Three).

4.3.2 Serological assays

4.3.2.1 HIV serology

HIV serological results were available from the NHLS, Department of Virology, University of Limpopo, Medunsa campus. The results are part of routine diagnosis by an independent team from the NHLS, Virology Diagnostic Laboratory, using Elecsys 2010 (Roche Diagnostics, Penzberg, Germany) and confirmed with Axsym anti-HIV 1/2 assays (Abbott Laboratories, North Chicago, IL, USA).

4.3.2.2 HBV serology

Serological markers (i.e. HBsAg, anti-HBs and anti-HBc) for the first line screening of HBV infection were performed in all 192 HIV/AIDS adult patients samples, using Elecsys 2010 assay (Roche Diagnostics, Penzberg, Germany) following manufactures instructions. The principle of the assays is summarized in the following sections:

4.3.2.2.1 Anti-HBs Elecsys assay

Principle of the assay

It is a sandwich assay with a total duration of 18 minutes. During the first incubation, anti-HBs in the sample (40µL), biotinylated HBsAg, and HBsAg, labelled with a ruthenium complex, react to form a sandwich complex. During the second incubation, the complex becomes bound to the solid phase via interaction of biotin and streptavidin, after addition of streptavidin-coated microparticles. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with Procell. Application of a voltage to the electrode then induces chemiluminescent emission, which is measured by a photomultiplier. Results are determined via a calibration curve, which is instrument-specifically generated by 2-point calibration, and a master curve provided via the reagent barcode.

Interpretation of the results

Samples with concentrations <10IU/L are considered non-reactive in the Elecsys Anti-HBs test. Samples with concentrations >10IU/L are considered reactive in the Elecsys anti-HBs test.

4.3.2.2.2 HBsAg Elecsys assay

Principle of the assay

It is a sandwich assay with a total duration of 18 minutes. For the first incubation, 50µL of sample, a biotinylated monoclonal HBsAg-specific antibody, and a monoclonal HBsAg-specific antibody labelled with a ruthenium complex, form a sandwich complex. During the second incubation, the complex becomes bound to the solid phase via interaction of biotin and streptavidin. After addition of streptavidin-coated microparticles, the reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with Procell. Application of a voltage to the electrode then induces chemiluminescent emission, which is measured by a photomultiplier. The Elecsys software determines results automatically, by comparing the

electrochemiluminescence signal obtained from the reaction product of the sample with the signal of the cut-off value previously obtained by HBsAg calibration.

Interpretation of the results

Samples with a cut-off index <1.0 are non-reactive, while samples with a cut-off index ≥1.0 are reactive, in the Elecsys HBsAg test.

4.3.2.2.3 Anti-HBc Elecsys assay

Principle of the assay

It is a competition assay of a total duration of 27 minutes. The assay begins with pre-treatment of 40µL of the sample with reducing agent. After addition of HBcAg, a complex is formed with anti-HBc antibodies in the sample. After addition of biotinylated antibodies and ruthenium complex labelled antibodies specific for HBcAg, together with streptavidin-coated microparticles, the still-free binding sites on the HBc-antigens become occupied. The entire complex becomes bound to the solid phase via the interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with Procell. Application of a voltage to the electrode then induces chemiluminescent emission, which is measured by a photomultiplier. The Elecsys software determines results automatically, by comparing the electrochemiluminescence signal obtained from the reaction product of the sample with the signal of the cut-off value previously obtained by anti-HBc calibration.

Interpretation of the results:

Samples with a cut-off index >1.0 are non-reactive in the Elecsys anti-HBc test.

Samples with a cut-off index ≤1.0 are reactive in the Elecsys Anti-HBc test.

4.3.3 HBV molecular techniques

4.3.3.1 Determination of HBV viral load

This was performed using COBAS TaqMan HBV 48 assay (Roche Diagnostics, Penzberg, Germany).

4.3.3.1.1 Principles of the procedure

The COBAS TaqMan HBV 48 assay is based on two processes: (1) manual specimen preparation to obtain viral DNA; and (2) automated PCR amplification of target DNA using HBV-specific complementary primers, and detection of cleaved dual fluorescent dye-labelled oligonucleotide detection probes that permit quantitation of HBV target amplified product (amplicon) and HBV Quantitation Standard DNA, which is processed, amplified, and detected simultaneously with the specimen. The Master Mix reagent contains primer pairs and probes specific for both HBV DNA and HBV Quantitation Standard DNA. The Master Mix has been developed to ensure equivalent quantitation of HBV genotypes A through G. The detection of amplified DNA is performed using target-specific and Quantitation Standard-specific dual labelled oligonucleotide probes that permit independent identification of HBV amplicon and HBV Quantitation Standard amplicons. The quantitation of HBV viral DNA is performed using the HBV Quantitation Standard. The HBV Quantitation Standard is non-infectious linearised plasmid that contains the identical primer binding sites as the HBV DNA target, and a unique probe binding region that allows HBV Quantitation Standard is incorporated into each individual specimen and control at a known copy number and is carried through the specimen preparation, PCR amplification and detection steps, along with the HBV target. The COBAS TaqMan 48 Analyzer calculates the HBV DNA titre in the test specimen by comparing the HBV signal to the HBV Quantitation Standard signal for each specimen and control. The HBV Quantitation Standard compensates for effects of inhibition and controls for the preparation and amplification to allow the accurate quantitation of HBV DNA in each specimen.

4.3.3.1.2 Specimen preparation

The COBAS TaqMan HBV 48 assay processes plasma and serum specimens and isolates viral DNA through a generic manual specimen preparation based on nucleic acid binding to glass fibres. The HBV particles were lysed by incubation at an elevated temperature with protease and chaotropic lyses/binding buffer that released nucleic acids and protected the released HBV DNA from DNases in plasma and serum. A known number of Quantitation Standard DNA molecules were introduced into each specimen along with the lyses reagent. Subsequently, isopropanol was added to the lyses mixture that was then centrifuged through a column with a glass fibre filter. Unbound substances, such as salts, proteins and other cellular

impurities, were removed by centrifugation. The adsorbed nucleic acids were washed and eluted with an aqueous solution. The disposables were allowed for a parallel processing of 12 specimens or multiple thereof. The processed specimen, containing HBV DNA and HBV Quantitation Standard DNA, was added to the amplification/detection mixture. The HBV target DNA and the HBV Quantitation Standard DNA were then amplified and detected on the COBAS TaqMan 48 Analyzer using the amplification and detection reagents provided in the Test kit.

4.3.3.1.3 PCR amplification

Target selection

Selection of the target DNA sequence for HBV depended on identification of regions within the HBV genome that showed maximum sequence conservation among all genotypes. Accordingly, the appropriate selection of the primers and probe is critical to the ability of the test to detect all clinically relevant genotypes of HBV. A region of the partly single-stranded circular DNA genome of HBV has been shown to have maximum conservation of DNA sequences among genotypes. The COBAS TaqMan HBV Test uses PCR amplification primers that define a sequence within the highly conserved pre-Core/Core region of the HBV genome.

Target amplification

Processed specimens were added to the amplification mixture in amplification tubes (K-tubes) in which PCR amplification occurred. The analyzer heated the reaction mixture to denature the double stranded DNA and expose the specific primer target sequences on the HBV circular DNA genome and the HBV Quantitation Standard DNA. As the mixture cooled, the primers annealed to the target DNA. The thermostable Thermus specie Z05 DNA polymerase (Z05) in the presence of Mn2+ and excess deoxyuridine (in the place of thymidine), extended the annealed primers along the target template to produce a double-stranded DNA molecule termed an amplicon. The analyzer automatically repeated this process for a designated number of cycles, with each cycle intended to double the amount of amplicon DNA. The required number of cycles was pre-programmed into the analyzer. Amplification occurred only in the region of the HBV genome between the primers; the entire HBV genome was not amplified.

Selective amplification

Selective amplification of target nucleic acid from the specimen was achieved in the COBAS TaqMan HBV Test by the use of AmpErase (uracil-N-glycosylase) enzyme and deoxyurudine triphosphate (dUTP). The AmpErase enzyme recognised and catalysed the destruction of DNA strands containing deoxyuridine, but not DNA containing deoxythymidine. Deoxyuridine is not present in naturally occurring DNA, but was always present in amplicon due to the use of deoxyuridine. Deoxyuridine was not present in naturally occurring DNA, but was always present in amplicon due to the use of deoxyuridine triphosphate as one of the dNTPs in the Master Mix reagent; therefore, only amplicon contains deoxyuridine. Deoxyuridine rendered contaminating amplicon susceptible to destruction by the AmpErase enzyme prior to amplification of the target DNA. Also, any non-specific product formed after initial activation of the Master Mix by manganese was destroyed by the AmpErase enzyme, thus improving sensitivity and specificity. The AmpErase enzyme, which was included in the Master Mix reagent, catalysed the cleavage of deoxyuridine-containing DNA at the deoxyuridine residues by opening the deoxyribose chain at the C1-position. When heated in the first thermal cycling step, the amplicon DNA chain broke at the position of the deoxyuridine, thereby rendering the DNA nonamplifiable. The AmpErase enzyme was inactive at temperatures above 550C, i.e. throughout the thermal cycling steps, and therefore did not destroy target amplicon formed during amplification.

4.3.3.1.4 Detection of PCR products

The COBAS TaqMan 48 HBV assay utilises real-time PCR technology. The use of dual labelled fluorescent probes provided for real-time detection of PCR product accumulation, by monitoring of the emission intensity of fluorescent reporter dyes released during the amplification process. The probes consist of HBV and HBV Quantitation Standard-specific oligonucleotides labelled with a reporter dye and a quencher dye. The HBV and HBV Quantitation Standard probes were labelled with different fluorescent reporter dyes. When the dual fluorescent dye-labelled probes were intact, the reporter fluorescence was suppressed by the proximity of the quencher dye due to Forster-type energy transfer effects. During PCR, the probe hybridised to a target sequence and was cleaved by the 5'-3' nuclease activity of the thermostable Z05 DNA polymerase. Once the reporter and the quencher were released and separated, quenching no longer occurred, and the fluorescent activity of the reporter dye was increased. The amplification of HBV DNA and HBV Quantitation Standard DNA were measured independently at different wavelengths. This process was repeated for a designated

number of cycles, each cycle effectively increasing the emission intensity of the individual reporter dyes, permitting independent identification of HBV DNA and HBV Quantitation Standard DNA. The intensity of the signals was related to the amount of starting material at the beginning of the PCR.

4.3.3.1.5 Interpretation of results

The results were reported as follows:

The HBV DNA titre is expressed in International Units (IU)/mL. The conversion factor between HBV copies/ml and HBV IU/ml is 5.82 copies/UI using the WHO HBV International Standard for NAT testing 97/746 (Roche diagnostics).

< 6.00E+00 IU/mL (<3.5 x 10 copies/ml) - Calculated IU/mL are below the range of the assay. Report results as"HBV DNA detected, less than 6.00 HBV DNA IU/mL".

> 1.10E+08 IU/mL or (>6.4 x 10^8 copies/ml) - Calculated IU/mL are above the range of the assay. Report results as “greater than 1.10E+08 HBV DNA IU/mL".

Target Not Detected – Cut off value for HBV below the limit for the assay or no cut off value for HBV obtained. Report results as "HBV DNA not detected".

4.3.3.2 HBV PCR assay

4.3.3.2.1 Extraction of HBV DNA

Total viral nucleic acid was extracted from 200μl serum using the High Pure Viral Nucleic Acid assay (Roche Diagnostics, Penzberg, Germany). Extractions were performed following the manufacturer’s instructions. In summary, 200μl of serum was aliquoted into 1.5 ml Eppendorf tubes, followed by addition of 200μl working solution (binding buffer supplemented with poly [A] carrier RNA) and 50μl proteinase K (dissolved in distilled water). Tubes were then mixed thoroughly and immediately incubated for 10 minutes at 72^0C for viral lyses to occur. After the incubation period, 100μl of isopropanol was added into the tubes and briefly mixed. The contents of the tubes were transferred into the High Pure filter tubes, which were attached to the collection tubes. This was followed by centrifugation for 1 minute at 8000xg.

The flow-through was discarded and the tubes were washed twice with 450μl wash buffer (10ml wash buffer [20 mM NaCl and 2 mM Tris-HCl, pH 7.5] mixed with 20 ml ethanol), and centrifuged for one minute at 8000xg. The final wash was centrifuged at 12000xg for 30 seconds to remove residual wash buffer. During washing, DNA bound to the matrix of the filter tubes while protein and other non-nucleic materials were washed away. The filter tubes were then combined with clean nuclease-free 1.5ml reaction tubes and 50μl elution buffer

(nuclease-free distilled water) was added to resuspend DNA from the matrix. The tubes were then centrifuged for 1 minute at 8000xg. The recovered nucleic acid was either used immediately for the detection of HBV DNA, or stored at –70^{o}C for later analysis within 2 months. A known positive and a negative control were always included in each set of 10 extractions.

4.3.3.2.2 HBV nested PCR assay

The amplification of HBV DNA was performed by a nested polymerase chain reaction that used two sets of primers flanking the YMDD motif of the HBV polymerase (Gutreund *et al.,* 2000; Selabe *et al,* 2007). The first round primers: P1, forward 5'-GTC TGC GGC GTT TTA TCA-3' (nucleotide positions 381-398 according to Eco RI restriction site), and P2, reverse 5'-GGA GTT CCG CAG TATGGA TCG G-3' (positions 1282-1261) amplified a 902 base pair (bp) product, and second round primers : P3, forward 5'-GGT ATG TTG CCC GTT TGT CC-3' (positions 458-477) and P4, reverse 5'-GGC GAG AAA GTG AAA GCC T-3' (positions 1103–1085) amplified a 646 bp product (Gutfreund *et al.,* 2000; Selabe *et al.,* 2007). Briefly, 5µl of extracted viral DNA was amplified in 50µl reaction mixtures containing the following: 10 X PCR buffer containing MgCl2 (Invitrogen, Carlsbad, CA), 10 pmol/µl of forward and reverse primers, 10mM dNTPs (Invitrogen), 5 U/µl Taq DNA polymerase (Invitrogen), and sterile distilled water. The PCR conditions were as follows: initial denaturation at 94^{0}C for 4 min, followed by 30 cycles each of denaturation at 94^{0}C for 2 min, annealing at 50^{0}C for 2 min, and extension at 72^{0}C for 2 min, with a final extension step at 72^{0}C for 7 min (Touchdown PCR thermocycler, Hybaid, Middlesex, UK). Positive and negative controls were included in all extractions and amplification reactions, and necessary precautions were undertaken to avoid contamination as previously reported (Mphahlele *et al.,* 2006; Selabe *et al.,* 2007).

4.3.3.2.3 Sensitivity test for the PCR assay

To ensure that the in-house PCR assay was sensitive enough to detect relatively low levels of HBV DNA, serial dilutions of a control specimen with a known HBV viral load (40,000 copies/ml) were made. These were followed by doubling dilutions from the point at which DNA was last detectable, until a dilution of 50 copies/ml was achieved. These dilutions were extracted, and PCR was performed. Further, the primers used in this study have been widely applied (Gutreund *et al,* 2000; Selabe *et al.,* 2007), and their sensitivity has been evaluated in our laboratory using HBV-positive specimen.

4.3.3.2.4 Detection and confirmation of the PCR products

Five microlitres of the nested PCR products mixed with 1µ of Blue/Orange 6× loading dye containing bromophenol blue, xylene cyanol, and orange G (Promega, USA) were run on a 2% ethidium bromide stained agarose gel along with a 100 bp DNA ladder (Promega, USA). Expected bands of 700 bp were observed with UV transillumination. Selected P-gene PCR products were directly sequenced using SpectruMedix SCE 2410 Genetic Analysis System (Spectru-Medix LLC, State College, PA).

4.3.3.2.5 Avoiding PCR contamination.

To avoid false positive PCR results that might be due to contamination or carry-over of amplified DNA sequences, the following measures were taken during the experimental procedures: Working areas were cleaned with 70% ethanol before and after each PCR experiment. Gloves and laboratory coats, strictly for the PCR preparations, were always worn during the PCR preparation procedures, and gloves were changed between the procedures. Nucleic acid extractions and PCR reactions were prepared at separate working stations. Separate sets of filtered tips and pippeting devices strictly for extraction and PCR preparations, were used. Tips and tubes were autoclaved before use. The pipettes were cleaned with 70% ethanol every time before and after they were used. Positive controls were diluted, so as to reduce the introduction of amplifiable molecules into the preparation area. In every PCR experiment, a master-mix that contained all the PCR reagents except the template (HBV DNA) was prepared and aliquoted into 0.5ml tubes. Extracted viral DNA was added last. This was done to minimise pippetting errors, the number of sample transfers and chances for sporadic contamination. Distilled water was aliquoted, and leftovers of the aliquots were discarded after every use.

4.4 Results

4.4.1 Total exposure to HBV in HIV patients

HBV serology was performed in 192 HIV-positive patients, and the total HBV exposure was 63.0% (121/192). HBV total exposure was defined as when a patient was positive for either one or more HBV serological markers. From the 192 patients, HBV DNA was detected in a total of 78 (40.6%), using in-house qualitative PCR assay, and from these, 69/78 (88.5%) were reactive for HBV DNA with a quantitative Cobas Taqman HBV assay (Table 4.1). This study shows a high exposure to HBV, as well as high active HBV infections in this cohort (Table 4.1).

Table 4.1: Overall distributions of HBV serology and HBV DNA positivity (N =192)

HBV serological profiles	Serology N = 192	Prevalence of HBV DNA in Various serological profiles			Distribution of HBV DNA between HBsAg-positives and HBsAg-negatives			
					HBsAg-positives (N = 44)		HBsAg-negatives (N = 148)	
		N	In-house PCR assay n (%)	Cobas Taqman HBV assay n (%)	In-house PCR assay n (%)	Cobas Taqman HBV assay n (%)	In-house PCR assay n (%)	Cobas Taqman HBV assay n (%)
All HBsAg +	44 (22.9)	44	44 (100)	41(93.2)	44 (100)	41 (93.2)	NA	NA
Isolated HBsAg +	31 (16.2)	31	31 (16.2)	28 (14.6)	31 (70.5)	28 (63.6)	NA	NA
Anti-HBs+; HBsAg+ only	1 (0.5)	1	1 (100)	1 (100)	1 (2.3)	1 (2.3)	NA	NA
All HBsAg -	148 (77.1)	148	34 (23.0)	28 (19,0)	NA	NA	34 (23.0)	28 (19.0)
All anti-HBs +	43 (22.4)	43	16 (37.2)	12 (27.9)	3 (7.0)	3 (7.0)	13 (8.8)	9 (6.1)
Isolated anti-HBs+	8 (3.1)	8	3(37.5)	3(37.5)	NA	NA	3 (2.0)	1 (0.7)
All anti-HBc +	73 (38.0)	73	33 (45.2)	31 (42.5)	12 (27.3)	12(27.3)	21 (14.2)	18 (12.2)
Isolated anti-HBc+	29 (15.1)	29	11 (37.9)	11 (37.9)	NA	NA	11 (7.4)	11 (7.4)
Anti-HBc+; HBsAg+	10 (5.2)	10	10 (100)	10 (100)	10 (23.0)	10 (23.0)	NA	NA
All anti-HBs -	149 (77.6)	149	62 (41.6)	57 (38.3)	41 (93.2)	38 (86.4)	21(14.2)	19(12.8)
Anti-HBs -; anti-HBc +	41 (21.4)	41	22 (53.7)	21 (51.2)	10 (22.7)	10 (22.7)	11 (7.4)	11 (7.4)
All anti-HBs+;anti-HBc+	31 (16.2)	31	12 (38.7)	10 (32.3)	2 (5.0)	2 (5.0)	10 (7.0)	8 (5.4)
anti-HBs+;anti-HBc+;HBsAg-	29 (15.1)	29	10 (34.5)	8 (27.6)	NA	NA	10 (7.0)	8 (5.4)
anti-HBs+;anti-HBc+;HBsAg+	2 (1.0)	2	2 (100)	2 (100)	2 (5.0)	2 (5.0)	NA	NA
anti-HBs-; anti-HBc-; HBsAg-	71 (37.1)	71	10 (14.1)	8 (12.7)	NA	NA	10 (6.7)	8 (5.4)
Total exposure (any of three markers +)	121 (63.0)	192	78 (40.6)					

Key: (+) designates positive and (-) designates negative; NA = for not applicable; N = Sample size

A total of 22.9% of patients (44/192) were HBsAg positive at baseline. HBsAg is the main serological marker for diagnosis of HBV infection, and indicates either an active or chronic state. A total of 56.8% (25/44) of these patients had a positive isolated HBsAg marker only. The HBsAg positive patients were confirmed for HBV infection by quantitating HBV DNA with Cobas Taqman HBV assay and qualitative in-house nested HBV PCR assays. Cobas Taqman assay detected 41(93.2%) of HBsAg-positives with detectable HBV DNA, while in-house nested HBV PCR assay detected HBV DNA in 100% (44/44) of the patients (Table 4.1). Cobas Taqman assay detected HBV DNA in 92% (23/25) of patients positive for HBsAg marker only.

Anti-HBs is an important serological marker for indicating protective immunity against HBV. The study detected 22.4% (43/192) of patients with anti-HBs marker. The majority of them were exposed naturally to HBV because of concurrent anti-HBc marker. This was detected in 72.1% (31/43) of patients with anti-HBs and anti-HBc only. However, a total of 3.1% (8/192) of patients had anti-HBs marker only. The phenomenon of anti-HBs marker as the only detectable marker is often associated with individuals who develop protective immunity against HBV due to HBV vaccination. However, the vaccination status of these patients was not established. Despite immunity (anti-HBs), 27.9% (12/43) of these patients had detectable HBV DNA with Cobas Taqman assay and 37.2% (16/43) with in-house HBV nested PCR (Table 4.1). This was also observed in 37.5% (3/8) of patients with anti-HBs immunity marker only, but having detectable HBV DNA with Cobas Taqman assay and also in-house HBV nested PCR assays (Table 4.1).

Anti-HBc is a serological marker for HBV natural exposure. It only shows that a patient was exposed naturally, and the patient may either be a chronic carrier or immune. A significant percentage of patients [38% (73/192)], had anti-HBc serological marker, and of these, 39.7% (29/73) had an isolated anti-HBc only, with another 39.7% (29/73) of patients being positive for “anti-HBc and anti-HBs only”. A total of 42.5% (31/73) of patients with anti-HBc had detectable HBV DNA with Cobas Taqman HBV assay, and 45.2% (33/73) with in-house HBV nested PCR assays. An interesting phenomenon was the detection of HBV DNA with Cobas Taqman HBV assay [(27.6%) 8/29] and 34.5% (10/29) with in-house HBV nested PCR in patients positive for “anti-HBs and anti-HBc only” (Table 4.1). This was an interesting occurrence due to anti-HBs, an immunity marker against HBV. However, the patients may have been at the stage of resolving natural HBV infection.

Two patients [2/192 (1.0%)] tested positive for all three HBV markers (i.e. co-existence of HBsAg, anti-HBs and anti-HBc). The patients had detectable HBV DNA with both Cobas Taqman HBV assay and in-house HBV nested PCR assay (Table 4.1). This could be attributable to early recovery from acute phase, or re-activation of latent HBV infection due to severe immunosuppression caused by HIV.

Finally, 37.1% (71/192) of patients were negative for all three HBV markers; however, 8/71 (12.7%) of these patients had detectable HBV DNA with Cobas Taqman HBV assay, and 10/71 (14.1%) with in-house HBV nested PCR assay (Table 4.1).

4.4.2 HBsAg negative patients

Although HBsAg is the main serological marker for diagnosis of HBV infection, as it indicates either an active or chronic state, this study detected 77% (148/192) of patients negative for HBsAg (Table 4.1). It was interesting to observe that 23.0% (34/148) of HBsAg negatives had detectable HBV DNA with in-house PCR assay, denoting occult HBV infection, and of these, 82.4% (28/34) had quantifiable HBV DNA with Cobas Taqman HBV assay (Table 4.1).

4.4.3 HBV atypical serology

A total of 23% (34/148) of patients with atypical serology had detectable HBV DNA with in-house HBV PCR assay, with 82.4% (28/34) of these having quantifiable HBV DNA with quantitative Cobas Taqman HBV assay (Table 4.1). As described from the literature review, these cases are referred to as occult HBV infection or "sero-silent" HBV infections, due to the detection of HBV DNA in the absence of or undetectable HBsAg (Mphahlele *et al.,* 2006). Different atypical serological patterns were observed, and HBV DNA was confirmed with Cobas Taqman HBV assay and in-house HBV nested PCR assay.

4.4.3.1 "Isolated" total anti-HBc serological pattern

Isolated total anti-HBc (IgM or IgG or both) was detected in 15.1% (29/192) of the study population. Of these, 37.9% (11/29) were HBV DNA positive, making up 32.4% (11/34) of occult hepatitis B infections. In all 32.4% cases, HBV DNA was confirmed with both in-house PCR and Cobas Taqman assays. Anti-HBc is a marker of natural exposure against HBV, but it appears that detection of HBV DNA in HIV-positive patients with anti-HBc serological marker is frequent.

4.4.3.2 "Isolated" anti-HBs serological pattern

While the vaccination status of the cohort was not established, yet, isolated anti-HBs was found in 3.1% (8/192) of the study population. Of these, 37.5% (3/8) were HBV DNA positive, making up 8.8% (3/34) of occult hepatitis B infections. While all three were detected by the in-house PCR assay, only one specimen had detectable HBV DNA with Cobas Taqman HBV assay (Table 4.1).

4.4.3.3 Both "anti-HBs and anti-HBc positive" serological profile

A total of 16.2% (31/192) of the study population was reactive for both anti-HBs and anti-HBc, meaning natural infections or exposure. Of these, 32.3% (10/31) were HBV DNA positive, making up 29.4% (10/34) of occult HBV infections. All but two patients were positive for HBV DNA with both assays (Table 4.1). Usually, this serological profile indicates past HBV infection. However, HIV-infected patients are more prone to lose anti-HBs immunity at a greater frequency than HIV-negative persons.

4.4.3.4 Negative for HBsAg, anti-HBs and anti-HBc serological markers

A total of 37.0% (71/192) sera were negative for all three HBV serological markers. Of these, 14.1% (10/71) were HBV DNA positive, making up 29.4% (10/34) of occult HBV infections. All but two patients were positive for HBV DNA with both assays (Table 4.1). This clearly shows that some HIV patients have active HBV infection without detectable HBV serology, and present diagnostic challenges – particularly in the developing world.

4.4.4 HBV viral loads quantitation

Assessment of HBV viral load should be performed in patients with at least a positive HBsAg marker to determine HBV replicative status. It is also important especially when patients are initiating HAART, for the correct choice of antiviral drugs. All 78 patients had detectable HBV DNA with qualitative PCR assay at baseline, and were also quantified for HBV viral load with COBAS TaqMan HBV assay (Roche diagnostics) to assess HBV replicative status. Quantitation of HBV viral load by COBAS TaqMan HBV assay demonstrated high HBV DNA viral loads in a significant number of patients (Table 4.2). Quantitation of HBV DNA levels is significant, since it is a predictive marker for initiation and response to HBV treatment. A high significant percentage [88.5% (69/78)] of patients had a detectable HBV DNA ranging between <10 and $\leq 10^8$ IU/mL. Interestingly, was the detection of patients who were highly infectious, as shown by HBV baseline viral load ranging between $10^4 \geq 10^8$IU/mL. A total of (14/78) 18% of patients had an overall HBV viral loads levels ranging between $10^4 \geq 10^8$IU/mL (Table 4.2).

Although the majority of patients had detectable HBV vireamia, a total of (9/78) 11.5% of patients had an undetectable HBV baseline viral load despite no previous exposure against any anti-HBV drugs (Table 4.2). The patients without detectable HBV DNA levels were observed in both HBsAg positive, (3/9) 33.3%, and HBsAg negative (6/9) 66.7%. They also had other additional serological marker, either anti-HBc, anti-HBs or negative for all tested HBV markers (Table 4.2).

Table 4.2: Distribution of HBV viral loads between HBsAg-positives and HBsAg-negatives

Serological profiles	**Number tested**	**Cobas Taqman HBV DNA quantitations**				
		Undetectable n (%)	**<10 n (%)**	**10 ≤ 200 n (%)**	**200 ≤ 10^4 n (%)**	**10^4 ≥ 10^8 n (%)**
All HBsAg +	**44 (100)**	**3(6.8)**	**2 (4.6)**	**8 (18.2)**	**19 (43.2)**	**12 (27.2)**
Anti-HBs+; HBsAg+ only	1 (2.3)	0	1 (2.3)	0	*0*	0
Anti-HBc+; HBsAg+ only	10 (22.7)	0	0	1 (2.3)	3 (6.8)	6 (13.6)
anti-HBs+; anti-HBc+; HBsAg+	2 (4.6)	0	0	0	1 (2.3)	1 (2.3)
Isolated HBsAg +	31 (70.5)	3 (6.8)	1 (2.3)	7 (15.9)	15 (34.1)	5 (11.4)
All HBsAg -	**34 (100)**	**6 (17.7)**	**5 (14.7)**	**12 (35.2)**	**9 (26.5)**	**2 (5.9)**
Isolated anti-HBs+	3 (8.8)	2 (5.9)	0	1 (2.9)	0	0
Isolated anti-HBc+	11 (32.4)	0	1 (2.9)	6 (17.7)	2 (5.9)	2 (5.9)
All anti-HBc+	21 (61.8)	2 (5.9)	2 (5.9)	8 (23.5)	7(20.6)	2 (5.9)
All anti-HBs +	13 (38.2)	4(11.8)	1 (2.9)	3 (8.8)	5 (14.7)	0
anti-HBs+;anti-HBc+	10 (29.4)	2 (5.9)	1 (2.9)	2 (5.9)	5 (14.7)	0
anti-HBs-; anti-HBc-; HBsAg-	10 (29.4)	2 (5.9)	3 (8.8)	3 (8.8)	2 (5.9)	0
Overall HBV viral loads	**78(100)**	**9 (11.5)**	**7 (9.0)**	**20 (25.6)**	**28 (35.9)**	**14 (18.0)**
All anti-HBs +	16 (20.5)	4 (5.1)	2 (2.6)	3 (3.9)	6 (7.7)	1 (1.3)
All anti-HBc +	33 (42.3)	2 (2.6)	2(2.6)	9 (11.5)	11 (14.1)	9 (11.5)
All anti-HBs -	62 (79.5)	5 (6.4)	4 (5.1)	18 (23.1)	22 (28.2)	13(16.7)

Key: (+) designates positive and (-) designates negative

4.4.5 Associations between HBV Baseline viral loads and HBsAg status in PCR-positive specimens

There was a statistically significant positive association between HBsAg-positivity and high viral loads (i.e. 10^4 to ≥ 10^8 IU/mL) (RR: 4.64; 95% confidence interval [CI]: 1.11, 19.35; chi-square p-value [p]: 0.015), with 27.2% (12/44) of PCR-positive HBsAg positives having high viral loads, compared to 5.9% (2/34) PCR-positive HBsAg negatives. The statistical significance of the association strengthened considerably when dropping the cut-off to 200 IU/mL (i.e. 10^2 to ≥ 10^8 IU/mL), although the strength of the association weakened (RR: 2.18; 95% CI:1.29, 3.67; p: 0.0008), with 70.5% (31/44) of PCR-positive HBsAg positives having moderate to high viral loads, compared to 32.4% (11/34) PCR-positive HBsAg negatives. Conversely, there was a statistically significant negative association between HBsAg-positivity and undetectable/low viral loads (i.e. <10 IU/mL) (RR: 0.35; 95% CI: 0.13, 0.92; p: 0.023), with 11.4% (5/44) of PCR-positive HBsAg-positives having undetectable/low viral loads, compared to 32.4% (11/34) of PCR-positive HBsAg-negatives. Chi-square for linear trend (undetectable/low viral loads in HBsAg-negatives vs. moderate/high viral loads in HBsAg – positives) was found to be statistically significant (p:0.00000).

4.5 Discussion

This study investigated the burden and diagnostic implications of HBV/HIV co-infections in patients who were candidates for HAART at DGMH, Tshepang HIV outpatient clinic. Overall, the study detected 22.9% of HBsAg positivity, an indication of high chronicity or acute HBV infection from this cohort of HIV patients initiating HAART. Of these 22.9% HBsAg-positives cases, a total of 93.2% had detectable HBV DNA, with the remaining 6.8% being undetectable with Cobas TaqMan HBV assay because they may have been acute or seroconverting to anti-HBs (immunity), and therefore had low HBV vireamia. However they were detected by in-house HBV nested PCR assay. Previous studies conducted in sub-Saharan Africa have shown that HBV infection is highly endemic (Ayoola, 1988; Kew, 1996; Kiire, 1996; Mphahlele *et al.,* 2002). It has been estimated that HBsAg carrier rates in the region range from 9.6% in South Africa to 20.6% in the Democratic Republic of the Congo, while HBV exposure rates range from 56.2% in Kenya to 91% in Senegal (Kiire, 1996). However, most of these studies are mainly from the general population. Burnett *et al.,* (2007) conducted a study on women attending antenatal clinics, and found no difference in the HBsAg prevalence between HIV-positives and HIV-negatives, with 6.2% (44/710) of the HIV-positives and 5.8% (41/710) of the HIV-negatives being HBsAg positive. However, the current study indicates a higher chronic or acute HBV infection from the cohort of HIV patients initiating HAART at DGMH.

The study also showed a high rate of HBV exposure in this cohort of HIV patients. A total of 63% of patients were detected as having one or more HBV serological markers, with a high proportion of patients having anti-HBc marker, 38%, which was an indication that the majority of these patients were exposed to HBV naturally. This should be expected, as the national immunisation programme against HBV only started in 1995 and targeted children <1 years of age. More significant, are high proportions of patients with detectable HBV DNA (40.6%). A previous study from our laboratory has found that the prevalence of HBV was 40.1% in HIV-positive and 31.7% in HIV-negative women attending antenatal clinics (Burnett *et al.,* 2003). However, current results showed that there was a high burden of HBV/HIV co-infection in DGMH HIV-positive patients. The variable results from Cobas Taqman assay and in-house PCR assay can be explained by the sensitivity of both test assays.

Detection of an "anti-HBc or anti-HBs alone" serological pattern is common in South African patients (Owiredu *et al.,* 2001; Mphahlele *et al.,* 2006), and the "anti-HBs alone" cases are not necessarily due to vaccination, as the majority of South African adults (with the exception of health care workers and others at high risk of HBV infection) are less likely to have received the hepatitis B vaccine. This study investigated adult patients who were not immunised, and therefore it should be expected that they were exposed to HBV naturally. A previous study from South Africa (Burnett *et al.,* 2007) also detected anti-HBc from 37.3% (265/710) of HIV-positives as compared with 28.6% for the HIV-negatives – both from women attending antenatal clinics. The study found no discernible trends over the three-year period in HBsAg, anti-HBc or anti-HBs prevalence, or in total HBV exposure. However, another study from South Africa, Mphahlele *et al.,* (2006), detected almost similar frequencies of anti-HBc (82%) for the HIV-negatives and 85% for the HIV-positives from 295 samples, indicating that both groups were equally exposed to HBV infection.

HBV/HIV co-infected patients are prone to lose immunity (anti–HBs) against HBV with greater frequency, due to immunosuppression because of HIV infections (Piroth *et al*, 2002). A total of 22.4% of patients with positive anti-HBs were detected, with 37.2% having detectable HBV DNA, despite immunity against HBV. Significant were a 3.1% of patients with anti-HBs marker only, but 37.5% of these having detectable HBV DNA. The detection of HBV DNA in patients having immunity against HBV can be explained by reactivation of past HBV infection, re-infection or occult hepatitis B infections (Piroth *et al.,* 2002; Mphahlele *et al.,* 2006).

Additionally, HIV immunosuppression may be associated with reactivation of HBV infection in persons who have lost detectable HBsAg, or HBeAg, or developing AIDS (Horvath and Raffanti, 1994; Mastroianni *et al,* 1998; Waite *et al,* 1988), or re-infection in patients who have lost protective anti-HBs or progressing to AIDS (Horvath and Raffanti, 1994). However, these results pose a significant question in the management of HBV in the co-infections with HIV, especially with inclusion of drugs with anti-HBV activity in the management of HIV in South Africa. Burnett *et al.,* (2007), found an upward trend in 'anti-HBs alone' in the HIV-positive group (2.5% [2/79] in 1999, 4.8% [6/125] in 2000, 5.4% [4/74] in 2001), accompanied by a downward trend in the HIV-negative group (2.5% [2/79] in 1999, 1.6% [2/125] in 2000, 1.4% [1/74] in 2001), neither trend being statistically significant.

While data on occult hepatitis B infections has been emerging in recent years, it has remained a controversial phenomenon, with conflicting clinical significance. It has been reported to be frequently detected in HIV-positive patients, both in developed and developing countries (Hofer *et al.,* 1998; Piroth *et al.,* 2002; Mphahlele *et al.,* 2006). This study detected anti-HBc marker only, a marker for natural exposure against HBV, in 15.1% of patients, and of these, 45.2% had HBV DNA. A previous study from our laboratory detected HBV DNA from 33.3% of patients with anti-HBc serological marker only (Mphahlele *et al.,* 2006). Persistence of isolated anti-HBc pattern and occult HBV infection has been well defined. It has been one controversial issue that has questioned the clinical relevance of an isolated positive test for anti-HBc. In HIV-positive patients, a variable fraction of persons (10 to 45%) with an isolated positive test for anti-HBc had detectable HBV DNA (Mphahlele *et al.,* 2006). Such atypical serologic findings have led to the recommendation that all patients with HIV infection undergo testing for HBsAg, anti-HBs, and anti-HBc. If the tests for HBsAg, anti-HBc, or both, are positive, these patients should be tested for HBV DNA, since therapy for both HIV and HBV infection may be needed. Patients without HBV DNA in serum (i.e. those who have anti-HBc alone) should be vaccinated against HBV, and may, like HIV-negative patients, have a primary or anamnestic response (McMahon *et al.,* 1992; Hofer *et al.,* 1998; Piroth *et al.,* 2002; Santos *et al.,* 2003; Shire *et al.,* 2004; Pogany *et al.,* 2005; Gandhi *et al.,* 2005; Mphahlele *et al,* 2006).

The phenomenon of 'anti-HBc only' is not rare in diagnostic settings (Jilg *et al.,* 1995; Grob *et al.,* 2000), and it is not clear how this serological profile should be interpreted and whether further molecular tests should be conducted routinely on these samples. Anti-HBc is found in individuals who have experienced natural infection with HBV. Conventionally, the presence of

anti-HBc in the absence of HBsAg is interpreted as evidence of a past HBV infection. A previous study from South African hospitalised patients tested for HIV and HBV, found 22% of HIV-infected people without HBsAg had detectable HBV DNA, compared with only 2·4% of HIV-uninfected people (31 of 140 versus two of 85) (Mphahlele *et al.,* 2006). Other studies have found this phenomenon as prevalent as up to 40% (Jilg *et al.,* 1995; Berger *et al.,* 2001).

In addition, this study detected a total of 16.2% of patients with anti-HBs and anti-HBc markers only, with 32.5% of these having detected HBV DNA. Usually, this serological profile indicates past HBV infection or natural exposure (Piroth *et al.,* 2002).

Surprisingly, the study also detected 14.1% (10/71) of patients having positive HBV DNA, making up 29.4% (10/34) of occult HBV infections, despite being negative for three markers tested (i.e. HBsAg, anti-HBs and anti-HBc). This clearly shows that some HIV patients have active HBV infection without detectable HBV serological markers, and presents diagnostic challenges particularly in the developing world were molecular assays are not always available. In contrast, previous study from our laboratory had seldom observed this phenomenon in HIV-negative patients, with only 5.7% (2/35) of HBsAg-negative being HBV vireamic (Mphahlele *et al.,* 2006). This study clearly supports the notion that HIV co-infection is a risk factor for occult HBV infection. Further, it appears from our findings that an "anti-HBc alone" serological pattern is an added risk factor for occult hepatitis B in HIV co-infected patients, as reported previously (Grob *et al.,* 2000).

Assessment of HBV viral load using molecular assays should be performed in all seropositive individuals to determine HBV replicative status, especially during initiation of HAART, for the correct choice of antiviral drugs. This study has shown that a significantly high number of patients had detectable HBV DNA with Cobas Taqman assay and in-house HBV PCR assay. The quantifications of HBV DNA showed that the majority of patients (53.9%) had HBV viral load ranging between 200 $\geq 10^8$ IU/mL, with an overall total of 88.5% of patients having detectable HBV DNA ranging between <10 $\geq 10^8$ IU/mL. The results confirm that there is higher chronic HBV carriage with replicative or active infections, which warrant the need for screening HBV before initiation of lamivudine-containing regimens. Secondly, it is also important to perform and monitor serum or plasma HBV DNA level to determine the impact and benefit due to HAART containing lamivudine. This is particularly important in the phase of sub-Saharan Africa region expanding HAART programmes, which includes an anti-HBV active drug (i.e. lamivudine), that has a double impact on the virological outcome of HBV and HIV in co-infected

individuals. This is also important in order to avoid the selection of lamivudine-resistant HBV strains. The implication being that the selection of HBV resistant strains may compromise the benefits of HAART because they can cause cross-resistance and the response to subsequent anti-HBV agents being impaired (Thio and Locarnini, 2007). While HBV can be treated with lamivudine, there are also reports of treatment complication in HBV/HIV co-infected individuals. This is in particular includes the addition and/or removal of lamivudine that have also been implicated in causing liver inflammation and, on rare occasions, liver failure (Drake *et al.,* 2004). The study also detected 1.0% of patients with co-existence of HBsAg, anti-HBs and anti-HBc in the serum (all tested HBV serological markers positive) and having detectable HBV DNA. This group of patients could be attributed to early recovery from acute phase or re-activation of latent HBV infection due to severe immunosuppression caused by HIV (Hatzakis *et al.,* 2006). Although very unlikely, it could also be due to anti-HBs or vaccine-escape mutants. Previous studies have associated the co-existence of HBsAg and anti-HBs in serum of HBV chronic carriers with vaccine-escape mutants. However, the amino acids pattern of HBV RT and the clinical settings associated with this serological profile remain largely unknown (Colson *et al.,* 2007). The co-existence of HBsAg and anti-HBs has been proposed to be associated with important clinical concerns, because HBsAg-mutated HBV strains may not be fully sensitive to vaccine-induced anti-HBs, with the potential risk of vaccine failure including contamination of presumably protected vaccinated individuals. The detection of such non-protective anti-HBs may also lead to misdiagnosis of chronic HBV infection if detection of HBsAg is not carried out concomitantly. Furthermore, due to the frame-shifted overlap between the open reading frames of HBsAg and HBV polymerase genes, mutations within the HBsAg gene might result in structural and functional alterations in the HBV RT with potential influence on viral replication capacity and efficacy of antiviral drugs (Torresi *et al.,* 2002; Sheldon *et al.,* 2005). In view of these potential implications, the frequency, clinical and virological settings and significance of the presence of concurrent HBsAg and anti-HBs in serum, remain largely unknown to date. A study from DRC, reported the detection of three HBV serological markers (Kashala *et al.,* 1994), and found that despite a similar prevalence of HBV exposure in HIV-positive and -negative pregnant women (n5500, 18 being HIV positive), there was a significant increase (11.1% in HIV-positive, 2.7% in HIV-negative), and also detected specimens that were positive for all three markers (HBsAg, anti-HBs, and anti-HBc), suggesting re-infection or reactivation.

4.6 Conclusions

Previous limited studies did not find a significant increase in the burden of HBV prevalence in HIV-positive individuals (Menendez *et al.* 1999; Candotti *et al.,* 2001). In those which found an increase, the increase was not as dramatic as reports from developed countries (Ogutu *et al.,* 1990; Pawlotsky *et al.,* 1995; Ahmed *et al.,* 1998). However, most of these studies were either conducted at the time when HIV infection was at relatively low levels, or small samples were used (Ogutu *et al.,* 1990; Pawlotsky *et al.,* 1995; Ahmed *et al.,* 1998). Conversely, most of the studies that have shown an increased prevalence of HBV in HIV-infected subjects, have been conducted in populations or countries where the HIV epidemic had either reached high levels, or have included larger numbers of HIV-positive individuals (Burnet *et al.,* 2003; Nakwagala *et al.,* 2002; Lodenyo *et al,* 2000; Shao *et al.,* 1993; Nacro *et al.,* 2001; Ter Meulen *et al.,* 1989).

This study has shown that the majority of patients with advanced HIV/AIDS infection have a high exposure rate or active HBV infection. The exposure rate was 63%, as defined by a positive HBV serology, and active infection was 40.6%, as defined by HBV DNA. Quantitation of HBV DNA showed that these patients had high HBV viral loads ranging from <10 ≥10^8 IU/mL, with 70.4% having HBV viral loads ranging between 200 ≥10^8 IU/mL. The high levels of HBV DNA confirm the significance of screening for HBV before initiation of HAART with anti-HBV activity.

The study also detected a high percentage of patients with atypical serological profiles, such as occult HBV infections. The results also indicate that HIV infection is a risk factor for occult HBV infections. High detection of HBV DNA with "anti-HBc only" suggests a possibility of reactivation in this population, due to immunosuppression due to HIV infections, while a high rate of HBV DNA in patients with anti-HBs and/or anti-HBs may suggest a high rate of re-infections. These results advocate for implementation of molecular techniques in detecting HBV infections in patients with anti-HBs and/or anti-HBc markers before initiation of HAART, especially because HBV DNA can be affected by lamivudine-containing HAART regimens. In addition, there is a risk for development of HBV drug resistant strains due to sub-optimal therapy in all patients with detectable HBV DNA. This poses a challenge to effective management of HBV/HIV co-infected patients in developing countries, where PCR assays are not widely available.

4.7 Implications

The presence of HBV DNA in HBsAg-negative samples has serious implications for the diagnosis of HBV, as well as in the management of HBV in endemic regions of the world. First,

these results call into question the current standard of testing HIV-positive persons for HBV infection by using HBsAg, anti-HBc and anti-HBs tests for initial screening, and further confirm that PCR remains the gold standard assay for detection of acute or chronic HBV infection. HBsAg is used as an indirect marker of active HBV infection, and has been independently, or together with other markers, used widely to estimate current or chronic HBV infections worldwide. Ideally, in HBV exposed, HIV-positive patients with a negative HBsAg test, HBV DNA test should be performed to ascertain the absence of active HBV infection. However, this is a challenge, not only to effective laboratory diagnosis and management of hepatitis B in HIV co-infected patients, but also to the total control of the burden of HBV infections in HIV endemic regions of the world, such as sub-Saharan Africa, where molecular-based HBV detection assays are not widely available. Countries in this region rely to a large extent on the use of serological assays, not only as first line screening assays, but also for definitive diagnosis of HBV.

These findings may also have implications in the context of the newly introduced Comprehensive HIV and AIDS Care, Management, and Treatment Programme for South Africa, because lamivudine is included as part of ARV therapy in 2 of 3 adult and 3 of 5 paediatric regimens, and screening for HBV is not mandatory before initiation of ARV therapy, and particularly in the phase of WHO advocating expansion of HAART programmes in the regions affected by both HBV and HIV. These regions largely use lamivudine-containing HAART regimens.

Due to the immunosuppression status of HIV patients, these can cause re-activation of latent HBV infections or re-infections in previously immune individuals. Therefore, all HIV-positive individuals with no immunity against HBV (<10IU/L), should be vaccinated before advancing to AIDS.

4.8 Further Research questions

From this study, a number of other investigations could still be pursued. These include, but are not limited to:

- Effect of underlying chronic hepatitis B on the outcome of HIV treatment
- Impact of HBV genotypes on the outcome of HAART
- Impact of HAART on occult HBV infection
- Impact of anti-HBV-containing HAART regimens on HBV DNA in African settings (this is addressed in part in the next chapter).

CHAPTER FIVE

5 INVESTIGATING THE IMPACT OF ANTI-HBV-CONTAINING HAART REGIMENS ON HBV DURING THE MANAGEMENT OF HBV/HIV CO-INFECTED PATIENTS, AND THE CO-EVOLUTION OF HBV AND HIV DRUG-RESISTANT STRAINS

5.1 Background

Studies from developing and developed countries have established that HIV infection modifies the course of HBV infection by increasing rates of chronicity, atypical serological presentations, prolonging HBV viraemia, and increasing liver-related morbidity (Benhamou *et al*., 1999; Thimme *et al.,* 2005; Mphahlele *et al*., 2006 and 2006; Selabe *et al*., 2007; Hoffmann *et al*., 2007). Hepatitis B virus infection in HIV-infected individuals may result in significant morbidity and mortality. More over, the management of each viral infection is complicated by the presence of the other virus. Response to HBV therapy is either diminished or likely to result in HIV drug resistance when used as monotherapy. Initiation of antiretroviral therapy may lead to clinically significant hepatic flares, hepatic decompensation and HBV drug resistance. Further clinical studies are required before accurate and informed therapeutic recommendations can be made. The interactions of HIV, HBV, and HAART complicate disease course and the management of patients co-infected with HIV and HBV. HBV co-infection does not substantially affect the course of HIV disease, but HIV co-infection does significantly alter the course of HBV disease. On the whole, early use of HAART is beneficial in co-infected patients who meet the criteria for HAART. Patients with low CD4 cell counts and high HBV viral loads at initiation of HAART are at increased risk for immune reconstitution hepatitis, and should be monitored closely (Lok and McMahon, 2004; Keeffe *et al*., 2006; Benhamou, 2006 and 2007).

The treatment of HBV and HIV co-infections depends on whether they will be treated concurrently or independently; hence, three possibilities in treatment of HBV/HIV co-infected patients exist (see Chapter Two, Section 2.4.3). In brief, treatment can be for patients who need anti-HIV therapy but not anti-HBV therapy, patients who need anti-HBV therapy but not anti-HIV therapy, or patients who need both anti-HIV and anti-HBV therapy.

5.2 Impact of HAART on HBV in HBV/HIV co-infected patients

The decision to treat HBV infection in co-infected patients is based on careful consideration of the need for combination antiretroviral therapy for HIV infection, the severity of liver disease, the likelihood of response, and potential adverse events (Hoff *et al.,* 2001; Bartholomeusz *et al.,* 2004; Hoffmann and Thio, 2007). The potential adverse events include: antiretroviral

hepatotoxicity, immune reconstitution, elevated liver enzymes and the development of drug resistance.

5.2.1 Antiretroviral hepatotoxicity

While initiation of HAART has been shown to result in a decline in plasma HIV RNA level and a rise in CD4 lymphocyte count, unfortunately, cases of severe hepatotoxicity have been reported, leading to a limited maximal therapeutic benefit of HAART, because of significant drug toxicity. This severe hepatotoxicity has been reported to occur in up to 10% of patients, mostly after commencement of antiretroviral therapy (Saves *et al*., 1999; Saves *et al*., 2000; Sulkowski *et al*., 2000). Severe hepatotoxicity was observed to be associated with mostly NNRTI regimen, especially in patients taking nevirapine, those with hepatitis B or C co-infection, and those co-administered protease inhibitors (Carr and Cooper, 1997). While HBV co-infections seems to be an independent risk factor for the development of HAART-related hepatotoxicity, however, the rate of related hepatotoxicity was observed to be approximately three times higher than in HBV-negative patients (Saves *et al*., 2000; Sulkowski *et al*., 2002).

5.2.2 Immune reconstitution

Studies have shown HAART to results in suppression of HIV replication; however, this has been compromised due to the report of HAART usually resulting in immune reconstitution. This is heralded by an increase in CD4 count and T-cell function (Notermans *et al*., 1999; Saves *et al.,* 2000; Sulkowski *et al.,* 2000; Hoff *et al.,* 2001). The immune restoration associated with HAART has been shown to be able to control HBV replication, but can also lead to increased immune-mediated liver injury. This has been associated with acute rises in serum aminotransferases known as hepatic flares, especially seen in HBV/HIV co-infected individuals (Carr and Cooper, 1997; Proia *et al.,* 2000; Hoff *et al.,* 2001). The hepatic flares usually occurs after a number of weeks during commencing HAART in individuals with high pre-treatment HBV viral loads (Hoff *et al.,* 2001; Saves *et al.,* 2000; Sulkowski *et al.,* 2000). Hepatic flares related to HAART have also been reported in a number of additional circumstances, including HBV reactivation (Carr and Cooper, 1997; Hoff *et al.,* 2001; Manegold *et al.,* 2001), development of lamivudine resistance (Liaw *et al.,* 1999) and following withdrawal of lamivudine in patients who have experienced immune restitution (Bessesen *et al.,* 1999).

5.2.3 Elevated liver enzymes

Liver enzymes elevations in HBV/HIV co-infected individuals on HAART are common, and important because of the impact on the tolerability of antiretroviral drugs (Sulkowski *et al.,* 2000;

Puoti *et al.,* 2003). There are several causes of liver enzyme elevations; hence, a determination of the aetiology responsible for elevation is important, because it guides the correct choice of therapy. Liver enzymes elevations causing agents can be divided into three categories: HAART-related, chronic hepatitis-B related, and diverse. The enzymes elevations due to HAART-related are as a result of toxicity from the drugs. These can be through liver injury due to direct drug toxicity, inhibition of mitochondrial DNA polymerase gamma, and idiosyncratic reactions, e.g.nevirapine and abacavir (Kaplowitz *et al.,* 2004).

HIV co-infection with chronic HBV has been shown to increase the risk of developing hepatotoxicity from antiretroviral drugs by three- to five-fold (Sulkowski *et al*., 2000; Puoti *et al*., 2003; Livry *et al.,* 2003). These was also observed with studies from Thailand and Taiwan, on HIV co-infection with HBV individuals, which showed an increase in hepatotoxicity to 15·3–16·0 episodes per 100 person/years compared with 4·5–8·0 episodes per 100 person/years in those without either chronic hepatitis B or hepatitis C (Law *et al.,* 2003; Sheng *et al.,* 2004). HBV - related liver enzyme elevations are detected during HBeAg seroconversion, which can be heralded by flares in ALT and AST. Secondly, individuals with chronic hepatitis B are at risk for acute infection with the hepatitis D virus which causes acute hepatitis. Thirdly, during acute hepatitis or fulminant hepatic failure which can occurs if HBV replication is suddenly uninhibited by discontinuation of an active anti-HBV agent or by emergence of drug-resistant HBV. Fourthly, in some individuals with chronic hepatitis B, antiretroviral therapy may lead to a paradoxical flare of hepatitis during immune recovery, caused by the immune reconstitution syndrome. This syndrome occurs because of increased immune activity against antigens from ongoing or resolved infections (Price *et al.,* 2001; Hirsch *et al.,* 2004; Drake *et al.,* 2004).

Region such as sub-Saharan Africa is concerned with the use of multiple hepatotoxic drugs. This is due to a higher chronic rate of hepatitis B infections and the use of anti-tuberculosis therapy (Hoffmann *et al.,* 2007). Tuberculosis therapy and HAART have been reported to likely cause liver enzyme elevations in HIV/HBV co-infected individuals than either therapy alone. In patients on tuberculosis therapy and HAART, chronic hepatitis B increases the risk for severe liver enzyme elevations three-fold above that for anti-tuberculosis therapy and HAART. Further studies are needed to determine if hepatotoxicity episodes cause progression of chronic liver disease as do chronic hepatitis B- associated flares (Wong *et al.,* 2000; Perrillo *et al.,* 2001; Hoffmann *et al.,* 2007).

5.2.4 Development of viral drug resistance

Initiation of HAART may lead to clinically significant HBV and HIV drug resistance. Response to HBV therapy is either diminished, or likely to result in HIV drug resistance when used as monotherapy (see Chapter Two, Section 2.5.5).

5.3 HIV treatment in South Africa

Of the seven agents used for treating chronic hepatitis B in the developing countries, only lamivudine is widely available in the South African public sector as part of HAART. The introduction of Comprehensive HIV and AIDS Care, Management, and Treatment Programme Guideline for South Africa has included lamivudine (anti-HBV agent) as part of ARV therapy in 2 of 3 adult and 3 of 5 paediatric regimens. However, routine screening for HBV is not yet mandatory before initiation of HAART, and indeed, most HIV treatment centres do not screen patients for HBV infection. Because HAART in South Africa contains an anti-HBV activity, treatment for HIV will potentially benefit both HIV and HBV. However, attention is not given to HBV, and clinicians in most cases are not aware of HBV-infected patients.

5.4 Study problem, hypothesis, purpose and specific objectives

5.4.1 Study problem

HBV patients co-infected with HIV require a detailed assessment and specific attention. Patients should initially be screened and identified as HBV-infected. However, in individuals with advanced immunodeficiency, HBsAg and anti-HBc assays may results in a decreased sensitivity; hence, occult hepatitis B infections are frequent. Assessment of HBV viral load using amplification assays should be performed in all seropositive individuals, to determine HBV replicative status. Guidelines for anti-HBV therapy recommended by the first European Consensus Conference on the Treatment of HBV in HIV co-infected patients have been developed especially for the developed countries (Alberti *et al.,* 2005). These however are not always followed by developing countries, especially in treating HBV in HIV co-infections, due to competing resources. Yet, the treatment responses achieved in developed countries may not necessarily be the same in developing countries such as South Africa. These can be attributed to the optimal time to initiate anti-HBV treatment as there is no conclusive data of when to initiate HAART with anti-HBV in HBV/HIV co-infected individuals. HIV co-infected patients with HBV replicative status and time for assessment and consideration for therapy. Based on available evidence, the consensus conference advised using the HBV DNA criteria applied to HBV mono-infected patients. South Africa introduced HAART for HIV/AIDS patients in state

hospitals in 2004 for the treatment and management of HIV, and the majority of these HIV/AIDS patients have been exposed to HBV or are HBV chronic carriers (Chapter Four). However, routine screening for HBV is not yet mandatory before initiation of HAART, and indeed, most HIV treatment centres do not screen patients for HBV infection. Because HAART in South Africa contains an anti-HBV activity, treatment for HIV will potentially benefit both HIV and HBV. However, attention is not given to HBV, and clinicians in most cases are not aware of HBV-infected patients. This study aims to highlight the significance of monitoring HBV in HIV co-infected South African patients receiving lamivudine-containing HAART at DGMH.

5.4.2 Hypothesis

HAART regimens containing anti-HBV activity such as lamivudine, may negatively impact on HBV and/or prognosis and outcome of HAART in HBV/HIV co-infected patients. Hence may impact on HBV, following the two possible scenarios:

(a) Co-infected patients will be exposed to HBV monotherapy (lamivudine) as part of HAART intended for HIV treatment, which may results in sub-optimal therapy to HBV.

(b) Sub-optimal therapy to HBV may result in:

- No benefit to HBV, leading to hepatic flares that will increase morbidity and mortality of co-infected patients, or reactivation of "silent" HBV carriers
- Partial benefit and subsequent risk of emergence of antiviral resistance

5.4.3 Purpose

To investigate the impact of anti-HBV-containing HAART on HBV during the management of HBV/HIV co-infected patients, as well as the co-evolution of HBV and HIV drug-associated resistant strains. It is important to understand HBV/HIV co-infection in South Africa, because of high number of hepatitis B chronic carries and expanding HAART containing anti-HBV programmes.

5.4.4 Specific objectives

This study will examine serial bleeds from the cohort of HBV/HIV co-infected patients, starting with baseline samples. The following parameters will be studied:

(a) Virological outcome of HBV and HIV to lamivudine-containing HAART regimens, by comparing HBV and HIV viral loads before and during therapy

(b) The degree of response to HIV and HBV (i.e. whether complete, partial or no response) to lamivudine-containing HAART regimens

(c) The degree of antiviral resistance to HIV and HBV before and during therapy

5.5 Study populations and methods

5.5.1 Study populations

A total of 78 baseline bleeds selected from a cohort of 192 HIV-positive patients initiating lamivudine-containing HAART at DGMH, Tshepang HIV outpatient clinic, were studied for HBV and HIV response against lamivudine-containing HAART regimens (see Chapter Four on details of the cohort). The study population comprised of 44 females and 32 males (gender missing on two patients). The average mean age was 35.8 years (range: 20 - 53) (Table 3.1).

5.5.2 Baseline serological and ALT profiles

All 78 patients' baseline bleeds were quantified for HBV DNA with COBAS TaqMan HBV 48 assay. They were selected for quantitations because they were positive for HBV DNA with in-house HBV PCR assay. Summarised serological profiles of patients' baseline sera and their total number of follow-up samples at 6, 12, 18 and 24 months, are presented in Table 5.1. The patients are stratified into two groups based on HBsAg status and the presence of other serological markers.

Table 5.1: Study population for HBV viral load quantitation in HBV/HIV co-infected patients (N = 78)

HBV serological Markers	*Baseline*	*Total follow-up (months)*			
		6	*12*	*18*	*24*
Overall bleeds obtained	78	32	38	26	19
All patients positive for HBsAg	44	16	21	12	9
Patients positive for HBsAg only	26	11	11	8	5
All patients positive for anti-HBs marker	16	8	9	8	6
All patients negative for HBsAg	34	16	17	14	10
All anti-HBc positive	32	14	14	11	7
Only anti-HBc positive	11	5	4	5	1
All HBV markers positive (HBsAg, anti-HBs, anti-HBc)	2	1	1	0	0
Anti-HBs and Anti-HBc positive only	10	6	6	6	13
All three markers negative	8	3	3	1	4

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5.5.3 CD4 cells

Values for CD4 cell (cells/μL) were obtained from the Department of Haematology, DGMH, NHLS, and were performed as part of routine HIV treatment and management. A total of 61 baseline CD4 cells levels were obtained, with 42 patients for 6 months, and 21 for 12 months.

5.5.4 Viral load testing assays

5.5.4.1 HBV viral loads

For the principle of HBV viral load assay, refer to Chapter Four, Section 4.3.3.1. The overall samples tested for HBV DNA during follow-ups (i.e. 6, 12, 18 and 24 months) are presented in Table 5.1.

5.5.4.2 HIV viral loads

HIV viral loads were obtained from the Department of Virology, DGMH, NHLS, as part of routine HIV treatment and management analysis. HIV viral loads were obtained from a total of 52 baseline sera, 39 and 21 sera for 6 and 12 months' follow-up respectively. No sera were obtained for 18 and 24 months' follow-ups.

Principle of NucliSens EasyQ HIV-1 viral load assay (BioMe'rieux; Netherlands).

The NucliSens EasyQ assay is the first commercially available real-time nucleic acid-based test for HIV-1 RNA measurement in routine diagnostic laboratories. The assay uses the NASBA amplification technology, followed by real-time detection with molecular beacons. Nucleic acid extractions (for EasyQ HIV-1) are done from 1 or 2 ml of plasma, using the NucliSens Extractor (BioMerieux, Boxtel, Netherlands).

Amplification and real-time detection of 48 samples took only 90 mins. with approximately 30 mins. hands-on time. The Nuclisens EasyQ assay, primers and probes are designed based on the well-conserved gag region of HIV-1. During the amplification process there is a constant growth in the concentration of amplicons to which the beacon can bind while generating a fluorescence signal. The overall fluorescence curve contains kinetic information on both amplicon formation and beacon binding. The quantitation is based on assessing the amplicon formation rate from the viral RNA relative to that from a fixed amount of calibrator RNA. The linear dynamic range of the assay runs from 50 IU/ml (1.7 log) to 3×10^6 IU/ml (6.48 log). Results are expressed as International Units per ml (IU/ml) for the EasyQ.

5.5.5 HBV and HIV genetic drug resistance

RNA extracted from plasma using High Pure Viral Nucleic Acid assay (Roche Diagnostics, Penzberg, Germany) was used for this investigation, following manufacturer's instructions (see Section 4.3.3.2.1).

5.5.5.1 HIV RT-PCR assays for pol gene

5.5.5.1.1 The synthesis of cDNA was done using the following protocol:

The cDNA was synthesised by adding 4µl of RNA to a primer/dNTP mix comprising of 1µl all of primer IN3 (anti-sense) (10 pmol/µl) and 1µl all of 10 mM dNTP and mix on ice. The reaction is denatured at 65˚ C for 5 minutes and cooled on ice for 1 minute for priming the RNA. Mastermix was prepared according to the manufacturer's protocol, (Invitrogen, USA). For 1 reaction mix: 2µl of 5x cDNA Synthesis Buffer, 0.5µl of 0.1M DTT, 0.5µl of RNaseOUT™ (40U/µl), 0.5µl of DEPC-treated water and 0.5µl of ThermoScript™ RT (15U/µl) were added to the primed RNA and incubated at 45˚ C for 1 hour. The reaction was terminated by heating at 85˚ C for 5 minutes. Excess RNA template was degraded by adding *E. coli* RNase H followed by incubation at 37˚ C for 20 minutes. The cDNA product was used immediately for PCR reactions or stored at 20˚ C for later use.

5.5.5.1.2 Expand Long Template PCR System for nested HIV PCR assay

The polymerase gene was amplified from cDNA following the University of Cape Town protocol (Vergne *et al.,* 2000; Johanne van Harmelen *et al,* University of Cape Town). The first round primers were expected to amplify 2400bp comprised of G25REV (5'-GCAAGAGTTTTGGCTGAAGCAATGAG-3') that anneal at position 1873 to 1898 and IN3 (5'-TCTATVCCATCTAAAAATAGTACTTTCCTGATTCC 3') that bind at position 2008 to 2042 of the pol gene. The second round primers targeted a 1770bp and consisted of AV150 (5-' GTGGAAAGGAAGGACACCAAATGAAAG-3') (2036 - 2062) and PolM4 (5'-CTATTAGCTGCCCCATCTACATA-3') (3870 – 3892). The first PCR reaction contained 1x buffer with 17.5 mM MgCl2, 10 pmol/µl of each primer, 0.075U of Expand long template enzyme, 350 µM of each dNTP and 5 µl of cDNA in a total volume of 45 µl. The cycling conditions were: initial denaturation of 94°C for 2 minutes, followed by 10 cycles each of denaturation at 94^0C for 10 seconds, annealing at 50^0C for 30 seconds, and extension at 68˚C for 2 minutes, plus 20 seconds cycle elongation for each successive cycle; then, 25 cycles of 94˚C for 15 seconds, 50˚C for 30 seconds and 68˚C for 2 minutes plus 20 seconds cycle elongation for each successive cycle. The final extension was performed at 68˚C for 7 minutes. The second round PCR reaction, performed under the same cycling conditions as the first round, contained 1 x buffer with 17.5 mM MgCl2, 350 µM of each dNTP, 10 pmol/µl of each primer, 0.0375U of Expand long template enzyme and 5µl of first round PCR product in a total volume of 100 µl. Amplification was carried out in a GeneAmp 2700 thermocycler (Applied Biosystems, Perkin Elmer). Positive and negative controls were included in all extractions and

amplification reactions, and necessary precautions were undertaken to avoid contamination. The nested product was verified by running on 1% agarose gel electrophoresis (see Section 4.3.3.2.4).

5.5.6 Sequence analysis of HBV and HIV PCR products

Principle of the sequencing methods

HBV and HIV PCR products were sent to Inqaba Biotech (Pretoria) or Stellenbosch University, Department of Genetics, for direct sequencing with Spectru- Medix SCE 2410 Genetic Analysis System (Spectru- Medix LLC, State College, PA) and ABI PRISM® 3100 Genetic Analyzer version 3.7. Both genetic analyzers are based on the Sanger-Coulson method of sequencing, using chain terminating dideoxynucleotides (ddNTPs) (Sanger *et a.l,* 1977). Genetic Analyzer is a multi-colour fluorescence-based DNA analysis system that uses capillary electrophoresis with 16 capillaries operating in parallel. The analyzer is fully automated from sample loading to data analysis, allowing both DNA sequencing and fragment analysis to be performed at medium-to-high throughput. Both dye primer and dye terminator cycle sequencing strategies are employed. With both methods, growing chains are labelled with four different dyes attached to "chain terminators" (ddATP, ddCTP, ddGTP and ddTTP) during cycling. Unincorporated dye terminators are removed with alcohol precipitation, and labelled fragments are separated according to the length using capillary electrophoresis. The analysis software performs base calling automatically. Fluorescent-labelled DNA fragments are separated according to size during capillary electrophoresis. The length and the relative concentration of each fragment can be estimated after electrophoresis.

5.5.6.1 Sequencing of HIV polymerase gene

The generation of HIV pol gene fragments and subsequent sequencing were done as described by Vergne *et al.* (2000). The polymerase fragment spans the entire protease and RT genes. PCR products were sequenced with the following primers:

PolM0 TCCCTCAGATCACTCTTTGGCA (sense) (position 2251 -2272)

PolM1 GTTAAACAATGGCCATTGACAGA (sense) (position 2610 – 2632)

PolM4 CTATTAGCTGCCCCATCTACATA (antisense) (3892 – 3870)

PolM9 ATTGAACTTCCCAGAAGTCTTGAGTT (antisense) (2823 -2798)

PolM8 CTGTATATCATTGACAGTCCAG (antisense) (3323 – 3302)

5.5.6.2 HIV sequence analysis

The nucleotide sequences were viewed using Chromas Pro version 1.32 (School of Health Science, Griffith University, Australia). Comparison, alignment and translations of sequences were done using BioEdit (Hall, 1999) and MAFFT (Katoh *et al.,* 2002). The sequences were compared with South African and other HIV polymerase gene sequence available from the GenBank. For drug resistance, sequences were submitted to HIV Drug Resistance Database (Stanford University, http://hivdb.stanford.edu) and Los Alamos Database for analysis (http://hiv-web.lanl.gov).

5.5.6.3 Sequencing of HBV polymerase gene

The generation of HBV polymerase gene fragments and subsequent sequencing were done as described by Selabe *et al.* (2007). A nested polymerase chain reaction that used two sets of primers flanking the YMDD motif of the HBV polymerase was used. PCR products were sequenced with the following primers:

P3: GGT ATG TTG CCC GTT TGT CC (sense) positions 458–477

P4: GGC GAG AAA GTG AAA GCC T (antisense) positions 1103–1085

5.5.6.4 HBV sequence analysis

The nucleotide sequences were viewed using Chromas version 1.45 (School of Health Science, Griffith University, Australia). Comparison, alignment and translations of sequences were done using BioEdit (Hall, 1999) and MAFFT (Katoh *et al.,* 2002). The sequences were compared with South African and other HBV polymerase gene sequence available from the GenBank.

5.6 Results

5.6.1 HBV virological response against lamivudine-containing HAART regimens

5.6.1.1 Overall HBV viral loads baseline and follow-up samples

As discussed in Chapter Two, Section 2.4.3, current treatment guidelines for HBV treatment recommend starting therapy in individuals positive for HBeAg with HBV DNA levels ≥2 x 10^4 IU/ml, mildly increased ALT (<2xULN), increased ALT (≥2xULN) and negative also HBeAg with: >2 x 10^3 IU/ml, normal or increased ALT (Loomba and Liang, 2007; Alberti *et al.,* 2005). The current study detected a significant number of patients (88.5% or 69/78) with detectable baseline HBV viral load, confirming chronic or active HBV infections as described in Chapter Four. Also interestingly, were patients detected with HBV vireamia ranging between 10^4 and ≥10^8 IU/mL (Table 5.2). The HBV viraemia between 10^4 and ≥10^8 IU/mL is considered as highly infectious (Matthews *et al.,* 2009). The quantitation of HBV DNA on follow-up samples

using COBAS TaqMan HBV assay also detected individual's with active HBV infections ($10^4 \geq 10^8$ IU/mL) during 24 months of follow-up (Table 5.2). This shows that individuals continue having persistent HBV DNA, despite exposure against lamivudine -containing therapy.

Table 5.2: Overall distribution of patients quantified for HBV DNA at different months

Months Sera collected	Number tested	HBV viral load detection range (IU/mL)				
		Undetectable N (%)	<10 N (%)	10≤200 N (%)	200≤ 10^4 N (%)	$10^4 \geq 10^8$ N (%)
Baseline (0)	78	9 (11.5)	7 (9.0)	20 (25.6)	28 (35.9)	14 (18)
6 months	32	14 (43.8)	7 (22.0)	1 (3.0)	8 (25)	2 (6.2)
12 months	38	21 (55.3)	7 (18.4)	4 (10.5)	1(2.6)	5 (13.2)
18 months	26	13 (50.0)	4 (15.4)	3 (11.5)	4(15.4)	2(7.7)
24 months	19	13 (68.4)	2(10.5)	1(5.3)	0	3(15.8)

The majority of patients had undetectable HBV DNA 55.3% (21/38) during 12 months' follow-up (Table 5.2 and Figure 5.1). Despite high percentage of patients with undetectable HBV DNA levels at 12 months (55.3%), a total of 13.2% were infectious, as confirmed by high HBV viral load ranging between 10^4 and $\geq 10^8$ IU/mL. This trend of virological responds, suggested that HBV/HIV co-infected patients may not necessarily achieve similar benefits against lamivudine-containing regimens (Figure 5.1).

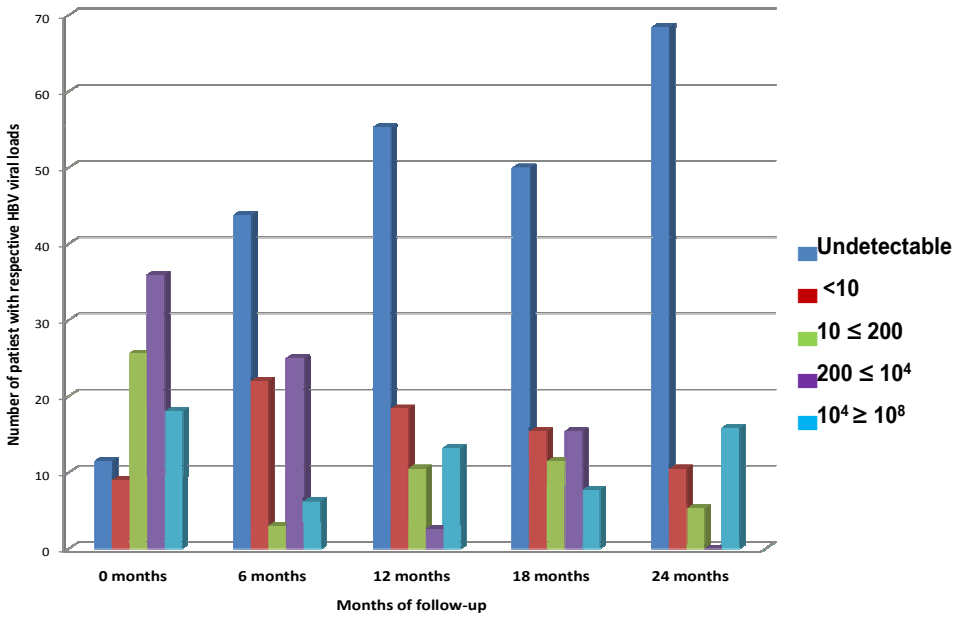

Figure 5.1: Overall distribution of patients HBV viral loads ranges at various months

During 18 months of' follow-up, a total of 26 serial bleeds were obtained. Of these, 50% (13/26) had detectable HBV DNA, with an equal percentage of patients suppressing vireamia to undetectable levels (50%). However, 7.7% were still highly vireamic as shown by viral load ranges between 10^4 ≥10^8 IU/mL (Table 5.3). At 24 months follow-up, serial bleeds were obtained from 19 patients (Table 5.2). Overall, a significant percentage of patients [68.4% (13/19)] had suppressed HBV DNA to undetectable levels, an indication of benefit due to lamivudine -containing regimen. However, there were some patients [31.6 (6/19)] having persistent HBV vireamia, with notable 15.8% (3/19) having vireamia raging between 10^4 ≥10^8 IU/mL (Table 5.2).

5.6.1.2 HBV virological outcome by HBsAg status

5.6.1.2.1 Variable HBV virological outcomes regardless of HBsAg status

Although the overall total number of patients with follow-up samples was variable, the trend during each term of follow-up was a decrease in HBV viral load from vireamic status to undetectable HBV DNA (Figure 5.1). However, a total of 18% (14/78) of patients showed various virological outcomes during exposure to lamivudine-containing HAART regimens (i.e. no response, partial response or possible reactivation). These degrees of virological responses were classified by HBV DNA and baseline serological markers (Table 5.3). Patients were classified as being “no virological response” when no significant change in HBV viral load was achieved during the follow-up period, “partial virological response” when patients experienced a decrease in HBV viral load during the first follow-up period, and then a sudden increase in HBV viral load at subsequent follow-ups, and “virological reactivation” when patients had no detectable baseline HBV DNA but with positive anti-HBs, but experienced a sudden increase in HBV viral load during subsequent follow-ups (Table 5.3).

A total of 42.9% (6/14) of patients were classified as having no response, (i.e. patients: 310, 265, 320, 289, 24B and 113) (Table 5.3). The HBV viral load levels were persistently high, despite exposure to lamivudine -containing HAART regimen. Distinctly were three patients, (113, 320 and 265), who had a persistent HBV DNA for 24 months of follow-up despite being exposed against lamivudine during HIV treatment. Both patients had baseline HBV viral load levels above 10^4 IU/mL, and continued to have high HBV viral load of above 10^5 IU/mL at 24 moths of follow-up. The results shows that patients with baseline HBV viral load levels above 10^4 IU/mL may persist having high vireamia, despite exposure against lamivudine (Table 5.3).

A total of 35.7% (5/14) of patients had partial virological response during treatment, with 20% (1/5) having positive baseline HBsAg marker (Table 5.3) (Patients: 24, 217, 317, 64 and 348). These patients had HBV baseline viral load ranging between <10 and >10^6 IU/mL, and HBV DNA became undetectable during 6 months or 12 months. However, the vireamia re-appeared and was detectable during follow-ups and persist at low levels, despite therapy with lamivudine (Table 5.3).

Table 5.3: HBV virological responses (i.e. no response, partial and reactivation) to lamivudine-containing HAART regimens (N = 14)

PN	CD4 cells (Months)			HIV viral load (Months)			HBV baseline Serology			ALT	HBV Viral Quantitations (Months)				
	BL	6	12	BL	6	12	HBsAg	Anti-HBs	Anti-HBc		BL	6	12	18	24
No HBV DNA responses															
113	67	Na	Na	750000	Na	Na	pos	neg	pos	52	1.99×10^4	Na	2.23×10^5	8.76×10^3	7.08×10^6
320	59	Na	Na	171533	<50	<50	neg	neg	pos	42	$>1.10 \times 10^8$	1.05×10^4	$>1.10 \times 10^8$	3.48×10^7	1.14×10^6
265	105	130	238	410000	<50	<50	pos	neg	neg	15	Above DL	7.36×10^2	1.52×10^5	2.49×10^4	2.53×10^5
289	7	79	Na	23000	<50	Na	neg	neg	pos	15	7.66×10^2	Na	Na	3.73×10^2	Na
310	Na	Na	Na	Na	Na	Na	pos	neg	pos	Na	7.44×10^4	3.34×10^2	Na	7.51×10^3	Na
24B	9	Na	Na	438585	Na	Na	pos	neg	pos	55	Above DL	7.58×10^3	Above DL	Na	Na
Partial HBV DNA responses															
317	6	106	Na	71000	50	Na	neg	neg	neg	61	1.68×10^2	Na	UND	2.91 x10	<6.00
24	281	236	Na	51000	4784	Na	neg	pos	pos	34	4.46×10^3	<6.00	>6.00	5.85 x10	Na
217	2	46	Na	Na	Na	Na	neg	neg	pos	18	1.14×10^6	UND	1.10 x10	Na	Na
64	15	540	Na	213140	<50	Na	neg	neg	pos	12	<6.00	UND	3.45 x10	Na	Na
348	Na	Na	Na	Na	Na	Na	pos	neg	neg	Na	2.74 x 10	UND	2.81 x10	Na	Na
HBV DNA Reactivations															
156	209	464	84	29000	<50	40	neg	pos	neg	33	UND	Na	UND	4.09×10^2	Na
131	54	264	Na	642499	50	Na	pos	neg	neg	18	UND	3.39×10^2	$>1.10 \times 10^8$	Na	Na
53	33	47	Na	Na	Na	Na	neg	pos	neg	17	UND	7.45 x10	<6.00	Na	UND

KEY: PN = patients; Sp = specimen; BL = baseline; ALT = alanine transferase; UND =undetected; DL = detection limits; Na = not available

Virological reactivation of HBV was observed in 21.4% (3/14) of patients (patients 131, 53 and 156). Both patients had undetectable baseline HBV DNA levels; however, follow-up samples had detectable HBV DNA, indicating a replicating virus. All three patients had occult hepatitis B infections, with 66.7% (2/3) being positive for anti-HBs at baseline (Table 5.3). The anti-HBs is an indicator of immunity against HBV, however, the other patient may have been losing immunity against HBV due to HIV infection.

5.6.1.2.2 HBV virologic outcome in HBsAg-positive patients

Overall, a total of 56.4% (44/78) of patients tested positive for HBsAg at baseline, with 93.2% (41/44) of patients having detectable HBV viral loads. A total of 27.2% of these patients had HBV viral load between $10^4 \geq 10^8$ IU/mL at baseline. Only 6.8% of patients had undetectable baseline HBV viral load. The majority of patients (43.2%) had viral load between $200 \leq 10^4$ IU/mL (Table 5.4).

Table 5.4: Distribution of HBV viral load from HBsAg -positive patients (N = 44)

Months Sera collected	**Number tested**	**HBV viral load detection ranges(IU/mL)**				
		Undetectable N (%)	**<10 N (%)**	**10≤200 N (%)**	**200≤ 10^4 N (%)**	**$10^4 \geq 10^8$ N (%)**
Baseline (0)	44 (100)	3(6.8)	2 (4.6)	8 (18.2)	19 (43.2)	12 (27.2)
6 months	16	6(37.5)	3 (18.75)	0	6(37.5)	1(6.25)
12 months	21	14(66.7)	1(4.8)	1(4.8)	1(4.8)	4(19.1)
18 months	12	6(50)	3(25)	0	2(16.7)	1(8.3)
24 months	9	5(55.6)	1(11.1)	1(11.1)	0	2(22.2)

Despite being HBsAg-positive, the majority of patients converted to undetectable HBV DNA, showing a benefit of receiving lamivudine-containing regimen. A total of 55.6% suppressed HBV DNA to undetectable level, as compared to 37.5% baseline. However, a total of 22.2% of patients had detectable HBV DNA ranging between $10^4 \geq 10^8$ IU/mL. The overall trend was the majority of patients becoming undetectable or decreased to ≤200 IU/mL after 24 months (Table 5.4 and Figure 5.2).

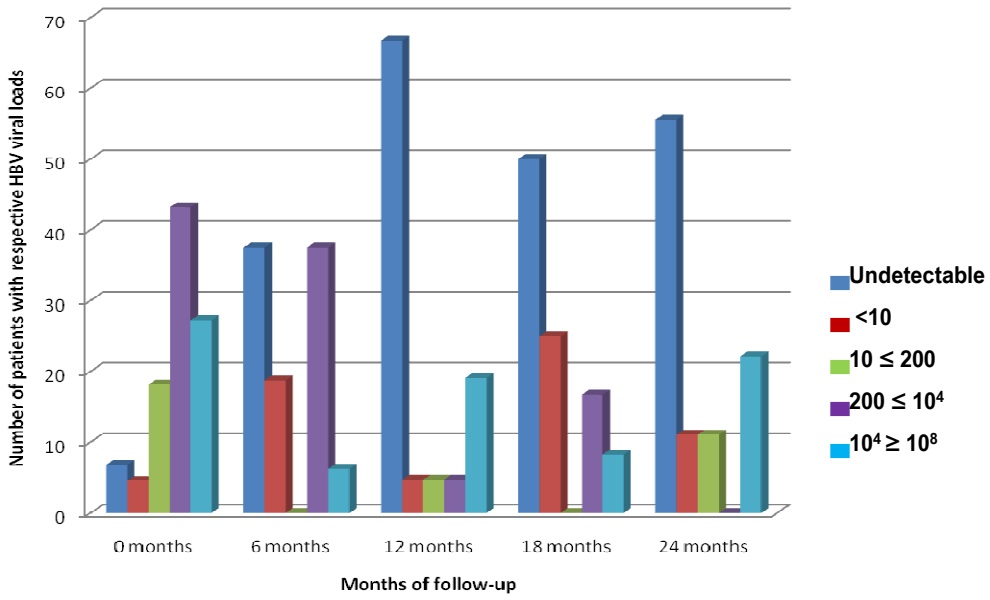

Figure 5.2: Sensitivity of HBV DNA to lamivudine-containing HAART regimens in HBsAg positive individuals.

5.6.1.2.3 HBV virologic outcome in HBsAg-negative patients

A total of 44% (34/78) of patients tested negative for HBsAg marker at baseline, but positive for HBV DNA with in-house PCR assay. These individuals represent classical cases of occult hepatitis B infection. Furthermore, quantitations of HBV DNA with Cobas Taqman assay detected HBV viral loads in 82.4% (28/34) of patients (range: <10 ≥10^8 IU/mL) despite being HBsAg -negative at baseline (Table 5.5).

Table 5.5: Distribution of HBV viral load in HBsAg -negative patients (N =34)

Months Sera collected	**Number tested**	**HBV viral load detection (IU/mL)**				
		UN N (%)	**<10 N (%)**	**10≤200 N (%)**	**200≤ 10^4 N (%)**	**10^4≥10^8 N (%)**
Baseline (0)	34 (100)	6 (17.7)	5 (14.7)	12 (35.2)	9 (26.5)	2 (5.9)
6 months	16	8 (50)	4 (25)	1(6.25)	2(12.5)	1(6.25)
12 months	17	7(41.2)	6(35.3)	3(17.7)	0	1(5.9)
18 months	14	7(50)	1(7.1)	3(21.4)	2(14.3)	1(7.1)
24 months	10	8(80)	1(10)	0	0	1(10)

Occult HBV infection has been described mostly with low- levels of HBV viral load, mostly less than 10 000 copies/mL (Mphahlele *et al.,* 2006). Only 5.9% (2/34) of patients had high HBV viral load levels, hence highly infectious, as confirmed by HBV viral load ranging between 10^4 and $\geq 10^8$ IU/mL. These indicate a persistent infectious virus despite negative HBsAg, which raises a concern for occult Hepatitis B infections especially in HIV positive patients.

Baseline HBV DNA quantitations showed a total of 17.7% (6/34) of patients having undetectable HBV DNA (Table 5.5). The HBV virological outcome was 80% (8/10) as the majority of patients responded against lamivudine-containing HAART regimen by suppressing HBV DNA to an undetectable level during 24 months' follow-up (Table 5.5). An overall trend was an increase in patients with undetectable HBV DNA over time of follow-up, despite two patients who had persistent HBV DNA while being exposed against HAART regimen containing lamivudine. The two patients had HBV viral load less than10 IU/mL and 10^4 $\geq 10^8$ IU/mL (Figure 5.3).

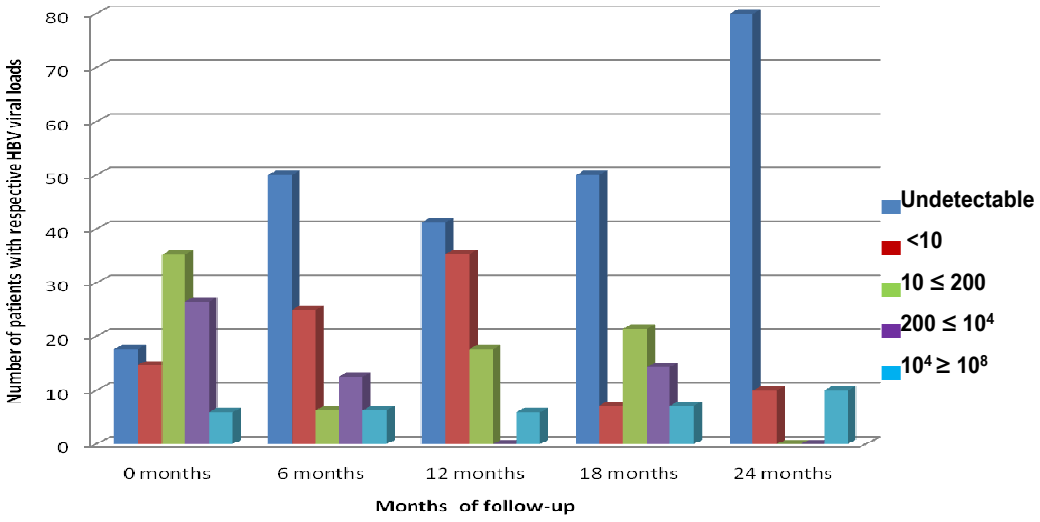

Figure 5.3: Sensitivity of HBV DNA to lamivudine-containing HAART regimens in HBsAg -negative individuals.

5.6.1.3 Concurrent response of HBV and HIV to lamivudine-containing HAART regimens

Since the patients were candidates for HAART, most had high viraemia for HIV at baseline, consistent with criteria for initiating HIV treatment. The study investigated concurrent virologic response to lamivudine-containing HAART regimens on both HBV and HIV viral

loads during treatment. A total of 94.1% (49/52) had HIV viral load ranging from 300 ≥10^5 copies/mL at baseline (Table 5.6). Only 3.9% (2/52) had HIV viral load ≤ 50 copies/ml. A significant benefit due to HAART was observed during 6 months' follow-up, with the majority of patients (80 % or 32/40) converted to undetectable (<50 copies/mL) HIV vireamia. The remaining patients, (20%) had HIV vireamia ranging between 50 ≤ 10^5 copies/mL (Table 5.6).

Table 5.6: Correlations between HBV and HIV viral loads during baseline (0) through 12 months of receiving HAART

Characteristics	**Exposure to lamivudine-containing HAART**		
	Baseline	6 Months	12 Months
Plasma HIV RNA viral load ranges(copies/ml)	N =52	N =40	N =23
≤50 (Undetectable)	2 (3.9)	32 (80)	21 (91.3)
50 ≤ 300	1 (1.9)	4 (10)	1 (4.4)
300 ≤ 10^5	25 (48.1)	4 (10)	1 (4.4)
>10^5	24 (46.1)	0	0
HBV viral load ranges (IU/ml)	**N =78**	**N=32**	**N = 38**
undetectable	9 (11.5)	14 (43.8)	21 (55.3)
<10	7 (9.0)	7 (22.0)	7 (18.40
10 ≤200	20 (25.6)	1 (3.0)	4 (10.5)
200 ≤10^4	28 (35.9)	8(25)	1 (2.6)
10^4 ≥10^8	14 (18)	2 (6.2)	5 (13.2)

Significantly, were 12 months follow-up of HIV viral load results. The overall majority of patients [91.3 % (21/23)] had suppressed HIV viral load to undetectable levels. Only two patients had HIV vireamia between 50 ≤ 10^5 copies/mL. When comparing the response, due to lamivudine -containing regimen, between HIV with HBV during 0 -12 months, a significant number of patients showed a benefits by converting from high viral load to undetectable vireamia. Briefly, a total of 56.25% (18/32) of patients had detectable HBV DNA during 6 months follow-up period, as compared to 43.75% of undetectable HBV vireamia. At 12 months follow-up period, HIV viral load was undetectable in 91.3% (21/23), with only 8.8% of patients still having HIV viraemia ranging between 50 ≤ 10^5 copies/mL. However, HBV viraemia was undetectable in 55.3%, with 44.7% of patients still having persistent vireamia.

Beside HIV viral loads not being available at 18 and 24 months for all patients studied, the indication was that HAART does benefit HIV patients from as early as 6 months by suppressing viral load, to undetectable levels (Table 5.6 and Figure 5.4).

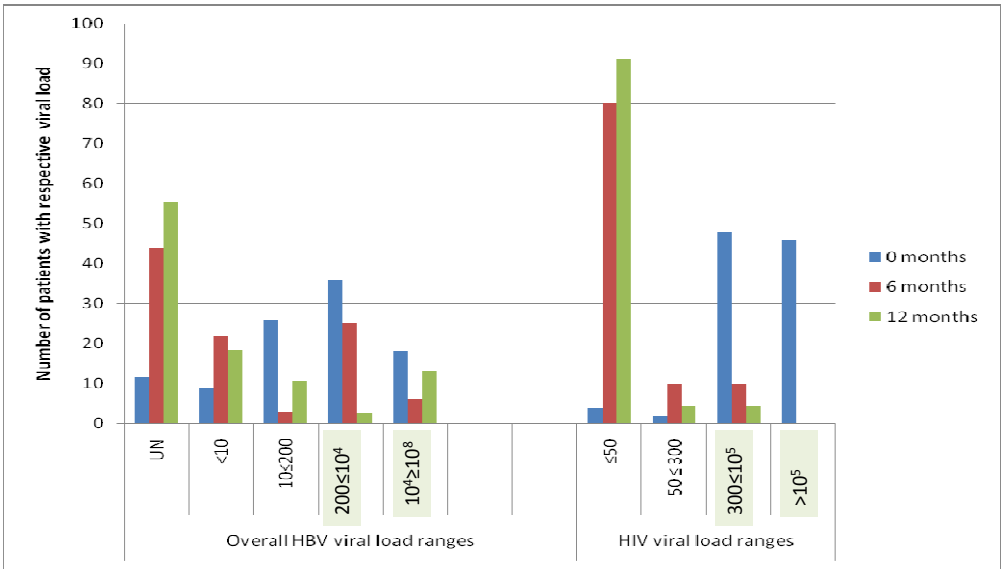

Figure 5.4: Correlations between HBV and HIV viral loads in patients receiving HAART at 0, 6 and 12 months

5.6.1.4 Correlation of CD4 cells with HBV and HIV virologic outcomes during HAART

The CD4 cells and HIV viral loads are important markers when initiating and monitoring HAART progress. The study correlated HIV and HBV viral loads with CD4 cells at different intervals, from baseline to 12 months follow-up period (Table 5.7 and Figure 5.5). A total of 78 baseline bleeds with available HIV viral loads and CD4 cells were correlated with HBV viral loads. The baseline CD4 cells and HIV viral load information were obtained from 61 and 52 patients respectively. As expected, the majority of patients, (87%) had CD4 cells count of ≤200 cells/ml at baseline, with only 13% having CD4 cells >200 cells/ml (Table 5.7). This can be expected, as the majority of patients were candidates for HAART.

Table 5.7: Correlations between CD4 cells, HBV and HIV vireamia during 12 months of HAART

Characteristics	**Exposure to lamivudine-containing HAART**		
	Baseline	6 Months	12 Months
Plasma HIV RNA viral load ranges(copies/ml)	**N =52**	**N =40**	**N =23**
≤50 (Undetectable)	2 (3.9)	32 (80)	21 (91.3)
50 ≤ 300	1 (1.9)	4 (10)	1 (4.4)
300 ≤ 10^5	25 (48.1)	4 (10)	1 (4.4)
>10^5	24 (46.1)	0	0
HBV viral load ranges (IU/ml)	**N =78**	**N=32**	**N = 38**
undetectable	9 (11.5)	14 (43.8)	21 (55.3)
<10	7 (9.0)	7 (22.0)	7 (18.40
10 ≤200	20 (25.6)	1 (3.0)	4 (10.5)
200 ≤10^4	28 (35.9)	8(25)	1 (2.6)
10^4 ≥10^8	14 (18)	2 (6.2)	5 (13.2)
CD4 cells ranges (cells/ml)	**N = 61**	**N = 42**	**N =21**
<50	26 (43)	3(7.1)	0
50 ≤100	13(21)	4(9.5)	1(4.8)
100 ≤200	14(23.0)	14(33.3)	6(28.6)
>200	8(13)	21(50.0)	14(66.7)

During 6 to 12 month follow-ups, most of the patients had improved CD4 cells level to >200 cells/ml, 50% (21/42) and 66.7% (14/21) respectively (Table 5.7). HIV viral load was suppressed to undetectable levels and HBV DNA suppressed to undetectable levels in most patients (Table 5.7 and Figure 5.5). In general, these results indicate that lamivudine-containing HAART regimens which suppress HIV replication lead to an improved CD4 cell (>200 cells/ml), an indication of immunological response. The immunological response (i.e. CD4 cells increase) was found to be related to the suppression of HBV and HIV replication to undetectable vireamia levels, as a sign of virological response (Figure 5.5).

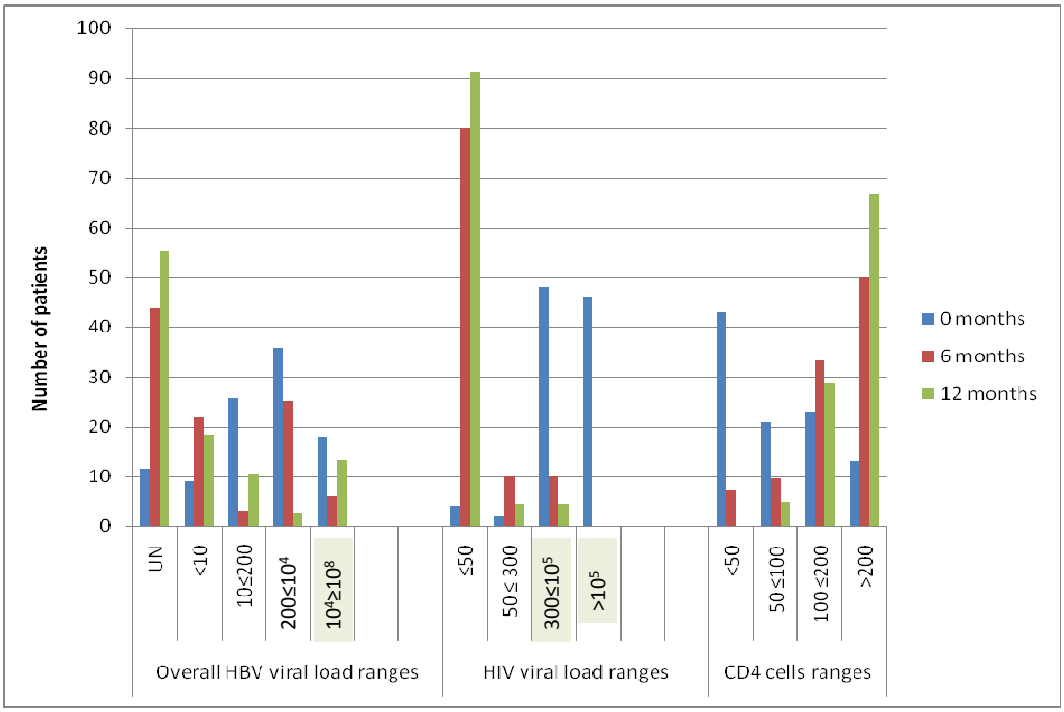

Figure 5.5: Correlations between CD4 cells, HBV and HIV viral loads during HAART

5.6.2 HBV and HIV drug resistance

5.6.2.1 HBV drug resistance

A total of 78 HBV PCR products from baseline bleeds were sequenced, targeting the polymerase region. A 646bp fragment of the polymerase gene was successfully sequenced. The sequences were analysed using Chromas Pro 1.45 and BioEdit Sequence alignment program for translations into amino acids. The mutations in this study were named according to revised nomenclature of the HBV polymerase gene by Stuyver *et al.* (2001).

Of the 78 baseline samples, only 1 HBV drug resistant strain, M204I, was detected, clearly indicating that there is less frequent detection of lamivudine-resistant mutations before therapy. Patient ZADGM164 was detected carrying M204I lamivudine-resistant mutant strain (Figure 5.6). This is in direct contrast to a previous study conducted in this laboratory, where mutations associated with lamivudine drug resistance were frequently observed in patients with and without HIV co-infection (Selabe *et al.,* 2007).

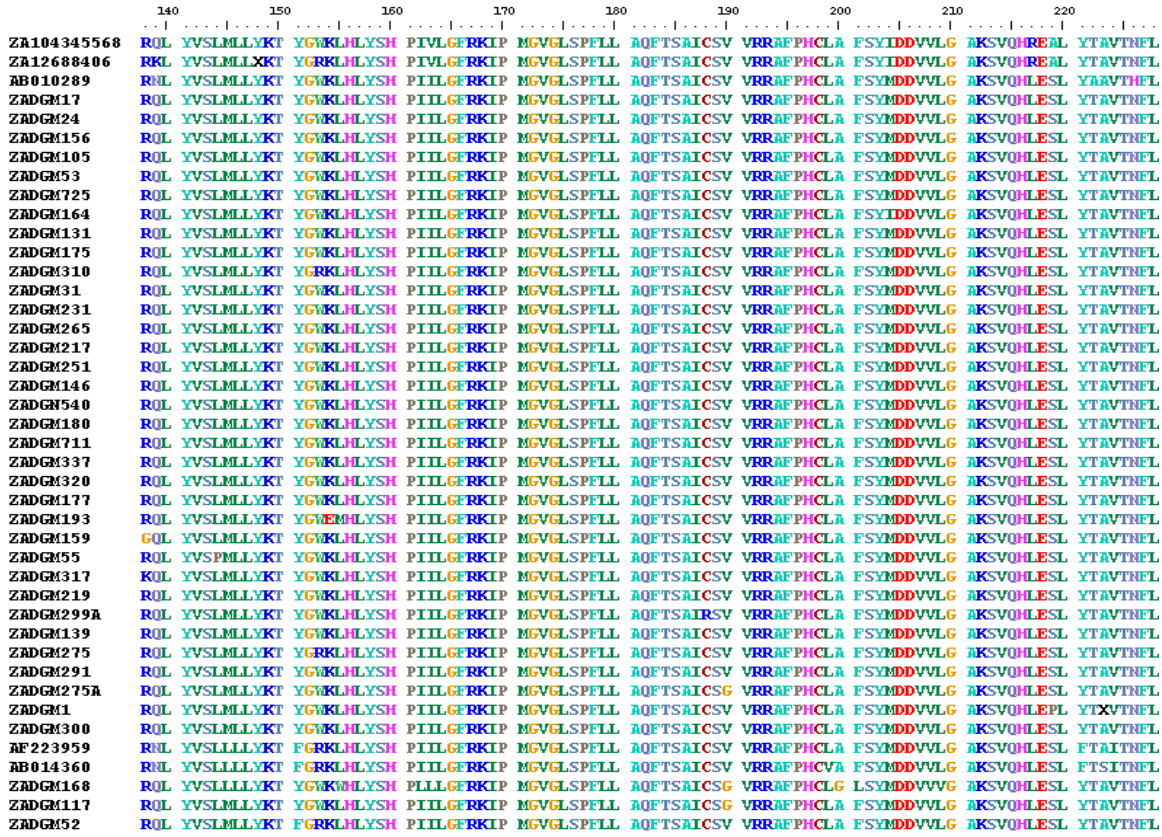

Figure 5.6: HBV polymerase amino acid sequences alignment. ZA DGM17 – ZADGM52 are study samples, with reference sequences from GenBank (i.e. AF223959, AB014363, AB010289, ZA1044345568, ZA12688406 and AB014360). Note patient with resistant strain (ZADGM164 at amino acids position 204).

5.6.2.2 HIV drug resistance

A total of 30 baseline sera previously sequenced for HBV polymerase lamivudine-associated resistance strains were sequenced targeting the HIV polymerase gene, which include reverse transcriptase (RT) and protease (PR) genes. The patients were later enrolled on ARV regimen 1a (lamivudine, stavudine and efavirenz) or 1b (lamivudine, stavudine and nevirapine), but none were on regimen 2 during the course of this study. The study detected primary HIV mutants against RT and PR in some patients, as shown in Table 5.8 and Figure 5.7. HIV-resistant mutations were identified according to the Stanford HIV Genotypic Resistance Interpretation Algorithm. The program is based on a subtype B consensus and compares codons of query sequences with resistance coding nucleotides. The reference resistant sequences in the database are derived from patients failing therapy, and from phenotypic assays. Mutations were defined as primary mutants when they results in resistant on their own, while secondary mutants are those that reduce drug susceptibility

in association with other mutations. There was no concurrent evolution of lamivudine-associated resistant strains for both HBV and HIV.

5.6.2.2.1 Drug resistance mutations related to the reverse transcriptase inhibitors

Analysis of the RT 410 amino acids detected primary resistance mutations, nine major NRTI's primary mutants – M41L (1/30), E44A (1/30), V75M (1/30), F77L (1/30), V118I (1/30), M184V (1/30), L210S (1/30), T215Y (1/30), V90I (1/30), V75M (1/30) and V118I (1/30). Most of these primary mutants were detected on patient ZAP24C4DGM (Figure 5.7). Five major NNRTI's primary mutants were detected: K103N (3/30), Y318CFSY (1/30), E138Q (1/30), P225H (1/30) and K238T (1/30). The A98G (3/30), an NNRTI mutant, was also detected (Table 5.8). It occurs in about 1% of NNRTI-naive persons and causes low-level nevirapine resistance. The presence of each of these primary mutants can make HIV replicate in the presence of the respective drugs, NRTI's, NNRTI's and PI's.

Figure 5.7: Comparison of HIV RT amino acid sequences, 1- 300 bases, References sequences from GenBank and Los Almos HIV database (Consensus A, Consensus B,CBRU52953, CBR025, CZAAY772699, CZASK164131, CETU46016, BU598 and CIN21068) and clinical samples (ZA467DGM to ZAP24C4DGM). Note patient ZAP24C4DGM with YVDD mutant at position184.

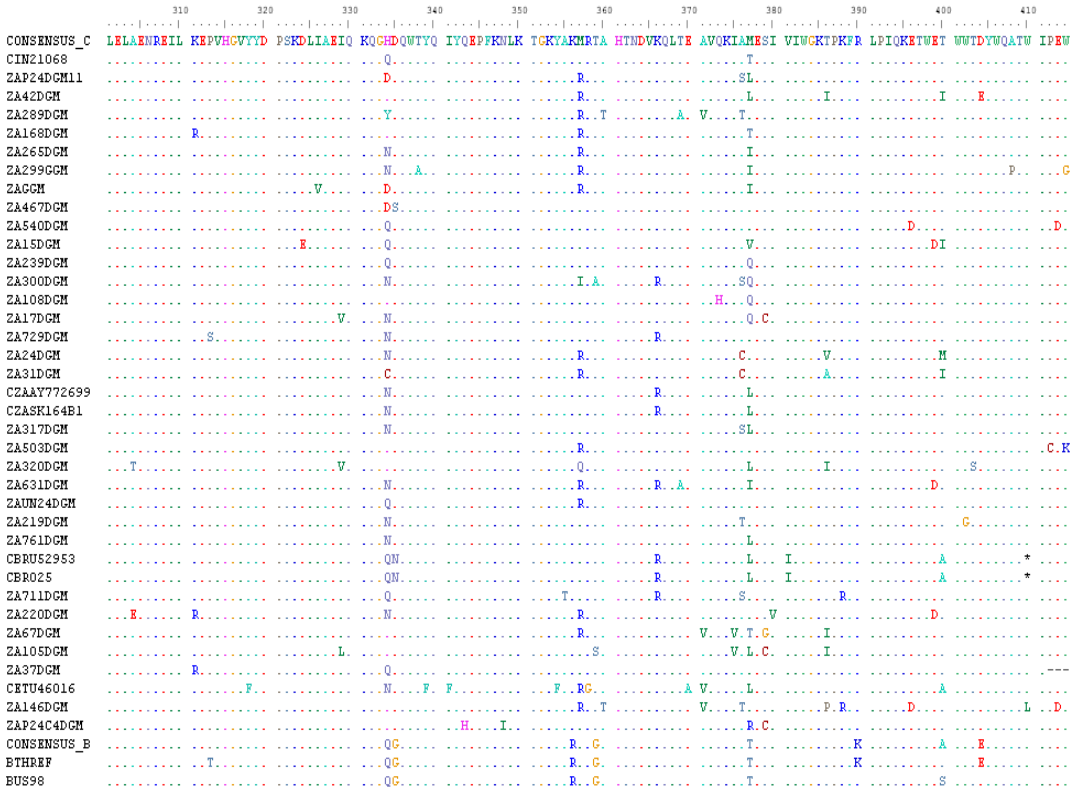

Figure 5.7: Comparison of HIV RT amino acid sequences continues (301 – 420 bases).

Table 5.8 HIV drug resistant mutations associated with polymerase gene (N = 30)

Patients names	Mutation s associated with Nucleoside-Reverse Transcriptase Inhibitor			Mutations associated with Non-Nucleoside Reverse Transcriptase Inhibitor			Mutations associated with Protease Inhibitor		
	Mutant	*Coding nucleotides*		*Mutant*	*Coding nucleotides*		*Mutant*	*Coding nucleotides*	
		Wild type	Mutant		Wild type	Mutant		Wild type	Mutant
ZAP24C4DGM	M184V	ATG	ATA/GTG	K103N	AAA	AAC	V82L	GTA/GTC/GTT	ATC
	M41L	ATG	TTG/CTG	E138Q	GAA/GAG	CAA/CAG			
	V75M	GTA	ATG	K238T	AAA	ACA			
	E44A/D	GAA	GAC	A98G	GCA	TCA			
	F77L	TTC	CTC						
	V118I	GTT	ATT						
	L210S	TTG	TGG						
	T215Y	ACC	TAC						
	N348I	AAC	ATC						
ZA711DGM				K103N	AAA	AAC	M36I	ATG/ATA	ATT/ATA
				P225H	CCT	CAT	I93L	ATG/ATT	CTG/CTT
				Y318CFSY	TAT	TTT	L89M	CTA/TTG	ATG/ATA
ZA146DGM				V90I			T74S	ACT/ACC/ACA/ACG	AGT/AGC
							I93L	ATG/ATT	CTG/CTT
ZA17DGM							V82L	GTA/GTC/GTT	ATC
							M36I	ATG/ATA	ATT/ATA
ZA15DGM				K103N	AAA	AAC	I93L	ATG/ATT	CTG/CTT
				A98G	GCA	TCA	M36I	ATG/ATA	ATT/ATA
ZA168DGM				E138A	GAG	GCG	M36I	ATG/ATA	ATT/ATA
ZA300DGM	A98S	GCG/GCC	TCG/TCC				M36I	ATG/ATA	ATT/ATA
							L89I	CTA/TTG	ATC
							I93L	ATG/ATT	CTG/CTT
ZA42DGM	V75M	GTA	ATG				L10I	CTC	ATC
ZA67DGM							T74S	ACT/ACC/ACA/ACG	AGT/AGC
ZA240DGM							L10I	CTC	ATC
ZA761DGM	V118I	GTT	ATT						
ZA729DGM							D30I	GAT	ATA
ZA631DGM							L10I	CTC	ATC
Other Protease Inhibitors polymorphisms									
T12S, I15V, G16E,L19I, N37S; R41K, D60E, Q61E, L63T I64L,H69K, L89M, I93L									

5.6.2.2.2 Drug resistance mutations related to protease inhibitors

The sequences of the PR gene from 30 baseline sera were analysed, to determine if there were any drug resistant mutations. Stanford HIV Genotypic Resistance Interpretation Algorithm queries for PR drug resistance related mutations revealed three classical drug resistance mutations – V82L (1/30), L10I (3/30) and D30I (1/30) (Table 5.8). All patients had polymorphisms occurring at the site associated with secondary drug resistance, this included T12S, I15V, G16E, L19I, M36I, N37S, R41K, D60E, Q61E, L63T I64L, H69K, L89M, and L93I (Figure 5.8). M36I and I93L are known secondary drug resistant mutations in non-subtype B infections (Bessong *et al.,* 2006).

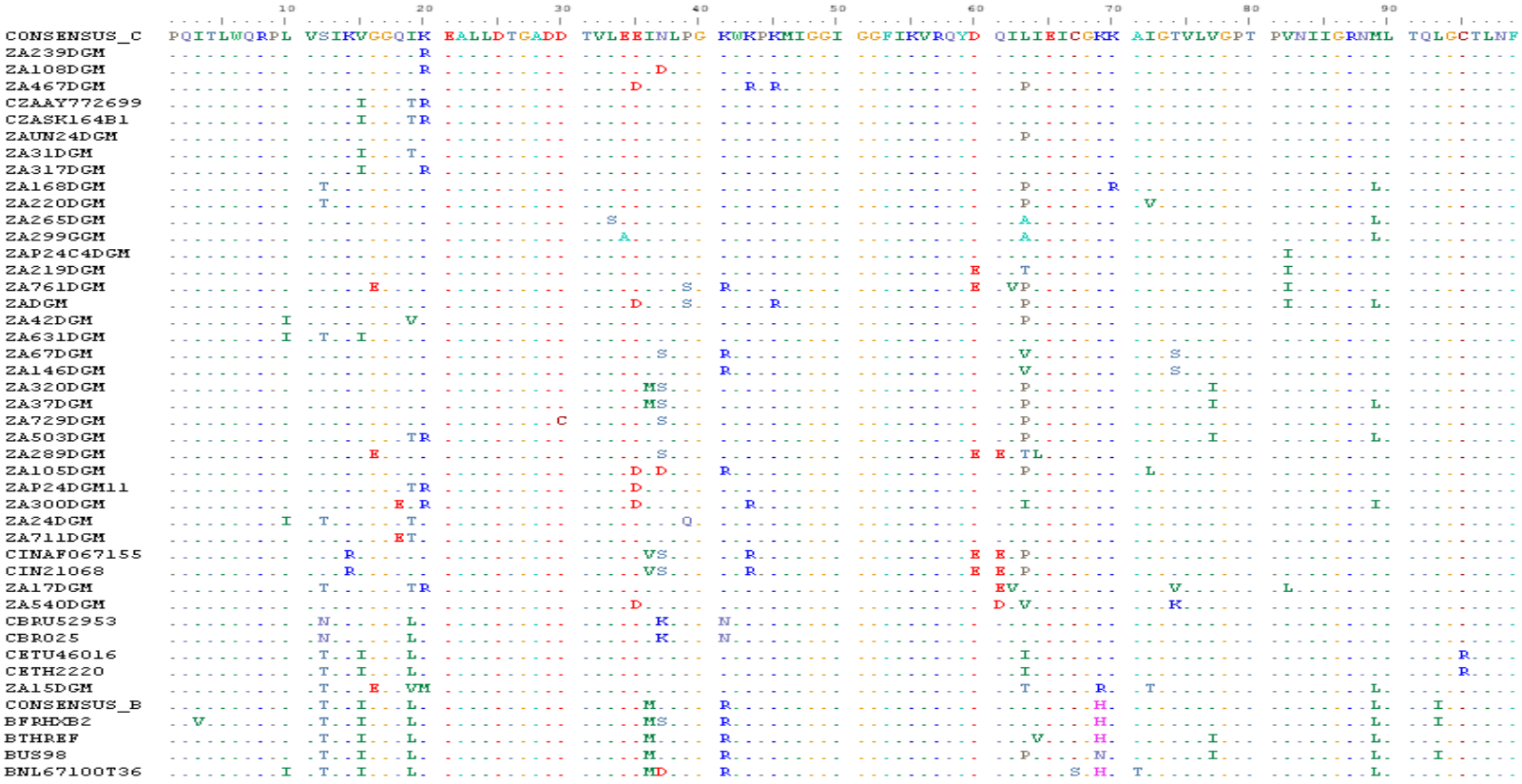

Figure 5.8: HIV protease gene amino acid alignment with mutations and polymorphism. Dots indicate identical amino acids and References sequences are from GenBank and Los Almos HIV database (Consensus A, Consensus B, CBRU52953, CBR025, CZAAY772699, CZASK164131, CETU46016, BU598 and CIN21068). Study samples areZA467DGM to ZAP24C4DGM.

5.7 Discussion

5.7.1 Overview of the results

The study investigated: (a) the virological outcome of HBV and HIV to lamivudine-containing HAART regimens in South African HIV patients, by comparing HBV and HIV viral loads before and during therapy; (b) the degree of response to HIV and HBV (i.e. whether complete, partial and no response) to lamivudine-containing HAART regimens; and (c) the development of antiviral resistance against HIV and HBV drugs, particularly lamivudine, before and during therapy, in the context of the South African setting, where HBV co-infection is not always screened before initiation of HAART.

Overall, the study was conducted on a total of 78 HIV-positive patients who were screened for baseline HBV serological markers, as part of this study. Quantitation of HBV DNA demonstrated that the majority of HIV patients co-infected with HBV (88.5%) are initiated on lamivudine-containing HAART regimens with detectable HBV vireamia. The study demonstrated that the initiation of lamivudine-containing HAART regimens results in suppression of HBV and HIV vireamia, which is a positive virological outcome following the South African Comprehensive HIV and AIDS Care, Management, and Treatment Programme. This was observed when comparing baseline HBV and HIV viral loads before initiation of HAART and follow-up samples during treatment, with the majority of patients having suppressing HIV replication to an overall 91.3%, with an undetectable viraemia at 6 and 12 months' follow-up periods. HBV also sustained a virological response of 50% during18 and 68.4% for 24 months of follow-up. Despite the benefit from lamivudine-containing therapy, a total of 31.6% of patients had persistent HBV DNA during 24 months. Interestingly, were 15.8% of these patients having HBV viral load ranges between $10^4 \geq 10^8$ IU/mL, which is an indication of patients being highly infectious.

While the trend was most patients having suppressed HBV vireamia to an undetectable level, a total of 18% had HBV various degrees of virological response outcome during follow-up (0 to 24 months). More concerning, was the persistent and reactivation of HBV vireamia in this patients with various degrees of virological response as detected in both HBsAg-positive and occult hepatitis B infections. These findings highlight the need for HBV screening before/during any HAART-containing anti-HBV activity is administered for HIV treatment, and continued monitoring of HBV viral load as a measure of response against anti-HBV drugs is important.

Studies on HBV and HIV drug resistance indicated one patient baseline sample having a YIDD mutant HBV strain, which is associated with resistance against lamivudine. The follow-up samples from the same patients showed a wild strain, an indication of mutant reversion. Concurrent evolution of lamivudine associated resistant strains against HBV and HIV were not detected during the course of patients follow-up. Studies on HIV drug resistance indicated a high degree of baseline primary antiviral resistance strains against HIV RT and PR genes (M41L, E44D, V75M, F77L, V118I, M184V, L210S, T215Y, Y318FY, K238T, V82L, K103N, K238T, Y318FY and P225H), while follow-up serial bleeds had no detectable HIV viral load, as response against HAART was successful, despite pre-existing mutations.

5.7.2 Impact of HAART on virological outcome of HIV

From the total of 52 baseline HIV viral loads obtained, the majority (94.2%) of patients had high viral loads ranging between 300 >10^5 copies/ml. This was expected, since all HIV patients were HAART candidates; hence, the high viral load. The HIV response to lamivudine-containing HAART regimens was overall, 91.3% at 12 months follow-up period. The results clearly support the fact that HAART can lead to a successful suppression of HIV viral load within 6 to 12 months of initiation of treatment. Only two patients (8.8%) had detectable HIV viraemia during 12 months of follow-up; however, a follow-up viral load for 18 months was not obtained. A previous study from a developed country found that almost three-quarters (72.4%) of people starting d4T/3TC/NVP achieved confirmed viral load suppression of ≤500 copies/mL in a median of 2.0 months (Tam *et al*., 2007). These findings were also similar to other studies conducted from developing countries (Nekthananon *et al*., 2004; Idigbe *et al*., 2005). Our results showed that lamivudine-containing HAART regimens are successful in the suppression of HIV viral load within 6 to 12 months. These indicated a successful HIV management and treatment programme. However, a leading question is the sustainability of the regimens, both in terms of duration and the development of HIV drug resistance. Tam *et al.,* (2007) showed that 67.6% (211/312) of antiretroviral-naive patients initiated on d4T/3TC/NVP, terminated treatment after a median of 12.4 months and required an alternative therapeutic approach, mostly due to drug resistance. The high frequency of termination of d4T/3TC/NVP indicated the need for accessible second-line treatment for HIV patients, which may presents a financial challenge for developing countries like South Africa with problem of competing resources (Tam *et al*., 2007).

5.7.3 Impact of HAART on virological outcome of HBV in co-infected patients

In contrast to HIV virological outcome, the HBV viraemia indicated a sustained virological suppression in 68.4% of patients, with undetectable HBV viral load at 18 and 24 months of follow-up. The sustained virological suppression of HBV vireamia was observed during 18 and 24 months follow-up periods, despite a small sample number as compared to other previous followed-up months, (total sample size of 26 and 19 respectively). The same trends were the majority of patients suppressing HBV DNA to undetectable levels. Although there was a significant HBV response in reducing HBV DNA to undetectable levels during 18 to 24 months of follow-up, a total of 18% of patients had classical examples of patients with HBV DNA remaining detectable or having persistent viraemia during the same period of follow-up. These patients had partial virological response, reactivation or no virological response. The results were significant in showing that although HBV/HIV co-infected patients may respond to lamivudine-containing HAART regimens, there are patients who may experience partial or no response, or with possible reactivation, independently of HBsAg status. This poses a significant problem, especially when considering that the majority of South African HIV patients are either exposed to or are chronic carriers of HBV (Burnett *et al.,* 2005; Mphahlele *et al.,* 2006).

This study showed that HBV co-infected patients do benefit from lamivudine-containing HAART regimens during HIV therapy. Lamivudine is registered for treatment of chronic hepatitis B at a dosage of 100 mg daily, and for HIV treatment at a dosage of 300 mg daily. Thus, the use of lamivudine for HIV (i.e. at a higher dose) can potentially benefit HBV/HIV co-infected patients, while the use of lamivudine for HBV treatment may be sub-optimal for HIV in co-infected patients. The study is significant, because, to our knowledge, it is the first African study to report on HBV/HIV co-infections in patients receiving lamivudine-containing HAART regimens. However, studies from Asia reported the efficacy of lamivudine-containing HAART regimens between HBeAg-positive and HBeAg-negative patients. For this study, HBeAg marker was not tested and therefore cannot be used to explain patients with detectable HBV DNA at 24 months of follow-up (Fang *et al.,* 2003). However, studies from sub-Saharan Africa have shown that the majority of African chronic carriers are mostly HBeAg-negative, with few studies detecting patients who are HBeAg-positive (reviewed by Hoffmann and Thio, 2007; Kramvis and Kew, 2007).

5.7.3.1 Virological outcome in patients with atypical serological patterns

Currently, clinical relevance of occult hepatitis B is uncertain. The majority of occult hepatitis B infection cases are characterised with low levels of HBV viral load, mostly ≤10000 copies/ml (Bre'chot *et al.,* 2001; Hu, 2002; Mphahlele *et al.,* 2006). This study detected 44% (34/78) of patients with occult hepatitis B infections, with an overall 82.4% (28/34) of patients having detectable HBV DNA ranging between <10 ≥10^8 IU/mL. Overall, the majority of occult hepatitis B infected patients (80%) showed a significant HBV virological response against lamivudine-containing HAART regimen by suppressing HBV DNA to undetectable levels. However, an excerption of 20% of patients had persistent HBV viral load, with 10% of these having viral load ranging between 10^4≥10^8 IU/mL. These cases with persistent HBV viraemia poses a significant concern, since a previous study from our laboratory has shown the development of lamivudine -associated drug resistant HBV strains in drug-naïve occult HBV infected individuals (Selabe *et al,.* 2007). While the remaining 10% had HBV viral load >10 IU/mL, the impact of prolonged anti-HBV therapy on HBV status in HBV carriers with extremely low HBV replication is unknown, because such cases will not be the target of anti-HBV antiviral therapy at the present time. Fang *et al.,* (2003), is the only study that investigated HBV infected individuals with low vireamia and HBsAg -positive on three co-infected patients. The three co-infected patients remained HBsAg -positive after prolonged HAART with lamivudine (300 mg/day) for an average of 16 months. The results lead to a speculation of HBsAg gene being perhaps integrated into the host genome (Chen *et al.,* 1982), and thus no longer susceptible to antiviral therapy. However, these studies have not investigated the impact of sub-optimal therapy during lamivudine-containing HAART regimens, as is the case in this study.

This study showed that the majority of occult HBV infected patients achieved the same response as HBsAg-positive patients. Thus, the results showed that while current HIV treatment guidelines in South Africa do not consider HBV as important opportunistic pathogen in the treatment of HIV patients, lamivudine-containing HAART have double benefit for patients also co-infected with HBV. This study advocates the significance of monitoring HBV, since lamivudine can pose a risk for patients developing resistance strains, as other studies have shown that long-term exposure to lamivudine can yield to the emergence of HBV-resistant strains (Benhamou *et al.,* 1999).

5.7.4 Impact of lamivudine-containing HAART regimens on immunological outcome

Immunological treatment success is generally defined as an increase in CD4 cell count, while for HIV and HBV virological treatment success is usually understood as a decrease in viral

load to undetectable levels (e.g. 50 copies/ml or 9.5 IU/ml) (Alberti *et al.,* 2005; Benhamou, 2006 and 2007). As expected, the majority of the studied patients (87%) had baseline CD4 cells levels <200 cells/ml, as they were candidates for initiating HAART. These, however, changed during 6 to 12 months period of follow-up, due to immune restoration associated with HAART, and the patients' CD4 cell levels increased to ≥200 cells/ml, 50% (21/42) and 66.7% (14/21), respectively. This shows a benefit of receiving HAART and patients suppressing HIV replication. The study showed that HAART is associated with immune restoration, and correlated with HBV and HIV viral loads suppression, showing that high viral loads correlated very well with low CD4 count.

HIV infections results in immunosuppression, hence, HIV/AIDS patients are easily susceptible to opportunistic infections such as HBV, which are able to replicate and become highly infectious (Thio *et al.,* 2002). However, the higher levels of viral replication are suppressed when patients start benefiting from lamivudine-containing HAART regimens. The CD4 cells increases and the majority of patients are able to suppress both viruses. The immune restoration associated with HAART has been shown to improve control of HBV replication, but can also lead to increased immune-mediated liver injury, and this may increase risk of hepatic death in HBV/HIV co-infected patients, compared with HBV-mono-infected patients, although data is still contradictory (Thio *et al.,* 2002). Individuals with high levels of HBV DNA (> 4 or 5 log10 copies/mL) or those with low nadir CD4 count may be particularly at risk. A previous study conducted by Miailhes *et al*., (2007) found that HBeAg or HBsAg seroconversion correlates with a sustained HIV response during antiretroviral therapy, especially with regimens which contains lamivudine. The study also found that patients with an elevated baseline ALT level, the HBV response was correlated significantly with immunorestoration following HAART. Overall the study results suggested that HAART regimens with dual activity against HIV and HBV are necessary for the majority of patients co-infected with both pathogens to optimise the HBV response (Alberti *et al*., 2005; Nunez *et al.,* 2005; Levy and Grant, 2006).

Finally, a study by Miailhes *et al.* (2007); showed that different HBV degrees of virological response in some patients, during evolution of the immune system, and changes in CD4 cell count, is clearly associated with different phases of chronic hepatitis B infection. The profiles of patients were observed to shift from chronic active hepatitis B to immunotolerance, because their CD4 cell counts decreased due to failure of HAART, and therefore, a dynamic evolution of the chronic hepatitis B pattern is dependent on changes in the immune system.

5.7.5 HBV and HIV drug resistance

The study investigated baseline and follow–up samples for HBV (78 baseline) and HIV (30 baseline) drug resistance, to establish the evolution of drug-resistant strains over time during treatment with lamivudine-containing HAART regimens, especially mutants that confer resistance to lamivudine with dual activity against HBV and HIV.

5.7.5.1 Drug-resistance to HIV-1

Unexpectedly, high baseline drug-resistant HIV strains were detected, with follow-up samples having an undetectable viral load. One patient had M184V mutant within the HIV RTIs. The M184V mutant is the most studied and commonly occurring NRTI resistance mutation. It causes a high level of resistance to lamivudine and emtricitabine (FTC), in both HBV and HIV (Balzarini *et al.,* 1996). It has also been shown to have low-level resistance against didanosine (ddI) and abacavir, (ABC) and increased susceptibility to zidovudine (ZDV), stavudine (d4T), and tenofovir (TDF) (Whitcomb *et al.,* 2003). The mutant emerges rapidly in patients receiving lamivudine monotherapy or during HAART -containing lamivudine (Wainberg, 2004 and; Svedhem *et al.,* 2007). The M184V usually develops after M184I mutant that result from a G to A mutation (ATG [methionine] to ATA [isoleucine]). This is because HIV RT is more prone to G to A substitutions than to A to G substitutions (ATG to GTG [valine]) (Keulen et al., 1996; Nijhuis et al., 1997)

The 184 mutant has been shown to develop because of the steric conflict between the oxathiolone ring of lamivudine and the side chain of beta-branched amino acids such as valine and isoleucine. The position 184 perturbs inhibitor binding, resulting to a reduction in lamivudine incorporation (Wainberg, 2004; Svedhem *et al.,* 2007). Data from multiple lamivudine-containing dual NRTI regimens also suggest that lamivudine continues to exert a beneficial effect even in patients whose virus isolates contain M184V (Miller *et al.,* 2002; Diallo *et al.,* 2003; Vray *et al.,* 2003). The role of lamivudine in these situations may be to maintain selective pressure on the virus to retain M184V, which increases HIV-1 susceptibility to AZT, d4T, and TDF.

Other HIV major NRTI's mutants that confer resistance against HIV only were also detected. These include M41L, which usually occurs with T215Y. Together they confer intermediate-to-high level resistance to AZT/d4T and a lower level of resistance to ddI, ABC, and TDF (Marcelin *et al.,* 2005). E44A/D occurs in patients receiving multiple NRTIs. E44A/D causes low-level resistance to 3TC and probably to each of the other NRTIs when present with V118I

or one or more TAMs. V118I occurs in ~2% of untreated persons and with increased frequency in persons receiving multiple NRTIs (Romano *et al.,* 2002). V75I increases multinucleoside resistance caused by Q151M when present with F77L and F116Y; its effect in the absence of Q151M is not known. V75T/M/A causes d4T and possibly ddI resistance. The significance of V75S and L are not known, although the former only occurs in treated persons. F77L increases multinucleoside resistance caused by Q151M when present with V75I or F116Y. L210W contributes resistance to each of the NRTIs except 3TC and probably FTC. It usually occurs with the mutations M41L and T215Y. L210F/S occurs rarely and is of unknown significance. T215Y/F cause AZT and D4T resistance and limits the effectiveness of ABC, ddI, and TDF particularly when it occurs in combination with M41L and L210W. 215S/C/D/E/I/V represents transitions between wild type and the mutations Y and F. Most of these mutants do not decrease drug susceptibility. But their presence suggests that a resistant virus that harbours T215Y or F may have been transmitted (Violin et *al.,* 2004; Bessong *et al.,* 2005 and 2006).

The study also detected major NNRTIs mutants: K103N, in 3 patients (P225H; K238T and Y318F). The K103N confers a high-level resistance to NVP (50-fold), delavirdine (DLV) (50-fold), and EFV (25-fold). It is the most detected mutants affecting the NNRTI's and occur more commonly than any other mutation in patients receiving NNRTIs. P225H is associated with EFV resistance when present with other NNRTI mutations. It confers hyper-susceptibility to DLV. K238T is an NNRTI-selected mutation that usually occur in combination with other NNRTI-resistance mutations and which appear to confer intermediate levels of resistance to NVP and possibly DLV and EFV. K238R is a naturally occurring variant that is common in some non-B subtypes and does not reduce NNRTI susceptibility. Y318F is a rare mutation that causes high-level DLV resistance and low-level NVP resistance. It usually occurs with other NNRTI-associated mutations (Bacheler et al., 2001; Delaugerre et al., 2001).

Examination of HIV PR revealed two primary drug resistance mutations, V82L (1/30), and D30I (1/30). These mutations have been recognised to confer broad cross-resistance to most PI (Vandamme *et al.,* 2004). Others were secondary mutants, L10I (3/30) H69K, L89M, I93L (30/30). Resistance to PIs requires multiple mutations to accumulate; some of these 'secondary mutations' are actually polymorphisms found in wild-type virus that alone do not appreciably reduce susceptibility, but along with so-called 'primary mutations' can produce higher levels of resistance. V82I occurs in about 1% of untreated individuals with subtype B

HIV-1 and in 5-10% of untreated individuals with non-B isolates (Vandamme *et al.,* 2004). Although V82I occasionally emerges during PI therapy, preliminary data suggest that V82I confers minimal or no resistance to the available PIs. The majority of these mutants were detected in the RESIST study and are mostly associated with non-subtype B strains (Baxter et al., 2006).

5.7.5.2 Drug-resistance to HBV

HBV resistance studies are rare in South Africa. In this study, only one of 78 patients carried YIDD (M204I) lamivudine-associated resistant HBV strains at baseline. However, the mutant reverted back to wild-strain on follow-up. The M204I has been reported to be induced by lamivudine as monotherapy or lamivudine-containing HAART regimens, and it occurs within the YMDD motif in the major catalytic region of the C domain of RT of the HBV polymerase gene in which methionine (M) is replaced by either isoleucine (I) or valine (V). Changes in the amino acid sequence were designated M204I according to the genotype-independent nomenclature (Stuyver *et al.,* 2001). The appearance of mutations in the YMDD region has been associated with an increased in ALT levels, and the reappearance of circulating HBV DNA usually at levels lower than baseline, and this is associated with reduction in the HBeAg seroconversion rate (Aye *et al.,* 1996; Perriolo *et al.,* 1999).

YMDD mutants have also been detected in some asymptomatic HBV carriers and patients with chronic hepatitis B who have never received lamivudine treatment (Kobayashi *et al*, 2001; Kirishima *et al*, 2002; Heo *et al*, 2004; Ohishi *et al*, 2004; Selabe *et al.,* 2007). The first study which reported YMDD mutants before lamivudine therapy in Japanese asymptomatic hepatitis B chronic carriers was published by Kobayashi *et al.* (2001). The study found that all the patients with YMDD mutants had positive anti-HBe antibody. Subsequently, other studies reported that YMDD mutants can emerge before therapy (Kirishima *et al.,* 2002; Heo *et al.,* 2004; Ohishi *et al.,* 2004; Selabe *et al.,* 2007).

5.7.5.3 Implications of drug-resistance

The patients studied for HIV drug resistance were drug-naive, and the results showed a high resistance pattern, (9/30), especially for HIV patients against the RT gene (NRT's and NNRT's) starting lamivudine-containing HAART regimens at DGM hospital. The presence of secondary PI resistance mutations and polymorphism may increase the likelihood for development of resistance in the presence of drug pressure. Previous studies in HIV drug-naïve patients from the same area detected no major PI in 95% of patients studied, detecting mostly wild mutants

against RT: V118I in 8.5% and Y318F in .5.7%. The mutants V118I and Y318F are associated with resistance to lamivudine and nevirapine, respectively. The results suggested that major resistance mutations among the drug-inexperienced population in South Africa in this area may be rare (Bessong *et al.,* 2006). However, this study shows a high resistance rate and this poses a challenge for continuous monitoring of HIV patients for drug-resistance. From the 12 months' follow-up, the South African HIV ARV programme was found to benefit most HIV patients with pre-existing drug-mutations. However, while the regimens are working in some patients, the study cannot generalise for all HIV patients. It is well known that patients who adhere to treatment usually come for further medical check-ups, while problematic patients usually don't come for follow-ups. There is therefore a need for continuous surveillance of drug-resistance, especially since the patients studied were too small to generalise.

While the results showed that HBV-resistance is not yet a concern from patients receiving lamivudine-containing HAART regimens, the study, however, encourages health care workers to screen for lamivudine, since resistance against lamivudine-containing HAART regimens have been reported to develop over time (Benhamou *et al.,* 1999; Benhamou, 2007). While this study detected only one patient with a lamivudine-resistant strain, a previous study from our laboratory has confirmed the detection of lamivudine-resistant HBV strains in therapy-naive HBV carriers with and without HIV infection, and in occult HBV co-infected individuals, suggesting an early emergence of antiviral resistance (Selabe *et al*., 2007).

5.8 Conclusions

Guidelines developed for the treatment of HBV in co-infected patients with HIV, recommend the suppression of serum HBV DNA to undetectable levels. HBV DNA is an acceptable surrogate marker for anti-HBV treatment success (Sulkowski, 2008). While South African public hospitals do not offer anti-HBV drugs, this study has showed that 68.4% of patients can experience sustainable virological response when exposed to lamivudine-containing HAART regimens for treatment of HIV, clearly supporting the fact that such regimens can benefit both HBV and HIV patients in co-infected individuals. This is a significant findings considering most resource-limited areas such as South Africa do not treat HBV. Continue monitoring of patients is also important as small percentage will have various degrees of HBV virological response, ranging from partial, reactivation or no response at all which may impact on long term choice treatment.

The study accentuates the need for screening HBV serological markers, and the use of molecular assays in detecting HBV DNA. Molecular assays are important, especially in occult

hepatitis B infections, as the study has shown patients (15.8%) having persistent detectable baseline and follow-up viraemia ≥10^8 IU/ml, irrespective of HBsAg status. This result should be underlined; as previous studies involving HBV/HIV co-infected patients have reported the development of HBV -resistant strains due to an exposure to anti-HBV drugs, such lamivudine at a rate of 20% and 50% at 1 and 2 years, respectively (Benhamou *et al.,* 1999; Matthews *et al.,* 2006; Selabe *et al.,* 2007). The current study did not detect any HBV resistant strains despite exposure against lamivudine for 24 months during HIV therapy.

The study has proved that while almost all HIV patients (91.3%) and 68.4% of HBV co-infected individuals respond to lamivudine-containing HAART regimens, HBV co-infected patients (18%), however, may experience varying degrees of virological response to such regimens (i.e. from complete, partial, possible reactivation, and to no virological outcome). This is due to sub-optimal monotherapy; hence, monitoring such patients is important. However, in practice, this study's results correlate with other studies, which suggests that a HAART regimen exhibiting dual activity against HIV and HBV is necessary for the majority of patients co-infected with HIV and HBV, to optimise the HBV response (Alberti *et al*., 2005; Nunez and Soriano, 2005; Levy and Grant, 2006).

This study has shown that the possibility of reactivation of HBV from previously immune patients (21.4%), due to exposure to lamivudine-containing HAART regimens, may pose a significant problem. However, continued monitoring may be significant, especially considering that the majority of HIV South African patients are exposed to/or chronic carriers of HBV.
While there is a high degree of antiviral resistance against HIV, especially in the RT gene, and no resistance in follow-up samples, long-term follow-up is still necessary to establish the evolutionary pattern of antiviral resistance, since other studies from both developed and developing countries have reported resistant strains in therapy-naïve patients

5.9 Implications

While the South African Comprehensive HIV and AIDS Care, Management, and Treatments Programme has shown to indirectly benefit in treating hepatitis B infection, this may-be short-lived in some patients due to HBV varying degrees of response against lamivudine-containing HAART regimens. This may have serious implications for the management of HIV as South Africa and of other developing countries do not screen for HBV despite being an important opportunistic pathogen. Long-term exposure of HBV against lamivudine results in the

development of HBV drug resistant strains, and HAART regimens (1a and 1b) are mostly afforded and widely used by South Africa.

5.10 Recommendations

Screening and monitoring of: (i) HIV patients initiating/ and receiving HAART,(ii) any HBV reactivations during exposure to HAART-containing dual activity against HBV, (iii) HBV viral load, and (iv) long -term development of drug-resistant strains

5.11 Limitation

HBeAg is an important and indirect replication marker of HBV infection, and could not be tested in this study; hence it was therefore difficult to assess the results using this marker.

The follow-up samples were variable and there was variation in comparing HBV virological response at various time points.

5.12 Further research questions

From this study, a number of other investigations could still be pursued. These include, but are not limited to:

- Long-term implications for HBV/HIV co-infection in patients on HAART-containing anti-HBV activity (including viral load and ALTs)
- HBV reactivation/re-infection studies during exposure with lamivudine-containing HAART regimens in previously exposed HIV-positive individuals
- HBV and HIV genotypic and subtype responses against HAART-containing anti-HBV activity (addressed in part in the next chapter)

CHAPTER SIX

6 INVESTIGATING THE GENETIC DIVERSITY OF HBV AND HIV CIRCULATING IN PRETORIA AND EXPLORING THE CORRELATION OF HBV GENOTYPES WITH RESPONSE TO ANTI-HBV-CONTAINING HAART REGIMENS

6.1 Background

6.1.1 Global HBV and HIV infections, and HAART

About 400 million people are chronically infected with HBV worldwide. Available data estimates that about 65 million of approximately 400 million people that are chronically infected with HBV reside in Africa. Africa and Asia carry the global burden of HBV infection, with cirrhosis and HCC accounting for 2% of annual deaths in Africa (reviewed by Kramvis and Kew, 2007). Many of the countries that are affected by hepatitis B are also affected by a high HIV burden, leading to frequent HIV/HBV co-infection. The majority of these cases occur in areas of Africa and Asia where HBV prevalence is high, with the sub-Saharan African region being the most affected by the HIV pandemic (Hoffman and Thio, 2007; Modi and Fled, 2007). Both viruses exhibit an extraordinary genetic variability, with potential impact on diagnostic and drug design. The HIV genetic variability has also lead to complication in vaccine design and development (Kellam and Larder, 1995).

Report for developed countries has led to speculations of increased liver disease from chronic hepatitis B emerging as an even greater problem in Africa and Asia due to a growing number of individuals accessing HAART and high HBV endemicity (population prevalence of HBV greater than 8%) (Hoffmann and Thio, 2007; Modi and Fled, 2007; William, 2006). HAART has significantly aided in progressing towards combating the devastating HIV epidemic in sub-Saharan Africa. This has, however lead co-infection with chronic hepatitis B virus becoming an increasingly relevant issue. This is despite incomplete data of co-infections in developing countries, with numerous studies from developed countries proposing the response to treatment with nucleo(t/s)ide analogues (e.g. lamivudine), and the progression to cirrhosis and HCC may be closely associated with the infecting genotype (Buti *et al.,* 2002; Modi and Feld, 2007). It is thus important to understand the correlations of HBV genotypes and HIV subtypes in HIV/HBV co-infection in regions with high chronic hepatitis B endemicity and expanding antiretroviral programmes, especially in view of the implications of using HAART agents that also possess anti-HBV activity. There are, however, no studies from Africa looking at the effect of HBV genotype on disease progression in co-infected patients (Modi and Feld, 2007).

6.1.2 HBV genetic diversity

HBV has been classified into eight genotypes (A to H) based on an intergroup divergence of 8% or more in the nucleotide sequences (Kramvis and Kew 2005). The genotypes have been associated with different geographical areas: genotype A with Europe and sub-Saharan Africa, genotypes B and C with East Asia, genotype D with the Mediterranean and Middle East regions, genotype E with Western Africa, and genotype F with the Americas. Genotype G has been observed in France and the United States (Stuyver *et al*., 2000). Genotype H has recently been discovered (reviewed by Kramvis and Kew, 2007; Kay and Zoulim, 2007). Within each genotype there are four major subtypes, based on the allelic determinants of the HBsAg; these are adr, adw, ayr and ayw (Thio, 2003). The genotypes are further subdivided into sub-genotypes with a difference of at least 4% (Kay and Zuolim, 2007) (Table 6.1).

Table 6.1: Summary of HBV genotypes, subtypes and serotypes (Kay and Zoulim, 2007)

Genotype	*Subtype*	*Serotype*	*Geographical Distribution*
A	A1 (Aa)	adw2, ayw1	Africa, Asia
	A2 (Ae)	adw2, ayw1	Northern Europe, North America
B	B1 (Bj)	adw2	Japan
	B2 (Ba)	adw2, adw3	Rest of Asia
	B3	adw2, ayw1	Indonesia, China
	B4	ayw1, adw2	Vietnam, Cambodia
C	C1	adrq+, ayr, adw2, ayw1	Far East
	C2	adrq+, ayr	Far East
	C3	adrq-, adrq+	Pacific Islands
D	D1	ayw2, adw1, ayw1	Europe, Middle East, Egypt, India, Asia
	D2	ayw3, ayw1	Europe, Japan
	D3	ayw3, ayw2, ayw4	Europe, South Africa, USA
	D4	ayw2, ayw3	Australia, Japan, Papua New Guinea
E		ayw4, ayw2	Sub-Saharan Africa, UK, France
F	F Ia	adw4, ayw4	Central America
	F Ib	adw4	Argentina, Japan, Venezuela, USA
	F II	adw4	Brazil, Venezuela, Nicaragua
	F III	adw4	Venezuela, Panama, Columbia
	F IV	adw4	Argentina
G		adw2	USA, Germany, Japan, France
H		adw4	USA, Japan, Nicaragua

Three HBV genotypes have been described as the most prevalent in Africa. These are: A (with subtype A1or Aa), D, and E. Genotype A1 is found in Southern African countries, while genotype D prevails in Northern Africa and genotype E (a variant seen only in sub-Saharan Africa) is found in Western and Central Africa (Kramvis *et al*., 2005). HBV genotype A has also been previously reported as the main genotype circulating in South Africa (Kramvis and Kew, 2007). Previous studies from our laboratory on samples from Pretoria and surrounding areas have also reported HBV genotype A as the predominant genotype (Selabe *et al*., 2007)

6.1.3 Relationship of HBV genetic diversity with disease progression and response to therapy

Several studies have associated different HBV genotypes with the severity of liver diseases and therapeutic responses (Mayerat *et al.,* 1999; Kidd-Ljunggren *et al.,* 2002; Schaefer, 2005). The association have remained inconclusive due to country genotype specific. Some countries with genotypes A and D had suggested genotype A infections lead to more chronic hepatitis than genotype D infection (Mayerat *et al.,* 1999). Country like Spain have reported contradicting results on long-term follow-up on chronic HBV carriers, who showed a significant higher viral clearance with genotype A carriers than other genotypes (Sanchez-Tapias *et al.,* 2002). Other countries with genotypes B and C, has observed genotype C infections being progressing more rapidly to cirrhosis and HCC, with symptoms appearing around the age of 30 years for those patients infected at birth (Ding et al., 2001; Kao *et al.,* 2000; Sumi *et al.,* 2003). Sero-conversion from HBeAg to anti-HBe was earlier in genotype B than C. Genotype C was also associated with high HBV DNA levels than in genotype B infected patients (Kramvis and Kew, 2005). Interestingly, was also the incidence of HCC observed more in individuals of 45 years being similar for genotype C or genotype B infections (Sumi *et al.,* 2003). In the Amazon basin, genotype F infections are associated with fulminant hepatitis, but this occurs in the context of co-infection or superinfection with hepatitis delta virus (Quintero *et al*., 2001). Other studies have found patients infected with genotype D being more likely to have elevated and have severe disease (Kidd-Ljunggren *et al.,* 2004). Another study conducted in a cohort of HIV co-infected patients, found that infection with HBV of genotype G was strongly associated with increased liver fibrosis (Lacombe *et al*., 2006).

Another interesting association was that of the relation between HBV genotypes on the influence of the outcome of HBV treatment. In a trial of pegylated interferon (Janssen *et al*., 2005), patients infected with genotypes A and B had a higher rate of HBeAg loss (about 45%) as compared to patients with genotype C or D (about 26%). On the other hand, in a Japanese study, while genotype B and C carriers responded well to interferon treatment, genotype A carriers responded poorly (Kobayashi *et al*., 2002). The discrepancy was speculated to be due to the type of interferon used, subtypes (probably Aa in the Japanese study and Ae in the European study), or to the small number of treated patients in the Japanese study. Another study reported that patients infected with genotype A are more prone to development of lamivudine-resistant mutations than patients infected with genotype D during the first year of treatment, but there was no difference in mutation development when treatment was prolonged for 2 or 3 years (Buti *et al*., 2002).

Kidd-Ljunggren *et al.,* (2004) reported patients infected with genotype D and HBeAg-positive, were more likely to have elevated ALT levels, compared to anti-HBe-positive patients. Genotype D was associated with more severe disease in some patients (McMillan *et al*., 1996). From Asian studies, where genotypes B and C are more prevalent, severity of liver dysfunction was associated more in people infected with genotype C than B (reviewed by Kramvis and Kew, 2005). Studies from Africa have shown sero-conversion from HBeAg to anti-HBe was earlier in genotype B than C. Genotype C was also associated with higher HBV DNA levels than in genotype B infected patients. However, in HBeAg-positive patients, genotype C was associated with low HBV DNA levels than genotype A, B and D. Genotype B infected patients were more likely to have sustained biochemical remission after spontaneous HBeAg sero-conversion than patients infected with genotype C (Kramvis and Kew, 2005).

There are studies linking HBV genotypes with clinical outcomes. Most of these studies were however subjective, and based the relationship of clinical consequences with genotype A only (Tanaka *et al.,* 2004; Kew *et al.,* 2005). A study on Southern African HBV carriers found that the mean age of patients infected with genotype A was 6.5 years younger than those infected with genotype non-A ($P < 0.05$) (Kew *et al*., 2005). Moreover, HBV carriers infected with subgenotype A1 had significantly ($P < 0.05$) lower levels of HBV DNA in both HBeAg-negative and HBeAg-positive relative to those infected with either genotype A2 or D. The frequency of HBeAg-positivity was lower in those infected with subgenotype A1, especially in HBV carriers younger than 30 years (Tanaka *et al*., 2004).

6.1.4 HIV genetic diversity

South Africa is considered to have experienced two HIV-1 epidemics. The first wave was confined to the homosexual population, with HIV-1 subtype B as the circulating variant (Williamson *et al.,* 1995). Later, HIV-1 subtype C was reported among the heterosexual population (reviewed in Papathanasopoulos *et al.,* 2003). Subtypes A, D and E (A/E recombinant) have also been identified, although in much smaller proportions (Becker *et al.,* 1995; Williamson *et al.,* 1995; Van Harmelen *et al*., 1997). With HIV, as time passes and with better evaluation techniques, HIV intersubtype recombinants and viruses with mosaic genomes have been described, although it appears that their prevalence is very low. Intersubtype recombinants have been largely detected in sex workers and residing foreigners (Bredell *et al.,* 2000 and 2002; Papathanasopoulos *et al.,* 2002). From these results, HIV-2 has only been reported in persons of West African origin, a region where HIV-2 is endemic, and not among the indigenous population.

6.2 Study problem, hypothesis, purpose and objectives

6.2.1 Study problem

Although HBV is hyperendemic in Africa, information on the distribution of the genotypes in various regions is not widely available, and very little is known about the relationship of different genotypes with disease progression or response to anti-HBV therapy. Few studies have reported genotype A as the predominant one, as well as some recombinant HBV genotypes in Africa (Kramvis and Kew, 2007; Kay *et al*., 2007). Another significant part is the correlation of HBV genotypes with response to anti-HBV drugs being given to HIV patients as part of ARV treatment, as no studies have been conducted in Africa. This study therefore seeks to investigate HBV and HIV diversity in Pretoria and surrounding areas, and the correlation of HBV genotypes with response to anti-HBV drugs as part of the expanding ARV programmes for treatment of HIV in South Africa. This is significant, as there are no studies on the impact of lamivudine-containing HAART regimens on different HBV genotypes in HBV/HIV co-infected patients in sub-Saharan Africa.

6.2.2 Hypothesis

South Africa is expanding the distribution of HAART in public hospital, and there may be an impact in HBV and HIV virological response to anti-HBV-containing HAART regimens in HBV/HIV co-infected patients due to different HBV genotypes.

6.2.3 Purpose

The purpose of the study was to explore the genetic diversity of HBV and HIV strains circulating in Pretoria and surrounding areas and to further investigate the correlation of HBV genotypes with response to anti-HBV-containing HAART regimens in co-infected patients.

6.2.4 Specific objectives

To explore the genetic diversity of HBV and HIV strains circulating in Pretoria and surrounding areas

To investigate whether response to anti-HBV-containing HAART regimens in co-infected patients correlates with HBV genotype

6.3 Study population and methods

6.3.1 Study populations

A total of 78 sequences from baseline samples and 45 sequences from followed-up samples were investigated for the genetic changes in the HBV polymerase gene sequences during treatment. HBV polymerase sequences were studied for HBV genetic diversity studies and

correlations to HBV response against lamivudine-containing HAART regimens. For HIV genetic diversity, only 30 sequences from baselines samples were studied (see Chapters 3 and 5, Table 3.1 and Table 5.1 for demographic status and serological results).

6.3.2 Extraction of viral nucleic acid

Refer to Chapter Four, Section 4.3.3.2.1

6.3.3 HBV and HIV PCR assays

Refer to Chapter Four, Section 4.3.3.2.2 and Chapter Five section 5.5.5.1

6.3.4 HBV and HIV sequencing assays

Refers to Chapter Four, Section 4.5.3.2 and Chapter Five, Section 5.5.5

6.3.5 Genetic genotyping

Both HBV and HIV genotyping were conducted by NCBI genotyping tool, (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi) and BioAfrica virus genotyping tools (Oxford HBV Automated Genotyping Tool version 1.0) and REGA HIV Subtyping Tool (Version 2.0) (http://www.bioafrica.net/virus-genotype/html), and the results compared. Both genotyping programs are an online version. The programs are designed to use phylogenetic methods in order to identify the subtype of a specific sequence. The sequences are analysed for recombination using bootscanning methods. A bootscanning method relies on the alignment of a suspected recombinant sequence with a set of potential parental reference sequences (groups of sequences from the different subtypes, or consensus sequences created from sets of reference sequences). After optimal alignment, the alignment is broken into sequential, overlapping segments (or windows) which are fed to a program for phylogenetic analysis (any of the sequence programs of Phylip could be used. Bootstrapped phylogenetic trees are built for each segment and finally, the bootstrap value for placing the unknown with each of the reference sequences/sequence groups is tabulated and plotted along the genome (Soltis and Soltis, 2003).

6.3.6 Phylogenetic analysis

Phylogenetic analyses of HBV and HIV polymerase genes were analysed from derived nucleotide sequences by aligning with representative subtype and intersubtype reference sequences from the GenBank with BioEdit, MAFFT version 6 (Katoh and Toh, 2008), Mega 4 and neighbour joining phylogenetic trees generated with the PHYLIP program, making use of the maximum likelihood model to take care of differences in the transition and transversion rates. Mean genetic distances were measured by the Kimura-2 parameter model available in Mega 4 software. Trees were rooted with the SIVcpz reference strain.

6.4 RESULTS

6.4.1 HBV Sequence analysis

6.4.1.1 HBV NCBI genotyping tool and BioAfrica virus genotyping tool

A total of 78 HBV sequences were submitted to NCBI and BioAfrica virus genotyping for comparison. Both genotyping programs gave the same HBV genotyping results, with the 77 sequences being HBV genotype A, and one sample ZADGM 52 being HBV genotype G. Genotyping with BioAfrica virus genotyping tool (Oxford HBV Automated Genotyping Tool version 1.0) gave the results indicating even the percentage homology (Table 6.2).

Table 6.2: HBV genotyping sequences with BioAfrica virus genotyping tool

HBV Sequence name	*Length*	*Assignment*	*Percent homology*
ZADGM1	609bp	subtype A	100.0
ZADGM17	606bp	subtype A	99.0
ZADGM24	597bp	subtype A	100.0
ZADGM31	599bp	subtype A	99.0
ZADGM53	611bp	subtype A	100.0
ZADGM105	624bp	subtype A	99.0
ZADGM131-2	624bp	subtype A	99.0
ZADGM131	609bp	subtype A	97.0
ZADGM725	630bp	subtype A	99.0
ZADGM117	590bp	subtype A	100.0
ZADGM291	568bp	subtype A	100.0
ZADGM275A	615bp	subtype A	94.0
ZADGM275	610bp	subtype A	100.0
ZADGM55	609bp	subtype A	100.0
ZADGM231	621bp	subtype A	100.0
ZADGM337	622bp	subtype A	99.0
ZADGM711	618bp	subtype A	100.0

6.4.1.2 HBV phylogenetic analysis

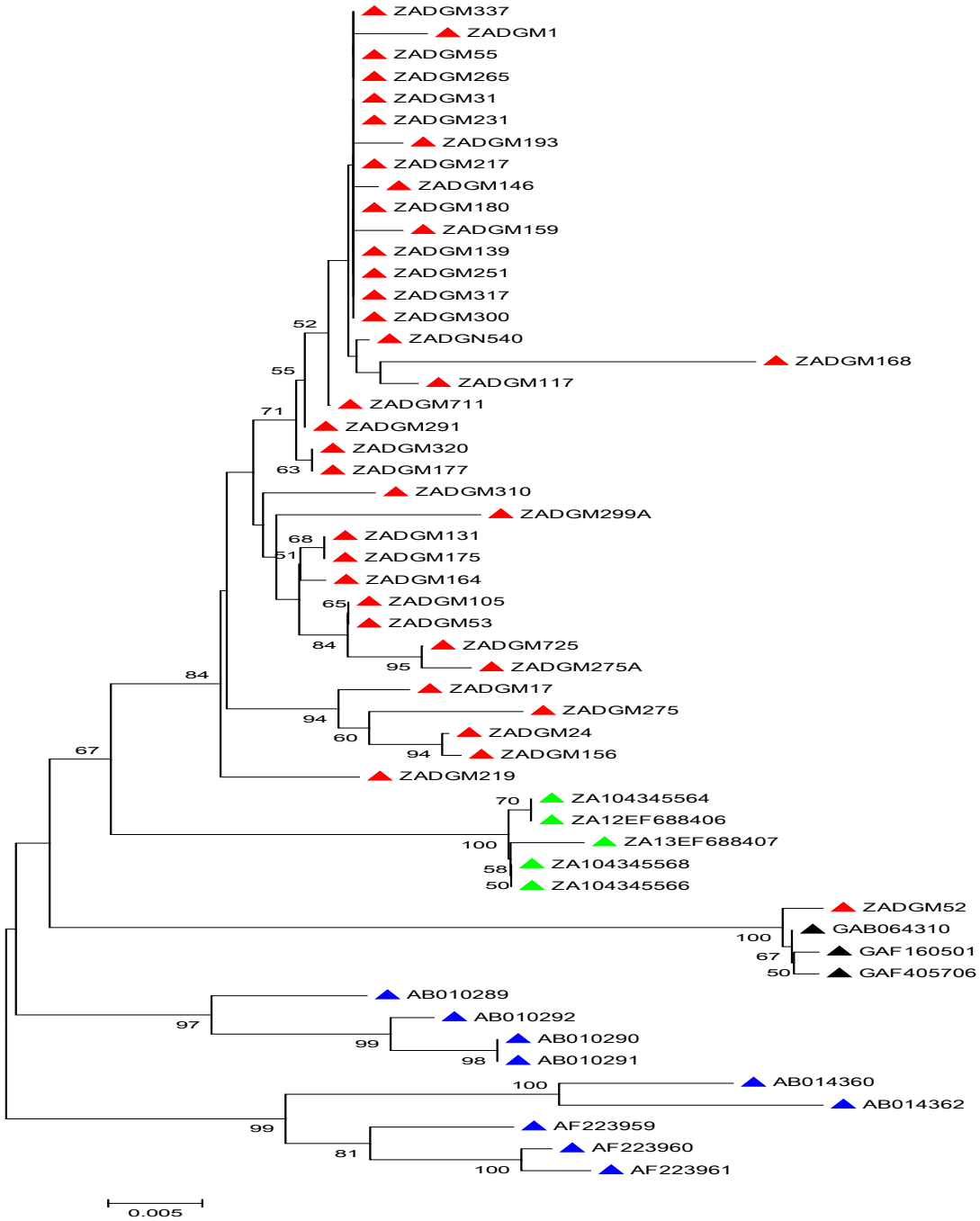

Figure 6.1: Phylogenetic analysis of HBV polymerase sequences. Red triangles refer to study samples; blue triangles represent reference strains HBV genotypes B, C, F; black triangles represent HBV genotype G, light green represents reference strains submitted to GenBank from our laboratory, genotype A (Selabe *et al*, 2007).

The HBV polymerase sequences from this study (red triangles, ZADGM 159 – ZADGM 219) formed a cluster on their own, separate from reference strains (blue triangles and light green

triangles) from South Africa and other regions. The study sequences clustered next to South African reference strains (ZA13EF688407, ZA104345568, ZA104345566, ZA1043455564 and ZA12EF688406), which were all genotype A. However, sample ZADGM52 did not cluster with other clinical samples, since it was found to be HBV genotype G. The sequence clustered with HBV genotype G reference strains from the GenBank (GAB0643310, GAF160501 and GAFA405706). Reference strains used were AB014360 – 62, and HBV genotype C, AB010289 genotype B and AF223962 genotype F. The bootstrap values are shown and the stringency was 1000. Bootstrapping values measure how consistent the data is supported, given taxon bipartitions. High bootstrap values (close to 100%) mean uniform support, i.e. if the bootstrap value for a certain clade is close to 100%, nearly all of the characters characteristic for this group agree that it is a group. "Stringency" is a term used to denote the degree of homology between the sequences; the higher the stringency, the higher percent homology between the sequences.

Nucleotides were further analysed for genetic relatedness using ClustalW 2 (http://www.ebi.ac.uk/clustalw2/). The strains had 87-100% homology between each other showing a great variability as they were mostly HBV genotype A (Table 6.5 see Appendix).

6.4.1.3 HBV GenBank accession numbers

HBV nucleotide polymerase sequences (most sequences were genotype A, except sequence ZADGM52, EF619364 being genotype G), were submitted to GenBank under the following accession numbers (Table 6.3):

Table 6.3: HBV polymerase sequences and GenBank accession numbers

HBV polymerase sequences and GenBank accession numbers			
HBV isolate	*GenBank accession number*	*HBV isolate*	*GenBank accession number*
ZADGM139	EF619390	ZADGM146	EF619389
ZADGM540	EF619388	ZADGM159	EF619387
ZADGM164	EF61386	ZADGM168	EF619385
ZADGM156	EF619384	ZADGM177	EF619383
ZADGM175	EF619382	ZADGM193	EF619381
ZADGM180	EF619380	ZADGM219	EF619379
ZADGM217	EF619378	ZADGM265	EF619377
ZADGM299A	EF619376	ZADGM300	EF619375
ZADGM251	EF619374	ZADGM310	EF619373
ZADGM317	EF619372	ZADGM320	EF619371
ZADGM711	EF619370	ZADGM337	EF619369
ZADGM231	EF619368	ZADGM55	EF619367
ZADGM275	EF61966	ZADGM275A	EF619365
ZADGM52	EF619364	ZADGM291	EF619363
ZADGM117	EF619362	ZADGM725	EF619361
ZADGM131	EF619360	ZADGM31	EF619356
ZADGM105	EF619358	ZADGM53	EF619357
ZADGM24	EF619355	ZADGM17	EF619354
ZADGM1	EF619353		

6.4.2 HIV sequence analysis

6.4.2.1 HIV NCBI genotyping tool and BioAfrica genotyping

A total of 30 HIV sequences were submitted to NCBI and BioAfrica virus subtyping, for comparison. Both subtyping programs gave the same HIV subtyping results, with the all sequences being HIV subtype C. The sequences had 100% homology to HIV subtype C reference strain, with both BioAfrica and NCBI genotyping tool (Table 6.4).

Table 6.4: HIV subtyping sequences with BioAfrica virus genotyping tool

Sequence ID	Length	Assignment	Percent homology
ZA467DGM	1548bp	HIV-1 Subtype C	100.0
ZA631DGM	1548bp	HIV-1 Subtype C	100.0
ZADGM	1547bp	HIV-1 Subtype C	100.0
ZA168DGM	1548bp	HIV-1 Subtype C	100.0
ZA265DGM	1547bp	HIV-1 Subtype C	100.0
ZA299GGM	1476bp	HIV-1 Subtype C	100.0
ZA317DGM	1548bp	HIV-1 Subtype C	100.0
ZA42DGM	1548bp	HIV-1 Subtype C	100.0
ZA24DGM	1548bp	HIV-1 Subtype C	100.0
ZA31DGM	1548bp	HIV-1 Subtype C	100.0
ZA239DGM	1548bp	HIV-1 Subtype C	100.0
ZA300DGM	1548bp	HIV-1 Subtype C	100.0
ZA108DGM	1548bp	HIV-1 Subtype C	100.0
ZA17DGM	1548bp	HIV-1 Subtype C	100.0
ZA320DGM	1548bp	HIV-1 Subtype C	100.0
ZA503DGM	1548bp	HIV-1 Subtype C	100.0
ZA729DGM	1548bp	HIV-1 Subtype C	100.0
ZA220DGM	1548bp	HIV-1 Subtype C	100.0
ZA15DGM	1548bp	HIV-1 Subtype C	100.0
ZA289DGM	1546bp	HIV-1 Subtype C	100.0
ZAUN24DGM	1548bp	HIV-1 Subtype C	100.0
ZA540DGM	1548bp	HIV-1 Subtype C	100.0
ZA219DGM	1548bp	HIV-1 Subtype C	100.0
ZA761DGM	1548bp	HIV-1 Subtype C	100.0
ZA67DGM	1548bp	HIV-1 Subtype C	100.0
ZAP24DGM11	1544bp	HIV-1 Subtype C	100.0
ZA37DGM	1536bp	HIV-1 Subtype C	100.0
ZA711DGM	1548bp	HIV-1 Subtype C	100.0
ZA105DGM	1548bp	HIV-1 Subtype C	100.0
ZA146DGM	1546bp	HIV-1 Subtype C	100.0
ZAP24C4DGM	1547bp	HIV-1 Subtype C	100.0

6.4.2.2 HIV phylogenetic analysis

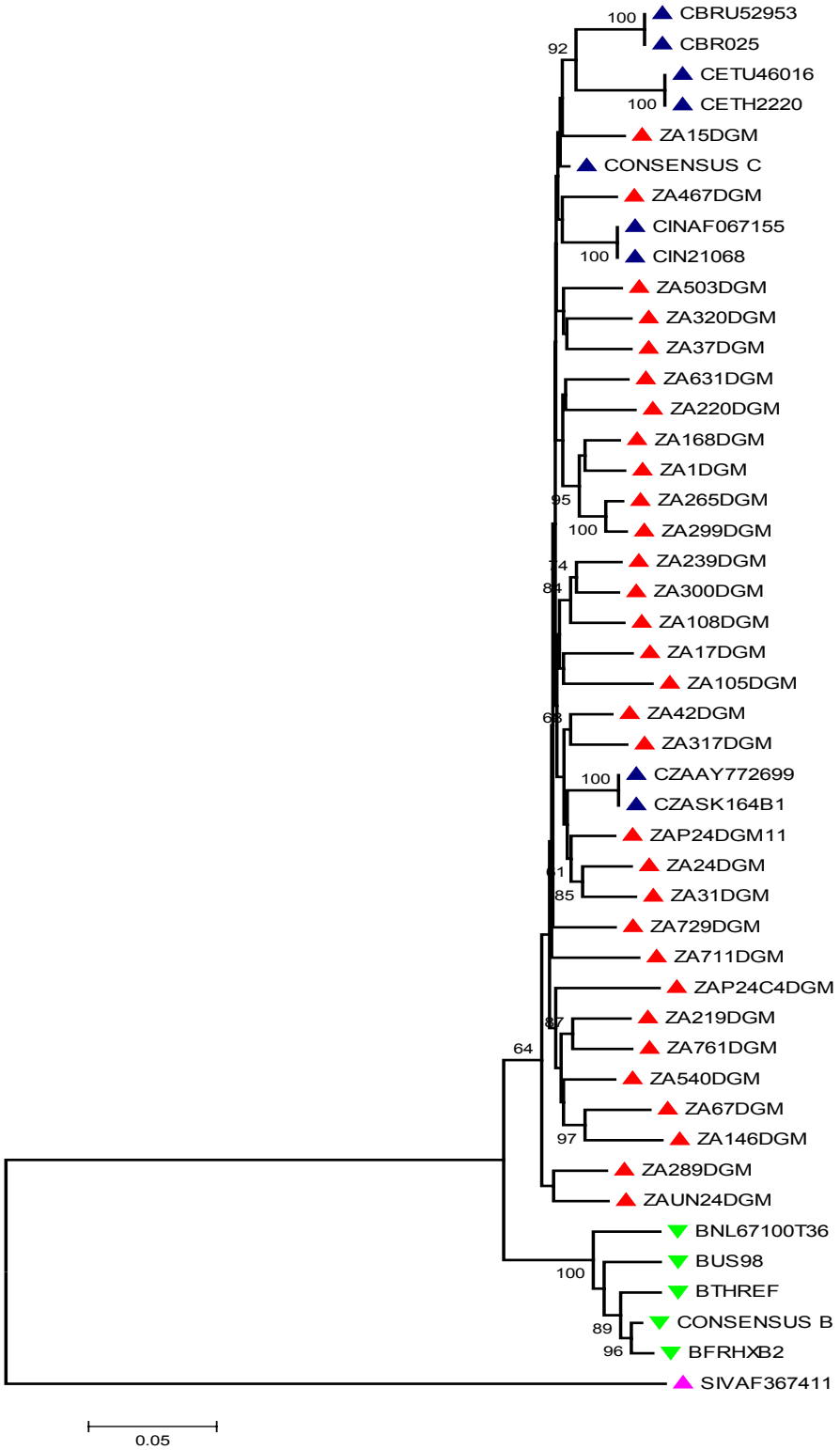

Figure 6.2: Phylogenetic analysis of HIV polymerase sequences. Red triangles refer to study samples; blue triangles represent reference strains HIV subtype C around the world and Consensus C from Los Almos; light green represents HIV subtype B with Consensus B from Los Almos. SIVA is an out-group.

The HIV polymerase sequences from this study (red triangles) intermingled with reference strains, HIV subtype C, blue triangles from South Africa (CZAAY772699 and CZASK164B1) and other regions [India (CINDAF067155 and CIN21068), Ethiopia (CETU46016 and CETH2220) and Brazil (CBRU52953 and CBR025)]. HIV subtype B formed a cluster separately from other sequences (light green triangles), with SIV sequences as an out-group. The bootstrap values are shown and the stringency was 1000.

Nucleotides were further analysed for genetic relatedness using ClustalW 2 (http://www.ebi.ac.uk/clustalw2/). These strains were variable between each other of 91-97% and 89% - 97% homology when compared with reference strains HIV Consensus B and C sequences (Table 6.6 see appendix).

6.4.3 Correlation between HBV genotypes and HIV subtypes with reference to HAART

A total of 45 follow-up samples were sequenced for HBV for any variable sequences between baseline and follow-up. Both baseline and follow-up samples were compared, and there were no sequence variations. Of the 18% of patients who had varied virological responses (discussed in Chapter Five, Section 5.6.1.2.1), no variation was found between baseline and followed-up sequences. All the patients were infected with HBV genotype A and HIV subtype C, with a total of 82% of patients having complete virological response against lamivudine-containing HAART regimens, as discussed in Chapter Six. Follow-up samples showed that the patients responded against lamivudine-containing HAART regimens.

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6.5 DISCUSSION

HBV is hyperendemic in sub-Saharan Africa and 2% of all annual deaths in Africa are due to clinical consequences of HBV infection which is high, considering that 25% of all annual deaths are caused by the clinical consequences of HIV infection (Kramvis and Kew, 2007). The study was set out to investigate the genetic diversity of HBV and HIV strains circulating in Pretoria and surrounding areas, and to further investigate the correlation of HBV genotypes or HIV subtypes with response to anti-HBV-containing HAART regimens in co-infected patients.

From 78 baseline samples, HBV genotyping results detected HBV genotype A from 77 (99%) samples and genotype G from one (1%) sample. Phylogenetic analyses with Mega 4 analysis program also grouped the clinical samples with HBV genotype G reference strains from GenBank. It showed 100% homology with reference HBV genotype G strains (GAB064310, GAF160501 and GAF405706). Genotype G has an insertion of 36 nucleotides in the core gene and was discovered in France and United States (Stuyver *et al*., 2000). Africa is one of the highly endemic regions for HBV, with five genotypes A-E identified. Genotype A in Kenya, genotype D in Tunisia, genotype A-D in South Africa and genotype E in Nigeria were reported as predominant genotypes in these countries. However, no study has reported on the detection of HBV genotype G in Africa (Kramvis and Kew, 2007; Stuyver *et al*., 2000; Borchani *et al*., 2000; Usuda *et al.*, 1999; Bowyer *et al*., 1997). Genotype G strains have been found to carry numerous mutations in the core promoter and precore region, and a unique 36-basepair insertion just downstream of the core start codon. The precore mutations preclude the expression of HBeAg, while the CP mutations may serve to enhance replication. The function of the insertion is unknown, but has been speculated to increase the expression of core (Kremsdorf *et al*., 1996).

Although little is known about the virological and clinical characteristics of HBV genotype G, one of its unique characteristics has been the frequent co-infection with the other HBV genotypes. In San Francisco, eight patients infected with HBV genotype G were co-infected with HBV genotype A (Kato *et al*., 2002; Kato *et al*., 2004), and all of the HBV genotype G isolates from Canada were also co-infected with HBV genotype A or C (Osiowy and Giles, 2003). However, from this study, co-infection with other HBV genotypes was not observed.

The study has confirmed that HBV genotype A is still the main genotype in South Africa. Previous studies conducted from our laboratory with samples around Pretoria and surrounding areas have also reported HBV genotype A as being the predominant genotype (Selabe *et al.*, 2007). HBV genotype A has also been previously reported as the main genotype circulating in South Africa (reviewed by Kramvis and Kew, 2007). There are a limited number of studies relating HBV genotypes to clinical outcomes from African countries, and most observations are subjective; however, studies relating to clinical consequences relative to genotypes in Africa

have been on genotype A. While this study did not find any correlation of HBV genotype A or G with respect to lamivudine-containing HAART regimens in South Africa, a study of southern African carriers of the HBV found that the mean age of patients infected with genotype A was 6.5 years younger than those infected with genotype non-A ($P < 0.05$) (Kew *et al*., 2005). Moreover, carriers infected with subgenotype A1 have significantly ($P < 0.05$) lower levels of HBV DNA in both the HBeAg and anti-HBeAg positive phases relative to those infected with either A2 or D. The frequency of HBeAg positivity is lower in those infected with subgenotype A1, especially in carriers younger than 30 years (Tanaka *et al*., 2004).

HIV subtype C was found to be the predominant subtype for this study. Phylogenetic analysis of the Pol gene showed that the isolates in this study were HIV – 1 subtype C, and were not distant, but intermingled with HIV subtype C from other countries. Previous studies from our laboratory have also detected subtype C to be the predominant subtype (Bessong *et al*., 2006). This finding was consistent with other studies in other regions of South Africa, where subtype C was the predominant subtype (Papathanasopoulos *et al.,* 2003; Bessong *et al*., 2005 and 2006). While South Africa continues to be one of the countries hardest hit by HIV and despite the high frequency of viral transmission, HIV-1 subtype C remains predominantly responsible for the epidemic

6.6 Conclusions

HBV genotype A (77/78 samples) was found to be the dominant genotype in South Africa. However, this study detected one patient (1/78) with HBV genotype G which has never been reported in Africa. HIV subtype C was found to be the prevalent subtype (30/30 samples). HBV genotypes and HIV subtype C were not observed to influence any treatment outcome with lamivudine-containing HAART regimens.

6.7 Further research questions

It would be interesting to perform full-length characterisation of the HBV genotype G sample.

CHAPTER SEVEN

7 COMBINED DISCUSSION, CONCLUSION AND RECOMMENDATIONS

7.1 Discussion

South Africa is one of the countries hardest hit by the HIV pandemic, and HBV co-infections are common, as both viruses share similar modes of transmission. With the dramatic scaling up of HAART in South Africa, there has been a move towards highly standardised treatment approaches (South Africa National Department of Health, 2004; WHO, 2006). However some drugs used as part of a combination ARV regimen for treatment of HIV have dual activity against HIV and HBV (e.g. lamivudine) and yet routine screening for HBV before initiating treatment for HIV is not a standard practice in South Africa. Hence, the study undertook to investigate: (1). the burden of HBV co-infection in HIV-positive patients enrolling for HAART at DGMH, (2) the impact of anti-HBV containing HAART regimens on HBV during the management of HBV/HIV co-infected patients, as well as the co-evolution of HBV and HIV drug-resistant strains, (3) and the genetic diversity of HBV and HIV circulating in Pretoria and exploring the correlation of HBV genotypes with response to anti-HBV containing HAART regimens.

To investigate the burden of HBV co-infections in HIV-positive patients, a total of 192 baseline sera of HIV-positive patients who were candidates for ARV therapy at DGMH were screened for HBV serological and molecular makers. The results indicated that the majority of South African HIV patients enrolling for HAART at DGMH are exposed to HBV infection and either have acute HBV infection or are HBV chronic carriers. A total of 63.0% of patients were found to have one or more HBV markers, with 40.6% having detectable HBV DNA as an indication of replication. The results pose a significant question on the use of anti-HBV-containing HAART in HIV patients, without HBV screening before initiation of treatment. Another significant finding in this study was the detection of occult HBV infections. The study detected 23% of 77% HBsAg-negative patients having occult hepatitis B infection. The results call into question the current screening methods of HBV since the majority of African countries may not afford application of molecular technology for initial screening of HBV. In developed countries, such atypical serologic findings have led to the recommendation that all patients with HIV infection undergo testing for HBsAg, anti-HBs, and anti-HBc. If the tests for HBsAg, anti-HBc, or both are positive,

these patients should be tested for HBV DNA, since therapy for both HIV and HBV infection may be needed. Patients without HBV DNA in serum (i.e. those who have anti-HBc alone) should be vaccinated against HBV, and may, like HIV-negative patients, have a primary or anamnestic response (Hofer *et al*., 1998; Piroth *et al*., 2002; Santos *et al*., 2003; Shire *et al*., 2004; Pogany *et al*., 2005; Gandhi *et al*., 2005; Mphahlele *et al*, 2006). HBV screening before initiation of therapy minimises the risk of HBV drug resistant strains.

To investigate the impact of anti-HBV-containing HAART regimens on HBV during the management of HBV/HIV co-infected patients, as well as the co-evolution of HBV and HIV drug resistant strains, a total of 78 patients were studied. Results indicated that HIV patients having detectable HBV DNA do benefit during lamivudine-containing HAART regimens for the management of HIV infections. A total of 68.4% of patients responded to HIV treatment, with undetectable HBV DNA during 18 to 24 months of follow-up. A total of 91.3% of HIV patients also responded to HAART, with undetectable HIV RNA during 6 to 12 months' follow-ups. However, 18% of patients had persistent HBV DNA, yielding various HBV virological responses against lamivudine-containing HAART regimens. This proportion of patients poses a question on the management of HBV and HIV co-infections, as guidelines on the use of HAART with anti-HBV activity from developed countries may not necessarily be followed in developing countries (Alberti *et al*., 2005).

In this study, some patients had virological reactivations during treatment, while other patients had persistent HBV DNA during treatment. The HBV reactivation may pose a significant problem, considering that the majority of South African HIV patients are either co-infected with, or exposed to, HBV. The study also indicates that while patients on lamivudine-containing HAART regimens benefit not only by suppressing HIV viral load, but also by improving immunologically (i.e. CD4 cell increases). There is conflicting data regarding the effect of chronic hepatitis B on CD4 lymphocyte recovery during HAART initiation in the HIV co-infected patients. Previous studies among the HIV-NAT cohort in Thailand, identified a lower mean increase in CD4 lymphocyte count in HIV/HBV co-infected (29 CD4 cells per µL) versus HIV-mono-infected (62 cells per µL) individuals after 4 and 8 weeks of HAART. However, by week 48, CD4 lymphocyte increases were similar, regardless of HBV status. In Nigeria, HIV RNA

suppression and absolute CD4 rise was similar between HBsAg-positive and HBsAg-negative patients initiating HAART. Results from an Italian cohort showed increasing divergence of mean CD4 lymphocyte count up to 36 weeks after HAART initiation between patients with and without chronic hepatitis B, with those with chronic hepatitis B having a lesser CD4 increase (p=0·03) (de Luca *et al*., 2002; Law *et al*., 2004; Idoko *et al*., 2007).

While the results showed that HIV baseline resistance was frequent, HBV resistance was not a concern from this cohort of patients. However, we cannot be complacent, since resistance against lamivudine-containing HAART regimens has been reported to develop over time, both in HBV mono- and HBV/HIV co-infected individuals (Benhamou *et al*., 1999; Benhamou, 2007), including patients from South Africa (Selabe *et al*., 2007). While this study detected only one patient with lamivudine-associated resistant strain, a previous study from our laboratory has, however, confirmed lamivudine-resistant HBV strains in therapy-naive HBV carriers with and without HIV infection, speculating an early emergence of antiviral resistance (Selabe *et al*., 2007).

Finally, this study explored the genetic diversity of HBV and HIV strains circulating in Pretoria and surrounding areas, as well as the correlation of HBV genotypes with response to anti-HBV-containing HAART regimens in co-infected patients. A total of 78 HBV and 30 HIV sequences were analysed, and their sequence diversity was compared and correlated with response to treatment. Results indicated that the HBV genotype A is still the most prevalent genotype circulating in South Africa. Of the 78 HBV sequences, 77 were genotyped as HBV genotype A and one sequence was genotype G. This is the first report from Africa on the detection of HBV genotype G, and, hence, further studies on this particular sequence are needed. Little data about HBV genotype G, and the correlation between the genotype and disease progression, is mostly from developed countries (Kato *et al*., 2002; Osiowy and Giles, 2003; Kato *et al*., 2004). Overall, there was no correlation between response to lamivudine-containing HAART regimens and HBV genotype or HIV subtype.

Studies on HIV sequences detected HIV subtype C as the only circulating subtype in South Africa, as previously reported (Engelbrecht *et al.,* 1999; Bredell *et al.,* 2000; Bessong *et al.,* 2005 and 2006).

7.2 Conclusions

The study has shown that the majority of patients with advanced HIV infection have high active HBV infection. The exposure rate was 63% as defined by a positive HBV serology, and active infection was 40.6% as defined by HBV DNA. Quantification of HBV DNA showed that these patients had detectable HBV viral loads, even as high as between 10^4 to $\geq 10^8$ IU/mL, irrespective of HBsAg status. The high levels of HBV DNA confirm the significance of screening for HBV before initiation of HAART with anti-HBV-containing regimens.

The results also indicate that HIV infection is a risk factor for occult HBV infections. The risk was also high especially in patients with anti-HBc marker as most individuals had detectable HBV DNA as high as $\geq 10^8$ IU/mL. An occult Hepatitis B infection poses a challenge to effective laboratory diagnosis of HBV in HIV-positive individuals especially from developing countries, where molecular assays are not widely available.

While the South African public sector does not offer anti-HBV drugs, this study confirmed that 68.4% of patients can experience a beneficial virological response when exposed to lamivudine-containing HAART regimens for the treatment of HIV. These clearly support the notion that such regimens can benefit both HBV and HIV patients in co-infected individuals, even in resource-limited areas such as South Africa; hence a careful consideration when initiating therapy with dual activity against HBV and HIV. Whether this virological response is temporary or sustained is, however, unknown at this stage. What is certain is that these patients require an effective monitoring programme, as (a) a small percentage (18%) experience variable HBV virological responses (partial, reactivation, or no response), (b) hepatitic flares are likely to develop if HAART is terminated, or the current HAART regimen is switched to another regimen without anti-HBV activity, and (c) resistant strains may develop over time and ultimately compromising alternate regimens due to cross-resistant. While there was a high baseline antiviral resistance against HIV, especially the RT gene, long-term follow-up with HBV will be necessary to establish the degree and evolution of antiviral resistance, since limited studies have reported the development of HBV lamivudine resistance strains in HBV drug naïve patients (Hoffmann and Thio, 2007; Selabe *et al.,* 2007).

HBV genotype A remains the dominant genotype in South Africa, but this study has detected HBV genotype G, which has never been reported in Africa. HIV subtype C was found to be the

prevalent subtype. HBV genotype or HIV subtype C were not seen to influence any treatment outcome, following treatment with lamivudine-containing HAART regimens.

7.3 Recommendations

Screening of HBV in HIV patients should be done before initiation of any anti-HBV-containing HAART regimens. The screening of HBV in HIV patients is also important, since some drugs included as part of HAART (e.g. nevirapine) can cause liver hepatotoxicity, and hence exacerbating HBV infections. This liver hepatotoxicity may lead to an increased morbidity and mortality due to liver complications especially with underlying HBV co-infections. Immunisation of HIV patients with low (< 10IU/L) or no immunity against HBV should be done, as this could be beneficial, although these patients may not respond optimally, or their immunity may wane faster, due to immunocompromised status. Monitoring of both HBV- and HIV-resistant strains should be conducted to detect early development of drug resistance.

7.4 Limitations of the study

The study could not conclude whether hepatitis B patients had current or chronic HBV infections as HBeAg and anti-HBe status were not tested. However, previous studies have shown the majority of South African individuals' acquire or being in contact with HBV during childhood (Kew, 1996; Kiire, 1996), hence chronic or reactivations due to immunosuppression will be appropriate. Further studies should be conducted to determine the impact of lamivudine-containing HAART regimens in HBV/HIV co-infected patients, comparing the virological outcome of HBeAg-positives and HBeAg-negatives. It was difficult to evaluate and correlate HBV virological response with ALT, as no follow-up ALT values were available. It is an important marker of liver damage. Finally, due to inconsistent total numbers of follow-up samples, it was difficult to compare the same number of patients over similar time points.

CHAPTER EIGHT

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CHAPTER NINE

9 APPENDIX

Table 6.5: HBV polymerase intersequence homology using ClustalW2(http://www.ebi.ac.uk/Tools/clustalw2/index.html)

SeqA	Name	Len(nt)	SeqB	Name	Len(nt)	Score(%)
6	ZADGM17	552	7	ZADGM24	552	98
6	ZADGM17	552	8	ZADGM156	552	98
6	ZADGM17	552	9	ZADGM105	552	97
6	ZADGM17	552	10	ZADGM53	552	97
6	ZADGM17	552	11	ZADGM725	552	96
6	ZADGM17	552	12	ZADGM164	552	96
6	ZADGM17	552	13	ZADGM131	552	97
6	ZADGM17	552	14	ZADGM175	552	97
6	ZADGM17	552	15	ZADGM131-2	552	96
6	ZADGM17	552	16	ZADGM310	552	96
6	ZADGM17	552	17	ZADGM31	552	97
6	ZADGM17	552	18	ZADGM231	552	97
6	ZADGM17	552	19	ZADGM265	552	97
6	ZADGM17	552	20	ZADGM217	552	97
6	ZADGM17	552	21	ZADGM300	552	97
6	ZADGM17	552	22	ZADGM251	552	97
6	ZADGM17	552	23	ZADGM146	552	97
6	ZADGM17	552	24	ZADGN540	552	97
6	ZADGM17	552	25	ZADGM180	552	97
6	ZADGM17	552	26	ZADGM55	552	97
6	ZADGM17	552	27	ZADGM711	552	97
6	ZADGM17	552	28	ZADGM337	552	97
6	ZADGM17	552	29	ZADGM320	552	98
6	ZADGM17	552	30	ZADGM177	552	98
6	ZADGM17	552	31	ZADGM193	552	97
6	ZADGM17	552	32	ZADGM159	552	97
6	ZADGM17	552	33	ZADGM317	552	97
6	ZADGM17	552	34	ZADGM219	552	97
6	ZADGM17	552	35	ZADGM299A	552	96
6	ZADGM17	552	36	ZADGM139	552	97
6	ZADGM17	552	37	ZADGM275	552	97
6	ZADGM17	552	38	ZADGM275A	552	96
6	ZADGM17	552	39	ZADGM291	552	98
6	ZADGM17	552	40	ZADGM168	552	94

6	ZADGM17	552	41	ZADGM1	552	96
6	ZADGM17	552	51	ZADGM52	552	91
6	ZADGM17	552	55	ZADGM117	552	95
7	ZADGM24	552	8	ZADGM156	552	99
7	ZADGM24	552	9	ZADGM105	552	97
7	ZADGM24	552	10	ZADGM53	552	96
7	ZADGM24	552	11	ZADGM725	552	96
7	ZADGM24	552	12	ZADGM164	552	96
7	ZADGM24	552	13	ZADGM131	552	97
7	ZADGM24	552	14	ZADGM175	552	97
7	ZADGM24	552	15	ZADGM131-2	552	96
7	ZADGM24	552	16	ZADGM310	552	96
7	ZADGM24	552	17	ZADGM31	552	97
7	ZADGM24	552	18	ZADGM231	552	97
7	ZADGM24	552	19	ZADGM265	552	97
7	ZADGM24	552	20	ZADGM217	552	97
7	ZADGM24	552	21	ZADGM300	552	97
7	ZADGM24	552	22	ZADGM251	552	96
7	ZADGM24	552	23	ZADGM146	552	96
7	ZADGM24	552	24	ZADGN540	552	96
7	ZADGM24	552	25	ZADGM180	552	96
7	ZADGM24	552	26	ZADGM55	552	96
7	ZADGM24	552	27	ZADGM711	552	96
7	ZADGM24	552	28	ZADGM337	552	96
7	ZADGM24	552	29	ZADGM320	552	97
7	ZADGM24	552	30	ZADGM177	552	97
7	ZADGM24	552	31	ZADGM193	552	96
7	ZADGM24	552	32	ZADGM159	552	96
7	ZADGM24	552	33	ZADGM317	552	96
7	ZADGM24	552	34	ZADGM219	552	96
7	ZADGM24	552	35	ZADGM299A	552	96
7	ZADGM24	552	36	ZADGM139	552	97
7	ZADGM24	552	37	ZADGM275	552	97
7	ZADGM24	552	38	ZADGM275A	552	96
7	ZADGM24	552	39	ZADGM291	552	97
7	ZADGM24	552	40	ZADGM168	552	94
7	ZADGM24	552	41	ZADGM1	552	96
7	ZADGM24	552	51	ZADGM52	552	91
7	ZADGM24	552	55	ZADGM117	552	94
8	ZADGM156	552	9	ZADGM105	552	96

8	ZADGM156	552	10	ZADGM53	552	96
8	ZADGM156	552	11	ZADGM725	552	96
8	ZADGM156	552	12	ZADGM164	552	97
8	ZADGM156	552	13	ZADGM131	552	96
8	ZADGM156	552	14	ZADGM175	552	96
8	ZADGM156	552	15	ZADGM131-2	552	96
8	ZADGM156	552	16	ZADGM310	552	96
8	ZADGM156	552	17	ZADGM31	552	96
8	ZADGM156	552	18	ZADGM231	552	96
8	ZADGM156	552	19	ZADGM265	552	96
8	ZADGM156	552	20	ZADGM217	552	96
8	ZADGM156	552	21	ZADGM300	552	96
8	ZADGM156	552	22	ZADGM251	552	96
8	ZADGM156	552	23	ZADGM146	552	96
8	ZADGM156	552	24	ZADGN540	552	96
8	ZADGM156	552	25	ZADGM180	552	96
8	ZADGM156	552	26	ZADGM55	552	96
8	ZADGM156	552	27	ZADGM711	552	97
8	ZADGM156	552	28	ZADGM337	552	96
8	ZADGM156	552	29	ZADGM320	552	97
8	ZADGM156	552	30	ZADGM177	552	97
8	ZADGM156	552	31	ZADGM193	552	96
8	ZADGM156	552	32	ZADGM159	552	96
8	ZADGM156	552	33	ZADGM317	552	96
8	ZADGM156	552	34	ZADGM219	552	96
8	ZADGM156	552	35	ZADGM299A	552	95
8	ZADGM156	552	36	ZADGM139	552	96
8	ZADGM156	552	37	ZADGM275	552	97
8	ZADGM156	552	38	ZADGM275A	552	96
8	ZADGM156	552	39	ZADGM291	552	97
8	ZADGM156	552	40	ZADGM168	552	93
8	ZADGM156	552	41	ZADGM1	552	95
8	ZADGM156	552	51	ZADGM52	552	90
8	ZADGM156	552	55	ZADGM117	552	94
9	ZADGM105	552	10	ZADGM53	552	99
9	ZADGM105	552	11	ZADGM725	552	99
9	ZADGM105	552	12	ZADGM164	552	99
9	ZADGM105	552	13	ZADGM131	552	99
9	ZADGM105	552	14	ZADGM175	552	99
9	ZADGM105	552	15	ZADGM131-2	552	98

9	ZADGM105	552	16	ZADGM310	552	98
9	ZADGM105	552	17	ZADGM31	552	98
9	ZADGM105	552	18	ZADGM231	552	98
9	ZADGM105	552	19	ZADGM265	552	98
9	ZADGM105	552	20	ZADGM217	552	98
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23	ZADGM146	552	33	ZADGM317	552	99
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23	ZADGM146	552	35	ZADGM299A	552	96
23	ZADGM146	552	36	ZADGM139	552	99
23	ZADGM146	552	37	ZADGM275	552	96
23	ZADGM146	552	38	ZADGM275A	552	96
23	ZADGM146	552	39	ZADGM291	552	99
23	ZADGM146	552	40	ZADGM168	552	96
23	ZADGM146	552	41	ZADGM1	552	98
23	ZADGM146	552	51	ZADGM52	552	91
23	ZADGM146	552	55	ZADGM117	552	96
24	ZADGN540	552	25	ZADGM180	552	99
24	ZADGN540	552	26	ZADGM55	552	99
24	ZADGN540	552	27	ZADGM711	552	99

24	ZADGN540	552	28	ZADGM337	552	99
24	ZADGN540	552	29	ZADGM320	552	99
24	ZADGN540	552	30	ZADGM177	552	99
24	ZADGN540	552	31	ZADGM193	552	99
24	ZADGN540	552	32	ZADGM159	552	99
24	ZADGN540	552	33	ZADGM317	552	99
24	ZADGN540	552	34	ZADGM219	552	97
24	ZADGN540	552	35	ZADGM299A	552	96
24	ZADGN540	552	36	ZADGM139	552	99
24	ZADGN540	552	37	ZADGM275	552	96
24	ZADGN540	552	38	ZADGM275A	552	96
24	ZADGN540	552	39	ZADGM291	552	99
24	ZADGN540	552	40	ZADGM168	552	96
24	ZADGN540	552	41	ZADGM1	552	98
24	ZADGN540	552	51	ZADGM52	552	91
24	ZADGN540	552	55	ZADGM117	552	96
25	ZADGM180	552	26	ZADGM55	552	99
25	ZADGM180	552	27	ZADGM711	552	99
25	ZADGM180	552	28	ZADGM337	552	99
25	ZADGM180	552	29	ZADGM320	552	99
25	ZADGM180	552	30	ZADGM177	552	99
25	ZADGM180	552	31	ZADGM193	552	99
25	ZADGM180	552	32	ZADGM159	552	99
25	ZADGM180	552	33	ZADGM317	552	99
25	ZADGM180	552	34	ZADGM219	552	97
25	ZADGM180	552	35	ZADGM299A	552	96
25	ZADGM180	552	36	ZADGM139	552	99
25	ZADGM180	552	37	ZADGM275	552	96
25	ZADGM180	552	38	ZADGM275A	552	96
25	ZADGM180	552	39	ZADGM291	552	99
25	ZADGM180	552	40	ZADGM168	552	96
25	ZADGM180	552	41	ZADGM1	552	98
25	ZADGM180	552	51	ZADGM52	552	91
25	ZADGM180	552	55	ZADGM117	552	96
26	ZADGM55	552	27	ZADGM711	552	99
26	ZADGM55	552	28	ZADGM337	552	99
26	ZADGM55	552	29	ZADGM320	552	99
26	ZADGM55	552	30	ZADGM177	552	99
26	ZADGM55	552	31	ZADGM193	552	99
26	ZADGM55	552	32	ZADGM159	552	99

26	ZADGM55	552	33	ZADGM317	552	99
26	ZADGM55	552	34	ZADGM219	552	97
26	ZADGM55	552	35	ZADGM299A	552	96
26	ZADGM55	552	36	ZADGM139	552	99
26	ZADGM55	552	37	ZADGM275	552	96
26	ZADGM55	552	38	ZADGM275A	552	96
26	ZADGM55	552	39	ZADGM291	552	99
26	ZADGM55	552	40	ZADGM168	552	96
26	ZADGM55	552	41	ZADGM1	552	98
26	ZADGM55	552	51	ZADGM52	552	91
26	ZADGM55	552	55	ZADGM117	552	96
27	ZADGM711	552	28	ZADGM337	552	99
27	ZADGM711	552	29	ZADGM320	552	99
27	ZADGM711	552	30	ZADGM177	552	99
27	ZADGM711	552	31	ZADGM193	552	98
27	ZADGM711	552	32	ZADGM159	552	98
27	ZADGM711	552	33	ZADGM317	552	99
27	ZADGM711	552	34	ZADGM219	552	97
27	ZADGM711	552	35	ZADGM299A	552	96
27	ZADGM711	552	36	ZADGM139	552	99
27	ZADGM711	552	37	ZADGM275	552	96
27	ZADGM711	552	38	ZADGM275A	552	96
27	ZADGM711	552	39	ZADGM291	552	99
27	ZADGM711	552	40	ZADGM168	552	96
27	ZADGM711	552	41	ZADGM1	552	98
27	ZADGM711	552	51	ZADGM52	552	91
27	ZADGM711	552	55	ZADGM117	552	97
28	ZADGM337	552	29	ZADGM320	552	99
28	ZADGM337	552	30	ZADGM177	552	99
28	ZADGM337	552	31	ZADGM193	552	99
28	ZADGM337	552	32	ZADGM159	552	99
28	ZADGM337	552	33	ZADGM317	552	99
28	ZADGM337	552	34	ZADGM219	552	97
28	ZADGM337	552	35	ZADGM299A	552	96
28	ZADGM337	552	36	ZADGM139	552	99
28	ZADGM337	552	37	ZADGM275	552	96
28	ZADGM337	552	38	ZADGM275A	552	96
28	ZADGM337	552	39	ZADGM291	552	99
28	ZADGM337	552	40	ZADGM168	552	96
28	ZADGM337	552	41	ZADGM1	552	99

28	ZADGM337	552	51	ZADGM52	552	91
28	ZADGM337	552	55	ZADGM117	552	97
29	ZADGM320	552	30	ZADGM177	552	100
29	ZADGM320	552	31	ZADGM193	552	98
29	ZADGM320	552	32	ZADGM159	552	98
29	ZADGM320	552	33	ZADGM317	552	99
29	ZADGM320	552	34	ZADGM219	552	98
29	ZADGM320	552	35	ZADGM299A	552	97
29	ZADGM320	552	36	ZADGM139	552	99
29	ZADGM320	552	37	ZADGM275	552	96
29	ZADGM320	552	38	ZADGM275A	552	97
29	ZADGM320	552	39	ZADGM291	552	99
29	ZADGM320	552	40	ZADGM168	552	96
29	ZADGM320	552	41	ZADGM1	552	98
29	ZADGM320	552	51	ZADGM52	552	91
29	ZADGM320	552	55	ZADGM117	552	96
30	ZADGM177	552	31	ZADGM193	552	98
30	ZADGM177	552	32	ZADGM159	552	98
30	ZADGM177	552	33	ZADGM317	552	99
30	ZADGM177	552	34	ZADGM219	552	98
30	ZADGM177	552	35	ZADGM299A	552	97
30	ZADGM177	552	36	ZADGM139	552	99
30	ZADGM177	552	37	ZADGM275	552	96
30	ZADGM177	552	38	ZADGM275A	552	97
30	ZADGM177	552	39	ZADGM291	552	99
30	ZADGM177	552	40	ZADGM168	552	96
30	ZADGM177	552	41	ZADGM1	552	98
30	ZADGM177	552	51	ZADGM52	552	91
30	ZADGM177	552	55	ZADGM117	552	96
31	ZADGM193	552	32	ZADGM159	552	98
31	ZADGM193	552	33	ZADGM317	552	99
31	ZADGM193	552	34	ZADGM219	552	97
31	ZADGM193	552	35	ZADGM299A	552	96
31	ZADGM193	552	36	ZADGM139	552	99
31	ZADGM193	552	37	ZADGM275	552	95
31	ZADGM193	552	38	ZADGM275A	552	96
31	ZADGM193	552	39	ZADGM291	552	98
31	ZADGM193	552	40	ZADGM168	552	96
31	ZADGM193	552	41	ZADGM1	552	98
31	ZADGM193	552	51	ZADGM52	552	91

31	ZADGM193	552	55	ZADGM117	552	96
32	ZADGM159	552	33	ZADGM317	552	99
32	ZADGM159	552	34	ZADGM219	552	97
32	ZADGM159	552	35	ZADGM299A	552	96
32	ZADGM159	552	36	ZADGM139	552	99
32	ZADGM159	552	37	ZADGM275	552	95
32	ZADGM159	552	38	ZADGM275A	552	96
32	ZADGM159	552	39	ZADGM291	552	99
32	ZADGM159	552	40	ZADGM168	552	96
32	ZADGM159	552	41	ZADGM1	552	98
32	ZADGM159	552	51	ZADGM52	552	91
32	ZADGM159	552	55	ZADGM117	552	96
33	ZADGM317	552	34	ZADGM219	552	97
33	ZADGM317	552	35	ZADGM299A	552	96
33	ZADGM317	552	36	ZADGM139	552	99
33	ZADGM317	552	37	ZADGM275	552	96
33	ZADGM317	552	38	ZADGM275A	552	96
33	ZADGM317	552	39	ZADGM291	552	99
33	ZADGM317	552	40	ZADGM168	552	96
33	ZADGM317	552	41	ZADGM1	552	98
33	ZADGM317	552	51	ZADGM52	552	90
33	ZADGM317	552	55	ZADGM117	552	96
34	ZADGM219	552	35	ZADGM299A	552	96
34	ZADGM219	552	36	ZADGM139	552	97
34	ZADGM219	552	37	ZADGM275	552	95
34	ZADGM219	552	38	ZADGM275A	552	96
34	ZADGM219	552	39	ZADGM291	552	98
34	ZADGM219	552	40	ZADGM168	552	94
34	ZADGM219	552	41	ZADGM1	552	96
34	ZADGM219	552	51	ZADGM52	552	91
34	ZADGM219	552	55	ZADGM117	552	95
35	ZADGM299A	552	36	ZADGM139	552	97
35	ZADGM299A	552	37	ZADGM275	552	95
35	ZADGM299A	552	38	ZADGM275A	552	96
35	ZADGM299A	552	39	ZADGM291	552	97
35	ZADGM299A	552	40	ZADGM168	552	94
35	ZADGM299A	552	41	ZADGM1	552	96
35	ZADGM299A	552	51	ZADGM52	552	90
35	ZADGM299A	552	55	ZADGM117	552	95
36	ZADGM139	552	37	ZADGM275	552	96

36	ZADGM139	552	38	ZADGM275A	552	96
36	ZADGM139	552	39	ZADGM291	552	99
36	ZADGM139	552	40	ZADGM168	552	96
36	ZADGM139	552	41	ZADGM1	552	98
36	ZADGM139	552	51	ZADGM52	552	91
36	ZADGM139	552	55	ZADGM117	552	97
37	ZADGM275	552	38	ZADGM275A	552	95
37	ZADGM275	552	39	ZADGM291	552	96
37	ZADGM275	552	40	ZADGM168	552	93
37	ZADGM275	552	41	ZADGM1	552	95
37	ZADGM275	552	51	ZADGM52	552	91
37	ZADGM275	552	55	ZADGM117	552	94
38	ZADGM275A	552	39	ZADGM291	552	97
38	ZADGM275A	552	40	ZADGM168	552	94
38	ZADGM275A	552	41	ZADGM1	552	96
38	ZADGM275A	552	51	ZADGM52	552	91
38	ZADGM275A	552	55	ZADGM117	552	95
39	ZADGM291	552	40	ZADGM168	552	96
39	ZADGM291	552	41	ZADGM1	552	98
39	ZADGM291	552	51	ZADGM52	552	91
39	ZADGM291	552	55	ZADGM117	552	97
40	ZADGM168	552	41	ZADGM1	552	95
40	ZADGM168	552	51	ZADGM52	552	88
40	ZADGM168	552	55	ZADGM117	552	94
41	ZADGM1	552	51	ZADGM52	552	90
41	ZADGM1	552	55	ZADGM117	552	96
51	ZADGM52	552	55	ZADGM117	552	87

Table6.6: HIV polymerase intersequence homology using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html)

SeqA	Name	Len(nt)	SeqB	Name	Len(nt)	Score(%)
1	CONSENSUS_B	1548	2	BFRHXB2	1548	98
1	CONSENSUS_B	1548	3	BTHREF	1548	97
1	CONSENSUS_B	1548	4	BUS98	1548	96
1	CONSENSUS_B	1548	6	CONSENSUS_C	1548	91
1	CONSENSUS_B	1548	7	CINAF067155	1548	90
1	CONSENSUS_B	1548	9	ZA467DGM	1548	90
1	CONSENSUS_B	1548	10	ZA168DGM	1548	90
1	CONSENSUS_B	1548	11	ZA42DGM	1548	90
1	CONSENSUS_B	1548	12	ZA239DGM	1548	90
1	CONSENSUS_B	1548	13	ZA300DGM	1548	90
1	CONSENSUS_B	1548	14	ZA1DGM	1548	90
1	CONSENSUS_B	1548	15	ZA265DGM	1548	90
1	CONSENSUS_B	1548	16	ZA299DGM	1548	90
1	CONSENSUS_B	1548	17	ZA108DGM	1548	90
1	CONSENSUS_B	1548	20	ZA317DGM	1548	90
1	CONSENSUS_B	1548	21	ZA729DGM	1548	90
1	CONSENSUS_B	1548	22	ZA17DGM	1548	90
1	CONSENSUS_B	1548	23	ZA24DGM	1548	90
1	CONSENSUS_B	1548	24	ZA31DGM	1548	90
1	CONSENSUS_B	1548	25	ZA540DGM	1548	90
1	CONSENSUS_B	1548	26	ZA320DGM	1548	89
1	CONSENSUS_B	1548	27	ZA503DGM	1548	90
1	CONSENSUS_B	1548	28	ZA15DGM	1548	89
1	CONSENSUS_B	1548	29	ZA631DGM	1548	90
1	CONSENSUS_B	1548	30	ZA220DGM	1548	90
1	CONSENSUS_B	1548	31	ZA289DGM	1548	89
1	CONSENSUS_B	1548	32	ZAUN24DGM	1548	90
1	CONSENSUS_B	1548	33	ZA219DGM	1548	90
1	CONSENSUS_B	1548	34	ZA761DGM	1548	90
1	CONSENSUS_B	1548	35	ZA67DGM	1548	90
1	CONSENSUS_B	1548	38	ZA711DGM	1548	90
1	CONSENSUS_B	1548	39	ZA105DGM	1548	89
1	CONSENSUS_B	1548	40	ZAP24DGM11	1548	89
1	CONSENSUS_B	1548	41	ZA37DGM	1548	90
1	CONSENSUS_B	1548	42	ZA146DGM	1548	89
1	CONSENSUS_B	1548	43	ZAP24C4DGM	1548	89
1	CONSENSUS_B	1548	44	CETU46016	1548	89
1	CONSENSUS_B	1548	45	CETH2220	1548	89
6	CONSENSUS_C	1548	7	CINAF067155	1548	97
6	CONSENSUS_C	1548	8	CIN21068	1548	97
6	CONSENSUS_C	1548	9	ZA467DGM	1548	97
6	CONSENSUS_C	1548	10	ZA168DGM	1548	97
6	CONSENSUS_C	1548	11	ZA42DGM	1548	96
6	CONSENSUS_C	1548	12	ZA239DGM	1548	97
6	CONSENSUS_C	1548	13	ZA300DGM	1548	96
6	CONSENSUS_C	1548	14	ZA1DGM	1548	96
6	CONSENSUS_C	1548	15	ZA265DGM	1548	97
6	CONSENSUS_C	1548	16	ZA299DGM	1548	96
6	CONSENSUS_C	1548	17	ZA108DGM	1548	96
6	CONSENSUS_C	1548	18	CZAAY772699	1548	97
6	CONSENSUS_C	1548	19	CZASK164B1	1548	97

6	CONSENSUS_C	1548	20	ZA317DGM	1548	96
6	CONSENSUS_C	1548	21	ZA729DGM	1548	96
6	CONSENSUS_C	1548	22	ZA17DGM	1548	96
6	CONSENSUS_C	1548	23	ZA24DGM	1548	96
6	CONSENSUS_C	1548	24	ZA31DGM	1548	96
6	CONSENSUS_C	1548	25	ZA540DGM	1548	96
6	CONSENSUS_C	1548	26	ZA320DGM	1548	96
6	CONSENSUS_C	1548	27	ZA503DGM	1548	96
6	CONSENSUS_C	1548	28	ZA15DGM	1548	96
6	CONSENSUS_C	1548	29	ZA631DGM	1548	96
6	CONSENSUS_C	1548	30	ZA220DGM	1548	96
6	CONSENSUS_C	1548	31	ZA289DGM	1548	96
6	CONSENSUS_C	1548	32	ZAUN24DGM	1548	96
6	CONSENSUS_C	1548	33	ZA219DGM	1548	96
6	CONSENSUS_C	1548	34	ZA761DGM	1548	96
6	CONSENSUS_C	1548	35	ZA67DGM	1548	95
6	CONSENSUS_C	1548	36	CBRU52953	1548	96
6	CONSENSUS_C	1548	37	CBR025	1548	96
6	CONSENSUS_C	1548	38	ZA711DGM	1548	96
6	CONSENSUS_C	1548	39	ZA105DGM	1548	95
6	CONSENSUS_C	1548	40	ZAP24DGM11	1548	97
6	CONSENSUS_C	1548	41	ZA37DGM	1548	96
6	CONSENSUS_C	1548	42	ZA146DGM	1548	95
6	CONSENSUS_C	1548	43	ZAP24C4DGM	1548	95
6	CONSENSUS_C	1548	44	CETU46016	1548	95
6	CONSENSUS_C	1548	45	CETH2220	1548	95
9	ZA467DGM	1548	10	ZA168DGM	1548	95
9	ZA467DGM	1548	11	ZA42DGM	1548	95
9	ZA467DGM	1548	12	ZA239DGM	1548	94
9	ZA467DGM	1548	13	ZA300DGM	1548	95
9	ZA467DGM	1548	14	ZA1DGM	1548	95
9	ZA467DGM	1548	15	ZA265DGM	1548	95
9	ZA467DGM	1548	16	ZA299DGM	1548	94
9	ZA467DGM	1548	17	ZA108DGM	1548	95
9	ZA467DGM	1548	20	ZA317DGM	1548	95
9	ZA467DGM	1548	21	ZA729DGM	1548	95
9	ZA467DGM	1548	22	ZA17DGM	1548	94
9	ZA467DGM	1548	23	ZA24DGM	1548	94
9	ZA467DGM	1548	24	ZA31DGM	1548	94
9	ZA467DGM	1548	25	ZA540DGM	1548	94
9	ZA467DGM	1548	26	ZA320DGM	1548	94
9	ZA467DGM	1548	27	ZA503DGM	1548	94
9	ZA467DGM	1548	28	ZA15DGM	1548	94
9	ZA467DGM	1548	29	ZA631DGM	1548	94
9	ZA467DGM	1548	30	ZA220DGM	1548	94
9	ZA467DGM	1548	31	ZA289DGM	1548	95
9	ZA467DGM	1548	32	ZAUN24DGM	1548	94
9	ZA467DGM	1548	33	ZA219DGM	1548	94
9	ZA467DGM	1548	34	ZA761DGM	1548	94
9	ZA467DGM	1548	35	ZA67DGM	1548	93
9	ZA467DGM	1548	38	ZA711DGM	1548	94
9	ZA467DGM	1548	39	ZA105DGM	1548	93
9	ZA467DGM	1548	40	ZAP24DGM11	1548	95
9	ZA467DGM	1548	41	ZA37DGM	1548	94
9	ZA467DGM	1548	42	ZA146DGM	1548	93
9	ZA467DGM	1548	43	ZAP24C4DGM	1548	93

10	ZA168DGM	1548	11	ZA42DGM	1548	95
10	ZA168DGM	1548	12	ZA239DGM	1548	95
10	ZA168DGM	1548	13	ZA300DGM	1548	94
10	ZA168DGM	1548	14	ZA1DGM	1548	97
10	ZA168DGM	1548	15	ZA265DGM	1548	97
10	ZA168DGM	1548	16	ZA299DGM	1548	97
10	ZA168DGM	1548	17	ZA108DGM	1548	94
10	ZA168DGM	1548	20	ZA317DGM	1548	94
10	ZA168DGM	1548	21	ZA729DGM	1548	94
10	ZA168DGM	1548	22	ZA17DGM	1548	94
10	ZA168DGM	1548	23	ZA24DGM	1548	94
10	ZA168DGM	1548	24	ZA31DGM	1548	94
10	ZA168DGM	1548	25	ZA540DGM	1548	94
10	ZA168DGM	1548	26	ZA320DGM	1548	94
10	ZA168DGM	1548	27	ZA503DGM	1548	95
10	ZA168DGM	1548	28	ZA15DGM	1548	94
10	ZA168DGM	1548	29	ZA631DGM	1548	95
10	ZA168DGM	1548	30	ZA220DGM	1548	94
10	ZA168DGM	1548	31	ZA289DGM	1548	94
10	ZA168DGM	1548	32	ZAUN24DGM	1548	94
10	ZA168DGM	1548	33	ZA219DGM	1548	94
10	ZA168DGM	1548	34	ZA761DGM	1548	94
10	ZA168DGM	1548	35	ZA67DGM	1548	93
10	ZA168DGM	1548	38	ZA711DGM	1548	93
10	ZA168DGM	1548	39	ZA105DGM	1548	93
10	ZA168DGM	1548	40	ZAP24DGM11	1548	94
10	ZA168DGM	1548	41	ZA37DGM	1548	94
10	ZA168DGM	1548	42	ZA146DGM	1548	93
10	ZA168DGM	1548	43	ZAP24C4DGM	1548	93
11	ZA42DGM	1548	12	ZA239DGM	1548	95
11	ZA42DGM	1548	13	ZA300DGM	1548	95
11	ZA42DGM	1548	14	ZA1DGM	1548	94
11	ZA42DGM	1548	15	ZA265DGM	1548	95
11	ZA42DGM	1548	16	ZA299DGM	1548	95
11	ZA42DGM	1548	17	ZA108DGM	1548	94
11	ZA42DGM	1548	20	ZA317DGM	1548	95
11	ZA42DGM	1548	21	ZA729DGM	1548	95
11	ZA42DGM	1548	22	ZA17DGM	1548	94
11	ZA42DGM	1548	23	ZA24DGM	1548	95
11	ZA42DGM	1548	24	ZA31DGM	1548	95
11	ZA42DGM	1548	25	ZA540DGM	1548	94
11	ZA42DGM	1548	26	ZA320DGM	1548	94
11	ZA42DGM	1548	27	ZA503DGM	1548	94
11	ZA42DGM	1548	28	ZA15DGM	1548	95
11	ZA42DGM	1548	29	ZA631DGM	1548	94
11	ZA42DGM	1548	30	ZA220DGM	1548	94
11	ZA42DGM	1548	31	ZA289DGM	1548	94
11	ZA42DGM	1548	32	ZAUN24DGM	1548	94
11	ZA42DGM	1548	33	ZA219DGM	1548	94
11	ZA42DGM	1548	34	ZA761DGM	1548	94
11	ZA42DGM	1548	35	ZA67DGM	1548	93
11	ZA42DGM	1548	38	ZA711DGM	1548	93
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32	ZAUN24DGM	1548	40	ZAP24DGM11	1548	94
32	ZAUN24DGM	1548	41	ZA37DGM	1548	93
32	ZAUN24DGM	1548	42	ZA146DGM	1548	93
32	ZAUN24DGM	1548	43	ZAP24C4DGM	1548	93
33	ZA219DGM	1548	34	ZA761DGM	1548	95
33	ZA219DGM	1548	35	ZA67DGM	1548	94
33	ZA219DGM	1548	38	ZA711DGM	1548	93
33	ZA219DGM	1548	39	ZA105DGM	1548	94
33	ZA219DGM	1548	40	ZAP24DGM11	1548	94
33	ZA219DGM	1548	41	ZA37DGM	1548	93
33	ZA219DGM	1548	42	ZA146DGM	1548	93
33	ZA219DGM	1548	43	ZAP24C4DGM	1548	93
34	ZA761DGM	1548	35	ZA67DGM	1548	94
34	ZA761DGM	1548	38	ZA711DGM	1548	93
34	ZA761DGM	1548	39	ZA105DGM	1548	94
34	ZA761DGM	1548	40	ZAP24DGM11	1548	94
34	ZA761DGM	1548	41	ZA37DGM	1548	93
34	ZA761DGM	1548	42	ZA146DGM	1548	93
34	ZA761DGM	1548	43	ZAP24C4DGM	1548	93
35	ZA67DGM	1548	38	ZA711DGM	1548	93
35	ZA67DGM	1548	39	ZA105DGM	1548	94
35	ZA67DGM	1548	40	ZAP24DGM11	1548	93
35	ZA67DGM	1548	41	ZA37DGM	1548	92
35	ZA67DGM	1548	42	ZA146DGM	1548	94
35	ZA67DGM	1548	43	ZAP24C4DGM	1548	92
38	ZA711DGM	1548	39	ZA105DGM	1548	93
38	ZA711DGM	1548	40	ZAP24DGM11	1548	94
38	ZA711DGM	1548	41	ZA37DGM	1548	93
38	ZA711DGM	1548	42	ZA146DGM	1548	92
38	ZA711DGM	1548	43	ZAP24C4DGM	1548	93
39	ZA105DGM	1548	40	ZAP24DGM11	1548	93
39	ZA105DGM	1548	41	ZA37DGM	1548	93
39	ZA105DGM	1548	42	ZA146DGM	1548	93
39	ZA105DGM	1548	43	ZAP24C4DGM	1548	92
40	ZAP24DGM11	1548	41	ZA37DGM	1548	94
40	ZAP24DGM11	1548	42	ZA146DGM	1548	92
40	ZAP24DGM11	1548	43	ZAP24C4DGM	1548	92
40	ZAP24DGM11	1548	44	CETU46016	1548	93
40	ZAP24DGM11	1548	45	CETH2220	1548	93
41	ZA37DGM	1548	42	ZA146DGM	1548	91
41	ZA37DGM	1548	43	ZAP24C4DGM	1548	92
42	ZA146DGM	1548	43	ZAP24C4DGM	1548	91