SEROPROFILE, GENETIC DIVERSITY AND DRUG RESISTANCE OF HEPATITIS B VIRUS AMONG HIV INFECTED INDIVIDUALS ATTENDING MAMA LUCY COMPREHENSIVE CARE CLINIC IN NAIROBI, KENYA

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Seroprofile, genetic diversity and drug resistance of hepatitis b virus among HIV infected individuals attending Mama Lucy Kibaki comprehensive care clinic in Nairobi, Kenya

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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This thesis has been submitted for examination with our approval as the University supervisors.

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DEDICATION

I dedicate this work to my husband Dr. Anthony Kebira and our lovely children, Janeel Jezreel, Joylean Jaasiel and Janviele Jean Kageha for giving moral support, prayers, endless love and enduring my absenteeism while in the laboratory doing the research. Lastly, to all HIV/HBV co-infected individuals in Nairobi County.

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ABBREVIATIONS AND ACRONYMS

3TC	Lamivudine
ADV	Adefovir dipivoxil
AIDS	Acquired Immunodeficiency syndrome
ART	Antiretroviral Therapy
ARVs	Antiretrovirals
BCP	Basal Core Promoter
cJNK	c-jun N-terminal kinase
CTL	Cytotoxic T Cells
ESLD	End-Stage Liver Disease
ETV	Entecavir
FDA	Federation of drug assay
FTC	Emetricitabine
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
HBsAb	Hepatitis B surface antibody
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus

HiDi	Highly deionized
IDUs	Injection Drug Users
IFN	Interferon
iNOS	Inducible Nitric Oxide Synthase
JAK	Janus Kinase
JNK	c-Jun N-terminal kinase
LdTt	Telbivudine
LHBS	Hepatitis B Virus large Protein
MAPK	Mitogenactivated Protein Kinase
MHR	Major Hydrophilic Region
NA	Nucleoside/Nucleotide Analog
NJ	Neighbor joining
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
Peg-INF	pegylated interferon
pgRNA	pregenomic RNA
PI 3-kinase	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C

Rb	Retinoblastoma gene
RNS	NO-derived reactive nitrogen species
RT	Reverse transcriptase
STAT	Signal Transducer and Activator of Transcription
TDF	Tenofovir Disofroxil Fumarate
UV	Ultra Violet
WHO	World Health Organisation
YMDD	Tyrosine-Methionine-Aspartate-Aspartate

ABSTRACT

Human immune deficiency virus (HIV) and Hepatitis B virus(HBV) coinfection is highly prevalent among high risk populations including pregnant women and infants. This poses a global public health challenge in laboratory diagnosis and is a major consideration for anti-HIV treatment. These viruses share common modes of transmission that is; through blood and body fluids. Further, there is little information on sero-profiles and circulating HBV genotypes in Kenya. This study aimed at determining seroprofiles, genetic diversity and drug resistance of HBV among HIV infected individuals attending comprehensive care clinic of Mama Lucy Kibaki Hospital Nairobi, Kenya. Ethical approval was sought from Kenyatta university ethics review committee and a cross-sectional study was conducted whereby the participants/guardians who gave consent/assent were included into the study. Their demographic data was collected using a questionnaire and 5ml of blood was collected from each participant using systematic sampling technique. The HBV seroprofiles were determined using the HBV-5 panel rapid diagnostic cassette according to the manufacturer's instructions (Healthaw Medical limited, Hangzhou, China) . Viral DNA was extracted using Qiagen® Miniviral DNA isolation kit and the HBV-*pol* gene amplified by nested PCR. The amplified products were sequenced using the Big Dye® sequence terminator kit (Applied Biosystem®) on an automated ABI 310 sequencer (Applied Biosystem, Foster City CA). The generated sequences of HBV were analysed for drug resistance and genetic diversity determined using Molecular Evolutionary Genetics Analysis (MEGA5). Four hundred participants were recruited and 293 were females, 107 were males with their age ranging between 4 months and 73 years. Of the 400 sera; (111) 27.8% were HBV immunized, 19 (4.8%) were recovery cases, 12 (3%) had acute disease, 10 (2.5%) were chronic, 9 (2.3%) had occult HBV and 7 (1.8%) asymptomatic. The prevalence of HBV/HIV was found to be 7.25% based on the presence of surface antigen. After the confirmation of HBV DNA by gel electrophoresis, 13 samples were successfully amplified, purified and sequenced.

All the 13 sequences were confirmed as HBV genotype A. Nucleotide drug resistance mutations were found in six (6) participants' samples. These were rtV173L, rtL180M, rtM204V which are major mutations associated with lamivudine, telbivudine and emtricitabine resistance. This study indicates that the utility of HBV seromarkers and infection staging are important in disease diagnosis. The findings confirm that, HBV genotype A remains the most predominant genotype circulating in Nairobi.This study proposes a need for a continuous surveillance of HBV genotype trends and evolution of drug resistance because the current findings have major implications on treatment of HBV in Kenya.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Hepatitis B virus (HBV) is the prototype member of the genus *Orthohepadnaviridae* of the family *Hepadnaviridae* that infects various avian and mammalian hosts) (Ganem and Schneider, 2001). It circulates in the serum as a Dane particle which is a round structure consisting of an envelope and an inner core of nucleocapsid protein, enclosing both polymerase and a partly double stranded circular viral DNA (Alexopolou *et al.*, 2005) of approximately size, 3.2 kb. Hepatitis B virus which is also referred as pregenomic RNA or pgRNA), its genome encodes four partially overlapped open reading frames (ORF): the *surface (preS1, preS2, S), core (precore, core), polymerase* and the '*X'* genes respectively(Ganem and Schneider, 2001) Since the viral polymerase lacks proofreading activity, the viral genome is highly variable (Seeger and Mason, 2000). Due to this limitation, the virus develops mutations which may not be fixed. Nevertheless, the highly overlapping nature of the open reading frames may not either fix them (Mizokami *et al.*, 1997).

These opposite aspects render the substitution rate of HBV to an intermediate level between RNA and DNA viruses (Mizokami *et al.*, 1997). Such a replication system leads to the virus developing mutations during replication, leading to diverse quesispecies of HBV (Datta, 2008). The nucleotide substitution rate of $1.4 – 5.0 \times 10^{-5}$ per site per year has been recorded, which is 10 fold superior to other DNA viruses. However, the rate of synonymous (silent) substitutions is found to be higher than the rate of non-synonymous substitutions, suggestive of a constrained evolution of the HBV genome (Ganem and Schneider, 2001) (Figure 1.1)

This virus mainly affects the liver, by infecting the hepatocytes leading to either acute or chronic disease. Globally two billion people are estimated to have been infected with Hepatitis B virus. Of these, 350-400 million people suffer chronic infection with estimated 240 million being HBV carriers (Muller *et al.*, 2015).

Therefore it is estimated that more than 600.000 HBV carriers who die every year from end-stage liver diseases including cirrhosis, fulminant hepatitis or hepatocellular carcinoma (HCC), therefore becoming a major global health problem.

Sub Saharan Africa, Asia and the Middle East have recorded the highest prevalence of HBV with an estimate between 5–10 % among the adult population who are chronically infected (Muller *et al.,* 2015). These rates are higher in these countries that western world where horizontal transmission is the most common route. Conversely, in western countries, sexual and parenteral (intravenous drug used) are the main routes of transmission. Nevertheless, since HIV and HBV share routes of transmission that is; through blood and body fluids co-infection has become relatively more frequent (Muriuki *et al.,* 2013). It is estimated that over four million (~10%) of the thirty six million people living with HIV worldwide are chronically co-infected with Hepatitis B virus. Most of these infections often occur in Africa and Asia with rates being up to 20% in some countries as compared to those from developed countries (<10%) (Alter, 2006).

The aim of this study was to determine the seroprevalence, genetic diversity and drug resistance of HBV in HIV infected individuals attending comprehensive care clinic of Mama Lucy Kibaki hospital. This hospital is situated in Umoja II estate off Kangundo road. Umoja II is in Embakasi sub County, Embakasi West Constituency in Nairobi County. The current situation of HIV/HBV coinfection has been recorded to be 6% (Muriuki *et al.,* 2013*).*

1.2 Statement of the problem

Hepatitis B virus (HBV) infection is serious public health concern worldwide. The information regarding the high exposure rate to HBV in Kenya is still elusive with recent reports indicating 6% and 12.5% HIV/HBV confection rate (Muriuki *et al.,* 2013; Kibaya *et al.,* 2015). However, the prevalence of active HBV infection is varied across different groups and regions in Kenya including those coexisting in the same geographic regions (Duarte *et al.,* 2010).

Hepatitis B virus genotype A, D and E have been detected in Kenya. Genotype A is the most predominant with low prevalence of sub-genotype A1 and A2 (Mwangi *et al.,* 2008). However, there is no recent data on the circulating HBV genotypes among HIV infected Kenyans and particularly in Nairobi. Like HIV-1, HBV replicates resulting into a large pool of quasi species during which HBV mutants are selected to become the dominant species. In addition, discontinuation of these drugs may potentially cause serious hepatocellular damage resulting from reactivation of HBV (Jang, 2004).

However, there is limited data on drug resistance that can guide patient management and tracking of HBV genotype circulation dynamics. Nevertheless, only a limited number of HBsAg mutations selected during antiviral treatment have been characterized. The diagnostic and public health implications of these mutations have also not yet been fully studied.

1.3 Justification of the study

Human immune deficiency virus (HIV) infections has been a growing public health problem globally. In Kenya, it has been shown that HIV/HBV coinfection increases morbidity and mortality beyond those caused by either infection alone (Muriuki *et al.,* 2013). Individuals co infected with HIV have higher levels of hepatitis B viremia, increased chances of disease progression and risk of developing liver

cirrhosis and liver cancer. However, very few studies have been conducted to track the infection rate and incidence of these viral strains within the country.

Despite the availability of vaccines for HBV, there is still increasing prevalence of this virus within the population, posing a challenge for clinicians in managing it especially in HIV/HBV co-infections. Human migration across the country(Kenya) and engagement in high risk behaviour like intravenous drug use and sexual commercial activities, the evolution of HBV/HCV HIV-1 might be changing and yet the distribution of HBV genotypes across population especially HBV in Kenya is still partially understood. The Kenyan HBV epidemic is predominantly of A, D and E genotypes. However, there is little information on HBV genetic diversity among HIV infected individuals. This study is therefore aimed at characterizing HBV infection stages, genetic diversity and assessing drug resistance mutations among HIV infected individuals for easy management of HIV/HBV infections.

1.4 Research questions

i. What are the seroprofiles of HBV among HIV infected individuals attending Mama Lucy Kibaki Hospital Comphrensive Care Clinic, in Nairobi?
ii. What are the circulating HBV genotypes among HIV/HBV infected individuals attending Mama Lucy Kibaki hospital Comprehensive Care Clinic in Nairobi?
iii. What are the HBV drug resistance mutations among HIV/HBV infected individuals attending Mama Lucy Kibaki hospital Comprehensive Care Clinic in Nairobi?

1.5 Objectives

1.5.1 Broad objective:

To determine the seroprofiles, genetic diversity and drug resistance of HBV among HIV infected individuals attending Comprehensive Care Clinic of Mama Lucy Kibaki hospital, Nairobi.

1.5.2 Specific objectives

i. To determine the seroprofiles of HBV among HIV infected individuals attending Comprehensive Care clinic of Mama Lucy Kibaki hospital, Nairobi.
ii. To determine the HBV genetic diversity among HIV infected individuals attending Comprehensive Care Clinic of Mama Lucy Kibaki hospital, Nairobi.
iii. To determine HBV drug resistance mutations among HIV infected individuals attending Comprehensive Care clinic of Mama Lucy Kibaki hospital, Nairobi.

CHAPTER TWO

LITERATURE REVIEW

2.1 The HBV structure

Hepatitis B virus (HBV) is an enveloped DNA virus with a diameter ranging between 42 - 47 nm surrounded by lipoprotein (Tiollais *et al.*, 1985). The viral genome of HBV is relaxed-circular, partially duplex DNA of 3.2 kb with the characteristic feature of lacking symmetry in its partial double stranded DNA (Seeger and Mason, 2000). The DNA contains a minus strand that is complementary to viral mRNAs and a partially completed plus strand (Lutwick and Robinson, 1977).

The genome is a compact and has small structure that is organized into four open reading frames (ORF). These encode the core/precore (*C* ORF), polymerase (*pol* ORF), envelope (*S* ORF), and *X* ORF. The biggest is the *pol* ORF which encodes the viral polymerase and its accessory functions. The *S* ORF is totally overlapped within the *pol* ORF and encodes the different envelope proteins. *C* ORF encodes the e antigen and the core (HBeAg and HBcAg) and partially overlapping the *pol* ORF is the *X* ORF which encodes the HBxAg (Thomas *et al.*, 2005). The viral DNA and DNA polymerase are contained in the icosahedral nucleocapsid composed of core protein (HBcAg), enclosed by the outer lipid membrane (envelope) in which large (LHBs), middle (MHBs) and small (SHBs) surface proteins are embedded (Kann, 2007) (Figure 2.1).

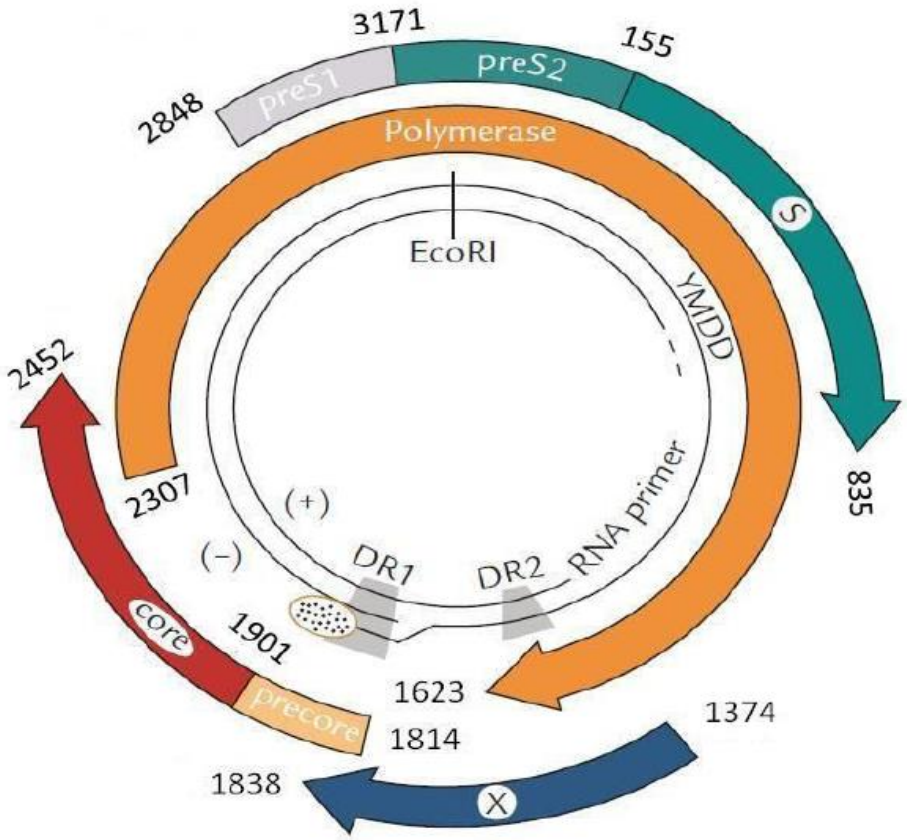

Figure 2.1: Structure and organization of hepatitis B virus genome (Lin & Kao, 2011)

2.2 The HBV replication cycle

The viral replication cycle is divided into several steps. First step begins when the virus infects the hepatocytes in the liver (Fig 1.2). Hepatitis B virus will attach to hepatocyte-specific preS1 receptor .The pre-S1 attached to the virus then interacts with sodium taurocholate cotransporting polypeptide (NTCP), which is a multiple trans-membrane transporter, is expressed mainly in the liver (Yan *et al.*, 2012). After attachment, the virus may enter the cell either by fusion and finally penetrating through plasma membrane or by endocytosis of the viral nucleocapsid followed by fusion to the cell. This is then followed by viral nucleocapsid release inside the cytoplasm (Urban *et al.*, 2010) where it is transported to the nucleus. At this point, the relaxed circular DNA (rcDNA) is then released into the

nucleoplasm (Kann *et al.*, 2007).The rcDNA is repaired by the viral polymerase at the nucleoplasm, which completes the synthesis of the positive strand. Thereafter the ligation of both positive and negative DNA strands occurs and the covalently closed circular DNA (cccDNA) is formed which serves as the transcriptional template for RNA polymerase II (Nassal, 2008). This trancription is directed by four different promoters (pre-S1, enhancer I/X, enhancer II/BCP, pre-S2/S,) that produce four different sized species of mRNA (Feitelson, 1992; Levrero *et al.*, 2009).

The largest one is the pre-genomic RNA (pgRNA) which encodes the HBcAg, HBeAg and the HBV polymerase. The smallest mRNA encodes the HBxAg whereas the remaining two encode the surface proteins. The pgRNA also serves as a template for reverse transcription which occurs after the genome is packaged into the core particle (Feitelson, 1992; Levrero *et al.*, 2009). The new nucleocapsid has two potential fates; it can either be re-imported to the nucleus to generate more cccDNA molecules (common occur at initial stage of infection) or it can be transferred to the endoplasmic reticulum (ER) where envelope proteins are incorporated before release from the cell. Mature virions containing HBsAg proteins are secreted along with spherical and filamentous enveloped sub-viral particles (SVPs), which lack a nucleocapsid and are non-infectious (Urban *et al.*, 2010).

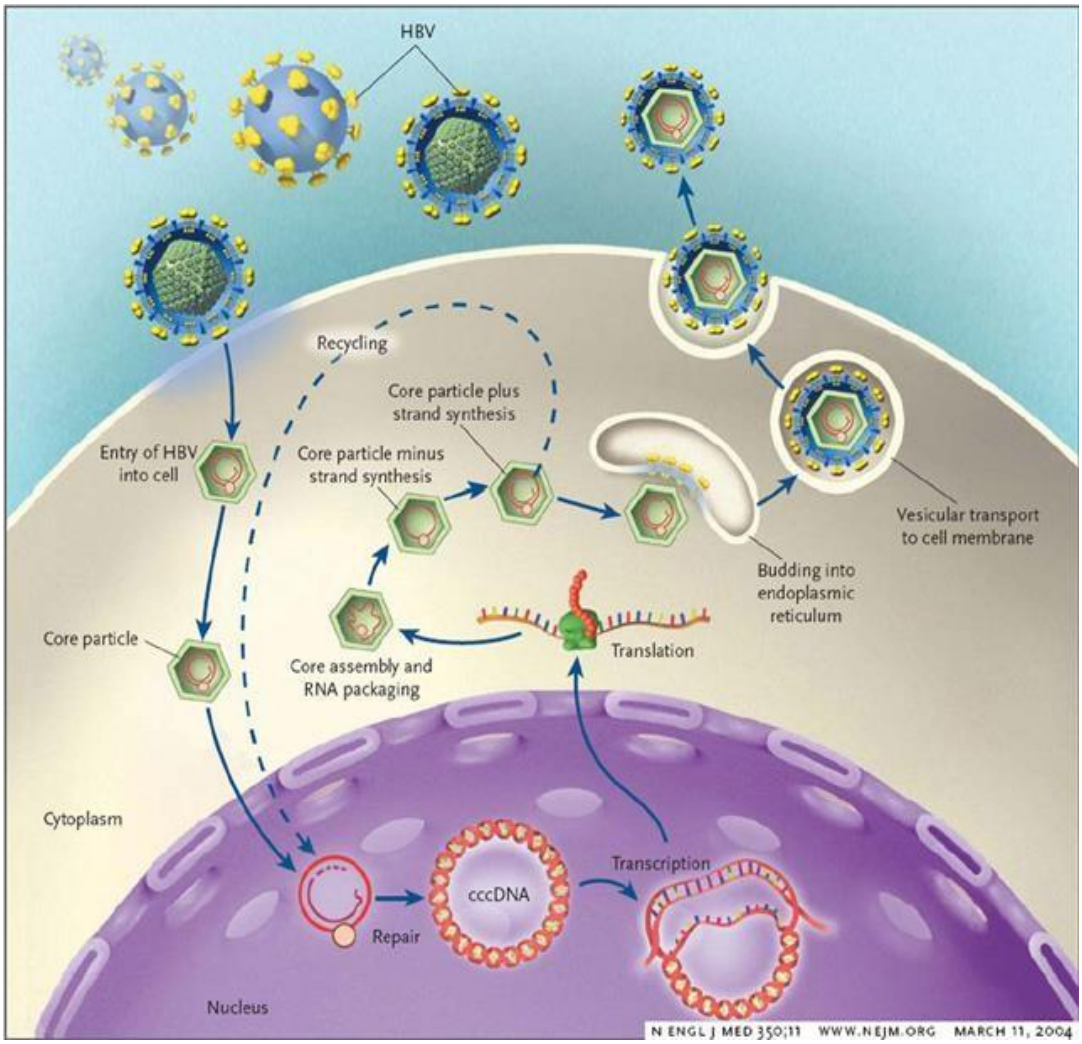

Figure 2.2: Hepatitis B virus replication cycle (Ganem & Prince, 2004)

2.3 Pathogenesis of HBV

Blood is the main carrier of HBV infection but other body fluids like semen and saliva have also been found to play role in viral transmission (Hou *et al.,* 2005).Additionally, in high endemic areas, vertical transmission is the most common roue with horizontal routes being common route during the preschool years. In low endemic areas, sharing of syringes between injections by drug users and unprotected sex are the main routes of transmission (Lavanchy, 2004).Other risk factors for HBV infection include; use of unscreened blood, tattooing, usage of non-sterilized instruments and multiple-dose vials in health care settings predisposes one to HBV infection. The outcome of HBV infection may vary across patients ranging from asymptomatic and self-limited infection to fulminant hepatitis and chronic disease. The risk of chronic infection is inversely proportional to age. Persistent infection has been reported in up to 90% of the infected infants at birth and 20-50% at infection between ages 1-5 (Shapiro, 1993).The risk of chronic infection in adults is less than 5% (Hyams, 1995).

2.3.1 Natural hhistory of HBV infection

The incubation period of HBV following successful infection typically is 8 to 12 weeks (Shepard *et al.,* 2006). At this stage, the clinical presentation will range from subclinical/asymptomatic carriage to acute self-limiting hepatitis or fulminant hepatitis to chronic hepatitis. Infection progression depends on the person's age, viral factors and the host immune response which could lead to liver cirrhosis and/or Hepatocellular carcinoma (HCC) (Shepard *et al.,* 2006). For instance, age depending at the time of infection, it has been shown that majority of perinatal or childhood infections are found to be asymptomatic that may proceed to chronicity. However, in adults the infection is usually acute and self resolving (Rodes *et al.,* 2003) (Figure 2.1).

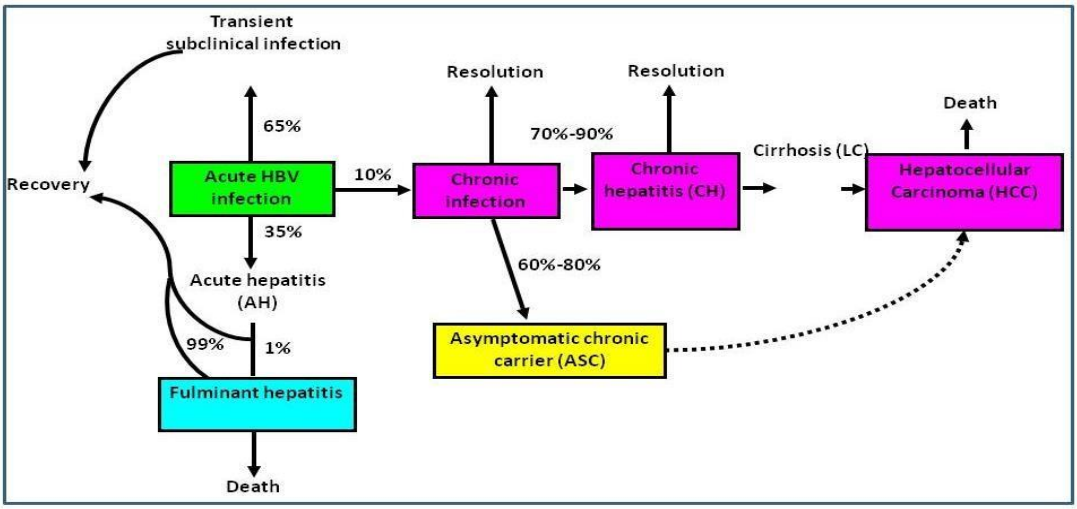

Figure 2.3: Natural history of Hepatitis B virus Infection (Feitelson, 1992)

2.3.2 Acute hepatitis

The acute phase of the HBV infection is observed after six months of infection. The clinical symptoms associated with acute infection include; jaundice, nausea, weight loss and flu-like illness. However, patients may also suffer from fever, urticaria and arthralgia. These symptoms generally subside within a few weeks along with disappearance of HBV DNA and seroconversion from HBeAg to anti-HBe. A large proportion of the cases are asymptomatic and the infection may pass without notice (Blackberg & Kidd-Ljunggren, 2000). Most patients with acute hepatitis B are HBsAg positive at presentation, but the critical test is IgM anti-HBc, which confirms acute HBV infection. If HBsAg is detected after six months of infection, the patient is considered to be a chronic carrier (Figure 2.2).

The risk for chronic disease is usually low in adults (Blackberg & Kidd-Ljunggren, 2000). Acute hepatitis may in some cases progress to fulminant hepatitis leading to liver failure (Fagan & Williams, 1990), a state that is associated with high mortality. Antiviral treatment with lamivudine or other nucleoside analogues is recommended in such cases, although liver transplantation would still be deemed necessary (Wang and Tang, 2009).

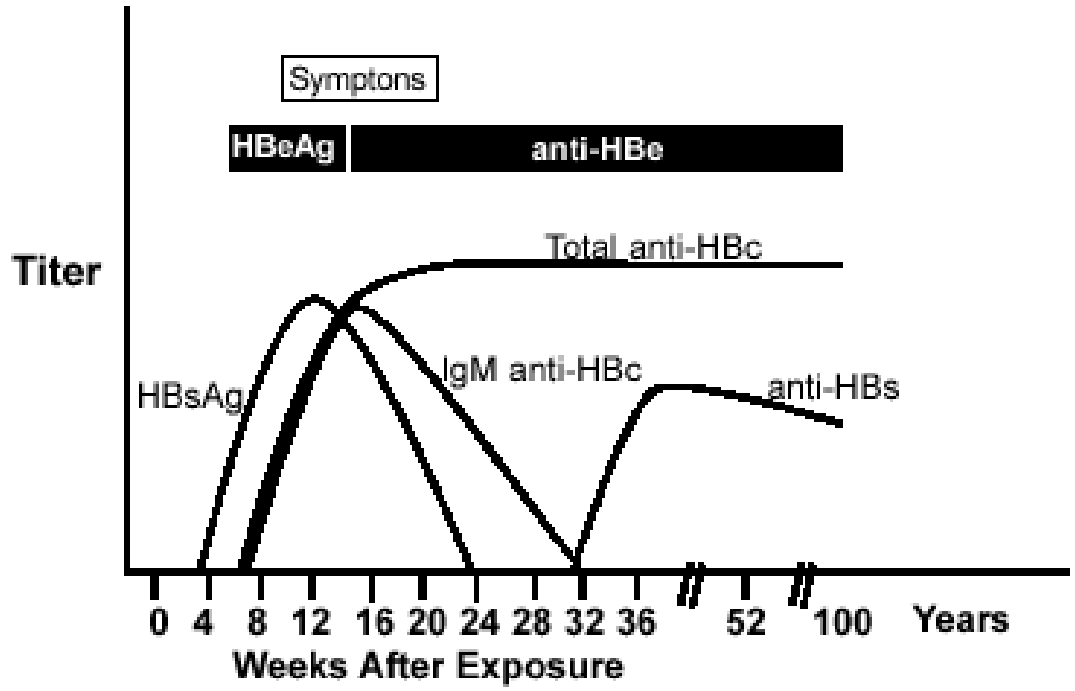

Figure 2.4: Serological HBV during infection (Paar, 2008)

2.3.3 Chronic hepatitis

Chronic HBV infection results during persistence of HBsAg for more than 6 months in the serum of an infected person, with no anti-HBc-IgM (Shepard *et al..*, 2006). The progression of chronic infection from acute or subclinical infection is dependent on host immune response but mainly the age of the patient. Ninety percent of infants infected develop chronic infection, while only 5% of adults will develop chronicity (Huang *et al.*, 2006). Patients chronically infected with HBV and lacking the anti-HBs, develop HBeAg, which is a marker of HBV replication and correlates with greater infectivity. Seroconversion to anti-HBe is usually associated with resolution of the infection and/or the development of BCP/PC mutations in the virus (Shepard *et al.*, 2006; Zhang *et al.*, 2016).

Chronic HBV infections can be divided into the following four stages; i) *immune tolerance phase*, is characterized by active viral replication and immune system

tolerance. In this initial phase, HBV DNA replicates at a high level and the HBs and HBe antigens are produced and detectable with very high detectable antibodies. ALT levels are normal, in this phase, which may last for 10-30 years hence no liver inflammation. This phase usually occurs in infants who were infected at birth or early childhood, and it can last for up to four decades (Yim & Lok, 2006).

ii) *Immune clearance phase,* this second stage is characterized by the immunologic response that causes inflammation and hepatic injury. As a result of viral clearance, there is seroconversion from HBeAg- positivity to anti-HBe, followed by moderate/severe necroinflammation of hepatocytes and elevated ALT levels. This usually occurs in the second or third decade of life in patients with perinatally acquired disease (Villeneuve, 2005; Yim *et al.,* 2006).

iii) *Inactive carrier state,* viral clearance in this third phase is accompanied by seroconversion of HBeAg, resulting in relatively low HBV DNA level and normalized ALT levels. With undetectable HBV DNA, there is no inflammation and anti-HBe persists. This phase can last a lifetime (Yim *et al.,* 2006).

iv) *Reactivation stage.* This stage is characterized by elevated ALT levels and the presence of HBV DNA, with the possibility of further liver damage leading to fibrosis and cirrhosis (Yim *et al.,* 2006).

Clinically, the e-antigen is important in chronic infection as it is regarded a marker for replication and indicative of ongoing HBV infection. When seroconversion occurs, it normally reflects remission of liver disease and viral clearance (Yim *et al.,* 2006).

2.3.4 Occult hepatitis

Occult hepatitis is defined as the presence of HBV DNA in the liver (with detectable or undetectable HBV DNA in the serum) of individuals testing HBsAg negative by currently available assays (Zhang *et al.,* 2015).HBsAg- negative individuals, with serum HBV DNA levels < 200 IU/ml, are considered to have true

occult Hepatitis (OBI) (Michalak *et al.*, 1994; Blackberg and Kidd-Ljunggren, 2000; Raimondo *et al.*, 2007). This is termed occult hepatitis B, and probably reflects that HBV persists life-long in a small proportion of the hepatocytes. It has been suggested that the molecular basis of OBI is related to the long term persistence of viral cccDNA in the nuclei of hepatocytes (Raimondo *et al.*, 2007). The clinical importance of this is not completely understood, but occult Hepatitis B has been associated with reactivation during immunosuppression, enhanced risk for liver cancer, interference with treatment response in patients with hepatitis C and a risk for transmission through blood transfusion and organ transplant (Michalak *et al.*, 1994; Schmeltzer & Sherman, 2010).

Essentially, OBI is found in five clinical contexts: (i) recovery from acute infection leading to seroconversion from HBsAg to anti-HBs, (ii) chronic HBV infection with mutant strains that have a mutation in the S region and this could result in diagnostic failure of HBsAg by routinely used assays, (iii) chronic infection without any marker except HBV DNA, (iv) chronic infection with HBsAg levels too low to be detected by serological assays the most common type occurring in endemic areas (Allain, 2004), (v) OBI in HIV-infected individuals. OBI has many impacts on different clinical aspects, including the possible transmission of infection, risk of reactivation and enhancing liver disease progression that can lead to HCC (Raimondo *et al.*, 2007). The clinical importance of OBI remains controversial, which is the main reason for the growing interest in this topic (Raimondo *et al.*, 2013).

2.3.5 Liver cirrhosis

Liver cirrhosis is defined as an advanced stage of liver fibrosis that is accompanied by distortion of the hepatic vasculature leading to portal hypertension, liver function impairment and HCC (Schuppan & Afdhal, 2008). Therefore, patients chronically infected with HBV can develop cirrhosis. Another risk factor includes alcohol consumption although viral hepatitis either HCV or HBV being the main causes of liver cirrhosis especially at chronic stage of their infections (Schuppan & Afdhal, 2008).

2.4 Hepatocellular carcinoma

Chronic HBV infection can lead to hepatocellular carcinoma (HCC).Globally more than 50% of HCC cases have been reported with high rates of 70-80% being reported in HBV endemic areas (Nguyen *et al.*, 2009). HBV is hepatotrophic virus just like hepatitis c virus and can cause both acute and chronic hepatitis infections. Therefore during infection, innate and adaptive immunity are established and activated in response in order to eliminate infection. An acute inflammation is a short process that is usually protective for the host with the purpose of eliminating the pathogen. Once this virus bypasses this immune response, they could establish a chronic active hepatitis leading to persistence infections hence creating an environment that favours carcinogenesis (Figure 2.3) (De Visser & Coussens**,** 2005).

During chronic inflammation, a variety of inflammatory cells like polymorphonuclear cells and other phagocytes are activated during this process, ending up releasing cytokines, chemokines and nitric oxide (NO) particularly, an inducible isoform of nitric oxide synthase (iNOS), and NO-derived reactive nitrogen species (RNS) (Szabo *et al.,* 1997). These free oxygen radicals cause DNA damage that leads to genetic diseases for instance damage of p53 gene(tumor suppressor gene) and retinoblastoma disrupts their roles on control of apoptotic mechanisms resulting in cancer.

On the other hand HBV DNA integration may cause chromosomal deletions, as found at the chromosomal region 17p11.2-12 causing the loss of the p53 gene. HBV DNA integration may cause disruptions or translocations, resulting in genetic instability (Robinsons, 1999). The HBV genome has itself some oncogenic activities, expressing from integrated HBV the X gene (HBx), which may contribute indirectly to carcinogenesis by activating pathways such as mitogenactivated protein kinase (MAPK), c-jun N-terminal kinase (JNK), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI 3-kinase), protein kinase B (PKB/Akt) and JAK/STAT signaling cascades (Diao *et al.*, 2001). It disrupts p53 pathway and alters the expression level of the retinoblastoma gene (Rb), affecting cell cycle progression

(Edamoto *et al.*, 2003). HBx is not considered a direct transforming gene and therefore, the tumor induction mechanism of HBV is believed to be indirect (Carrillo *et al.*, 2007).

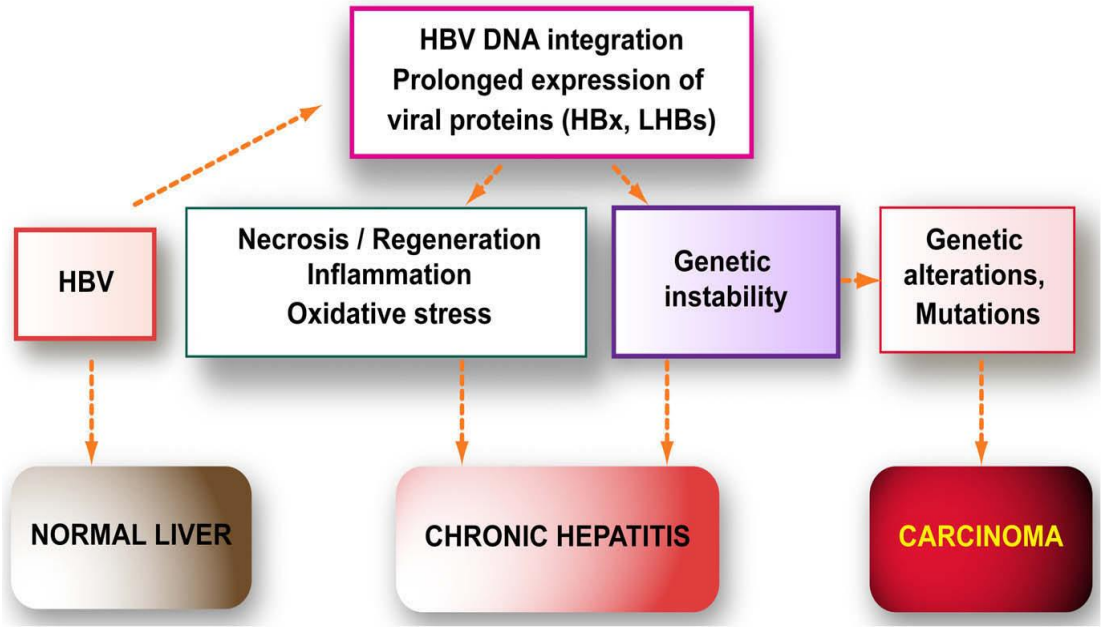

Figure 2.5: Chronic HBV infection and Hepatocarcinogenesis

HBV DNA integration into the host genome and persistent expression of viral proteins such as HBx and large envelope protein (LHBs) can activate cellular cancer-related genes, induce oxidative stress and genetic instability. In the inflammatory context triggered by host immune responses, the viral functions contribute to ceaseless hepatocyte destruction–regeneration, and provide a favorable ground for emergence of genetic and epigenetic alterations leading to hepatocyte transformation (Neuveut *et al.*, 2010)

2.5 Antiviral therapy and drug resistance

The main aim of treatment is to reduce the viral load and minimize possible risk of patient's disease progression to chronic stages with high risk of disease complications. This risk may include development of liver cirrhosis and possible development of hepatocellular carcinoma. Therefore interferon-α (IFN) and

pegylated interferon-α (peg-IFN) together with five nucleoside/nucleotide analogues (NAs) that target the viral polymerase have been approved for treatment of HBV infections. The five approved nucleoside/nucleotide analogues, include; lamivudine (3TC), adefovir dipivoxil (ADV) that are cytidine or thymidine L-Nucleoside analogues, entecavir (ETV) as a D-Cyclopentane, telbivudine (LdT) and tenofovir disoproxil fumarate (TDF) that are alkyl phosphanates.Other analogues that target various stages of viral development include; emtricitabine (FTC), clevudine and pradefovir (Figure 2.4) (Zhu *et al.*, 2009).

IFNs have antiviral effects and modulate the immune system. NAs directly interfere with the reverse transcription of hepatitis virus and thus have a strong antiviral effect on HBV. NAs are disadvantageous over IFN due to development of resistance mutations during treatment, reducing and abolishing the antiviral effect. Lamivudine has been approved for the treatment of chronic hepatitis B. This drug reduces viral load as well as inflammatory activity in liver that could favour liver cell transformation. The drawback of this treatment,therefore, is the appearance of lamivudine-resistant mutant (Kramvis & Kew, 2005). Therefore, with frequent usage of lamivudine as guided by WHO treatment guideline, has led to increased number of patients with lamivudine-resistant mutations (YMDD).

For instance, drug resistance mutation like rtM204V/I, developed up to 23% of patients within one year and 70% after five years of treatment (Lai *et al.*, 2003; Lok *et al.*, 2003). Due to this challenge, entecavir and tenofovir have replaced lamivudine as a preferred treatment due to the lower risk for resistance development. This effect is due to their high genetic barrier compared to lamivudine hence requiring more than one mutation to result in drug resistance. Due to this limitation, more NAs are under development with aim of reducing high rates of treatment failure due to low genetic barrier drugs like lamivudine (Koumbi, 2015).

According to WHO (2015), the HBV/HIV co infected patients are recommended for commencement of treatment immediately regardless of their CD4 count where they are given pegylated interferon for period of up to 48 weeks under monitoring. Based

on the liver biochemistry and viral loads, two active HBV drugs; entecavir and tenofovir are then initiated. In case of HIV co-infections, tenofovir plus emtricitabine or lamivudine are also given. Unless contraindicated, the drug of choice is Pegylated interferon as recommended by WHO. This intervention is aimed at reducing the risk of progressive chronic liver diseases, transmission to others individuals and prevention of long term complications such as liver cirrhosis and hepatocellular carcinoma and death (WHO, 2015).

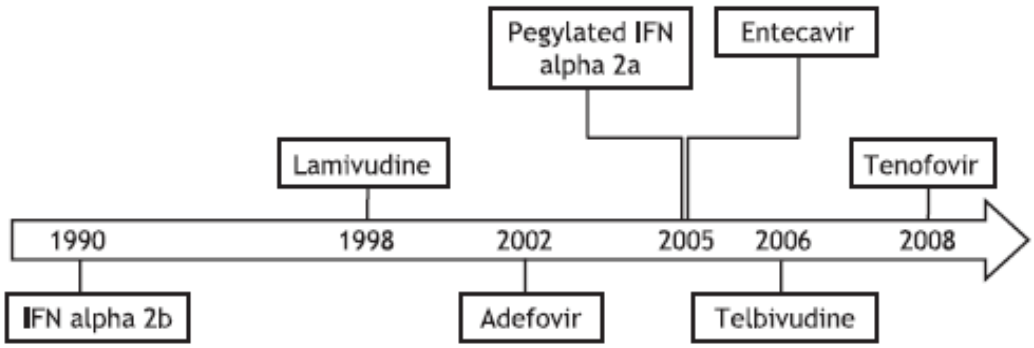

Figure 2.6: FDA Approved antiviral therapy for chronic hepatitis B over time (Locarnini, 2008)

2.6 The HIV/HBV co-infections

Human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV), are the three most common chronic viral infections all over the world, sharing similar transmission routes including sexual, blood-blood contact, and injecting drug usage (Saravanan *et al.,* 2007; Koziel *et al.,* 2007). Co-infection with HIV and HCV and/or HBV is very common in certain populations, such as intravenous drug users (IDUs) who often share the contaminated needles/syringes for intravenous drug injection (Zhou *et al.,* 2011).Previous reports shows that the

prevalence of HIV-HCV co-infection among IDUs can surpass 90% (Aceijas & Rhodes, 2007). Besides, the rates of HIV-HBV co-infection are reported to be as high as 10–20% in endemic countries with intermediate and high HBV infections (Aceijas and Rhodes 2007). An increasing number of studies have suggested that HIV can accelerate the natural course of chronic hepatitis C or hepatitis B. Previous studies have indicated that patients with chronic viral hepatitis co-infected with HIV will experience more rapid progression and are more likely to die of liver-related diseases compared with those without HIV infection. Inversely, the effect of HCV and/or HBV on HIV infection is less clear (Thio, 2010). Co-infection with HIV and hepatitis viruses has significantly increased morbidity and mortality of the HIV/AIDS patients (Thio, 2010). Therefore, it is critical to investigate the prevalence of co-infection with HIV and HCV and/or HBV, especially among the IDUs that are considered to be a high-risk population of co infections (Zhou *et al.,* 2011).

2.7 The HBV genotypes

Hepatitis B virus (HBV) is distributed worldwide and is endemic in many counties. Sub Saharan Africa carries a high burden of HBV infections and therefore is among one of the hyper endemic regions for this virus. Nevertheless, this virus has become a public health problem, with two billion people infected worldwide, including 350 million of them suffering from chronic HBV infection. HBV infection results in 500,000 to 1.2 million deaths per year caused by chronic hepatitis, cirrhosis, and hepatocellular carcinoma and is the 10^{th} leading cause of death worldwide (Lok *et al.,* 2002; Neuveut *et al.,* 2010). HBV persistence and transmission dynamics within these populations remains elusive and this involves genetic component of the virus (Santos *et al.,* 2010).

Due to its unique life cycle which requires an error-prone reverse transcriptase for replication, HBV constantly evolves, resulting in tremendous genetic variation in the form of genotypes, sub-genotypes, and mutations (Zhang *et al.,* 2016). HBV is divided into ten genotypes, A–J, with each genotype differing in sequence by more than 8% at the nucleotide level when compared to each other and less than 4%

intragenotype divergence. Migration and human behaviour have been found to play major role in distribution and pattern of HBV genotypes. Despite these factors, HBV genotypes and sub-genotypes show distinct geographical distributions (Santos *et al.,* 2010).

Genotype A for instance is distributed globally and is the main genotype found in Europe, North America, Africa and India whereas genotypes B and C are predominant in East and Southeast Asia (Santos *et al.,* 2010). Genotype A is divided into seven sub genotypes A1-A7 (Santos *et al.,* 2010; Zhang *et al.,* 2016). Among these HBV Sub genotypes; A1 circulates in Africa (Hannoun *et al.,* 2005), Sub genotype A2 in Europe while sub genotype A3 has been detected in Central and West Africa (Karbanov *et al.,* 2005). Sub genotype A4 has been reported in Gambia (Hannoun *et al.,* 2005), sub genotype A5 in Nigeria and also among African descendants in Haiti (Andernach *et al.,* 2009). Sub genotype A6 has been detected in Belgium but among African-Belgian patients of Congo and Rwanda origin (Pourkarim *et al.,* 2009) and A7 has been detected in Rwanda and Cameroon (Hubschen *et al.,* 2010).

Hepatitis B virus (HBV) sub genotype B1 is dominant in Japan, B2 is common in China and Vietnam, B3 is confined to Indonesia, and B4 is confined to Vietnam (Thedja *et al.,* 2011). B7, B8, and B9 have been found in an island in Southeast Asia (Huy *et al.,* 2004). HBV/C1 (Cs) is found mainly in Southeast Asia, whereas C2 (Ce) is predominant in East Asia (Lusida *et al.,* 2008). HBV/C3 was confined to Oceania, while C4 was exclusively found in Australia and regarded as the most divergent sub-genotype within HBV/C (Davies *et al.,* 2013). Sub-genotypes C5 and C7 were found in Philippines, while C6 and C8 to C16 were isolated from Indonesia (Sugauchi *et al.,* 2002). Genotype D which was previous divided into 4 sub genotypes (D1-D4) is mainly found in the Middle East and Mediterranean countries in Asia but it has been reported in Africa and Europe (Norder *et al.,* 2004; Mahatab *et al.,* 2008; Thedja *et al.,* 2011). However, new genotypes D5-D7 have been described in India (Chandra *et al.,* 2009), Indonesia (Utama *et al.,* 2009) and Mediterranean basin (Meldal *et al.,* 2009). HBV genotype E seems to be predominant in western-sub-Saharan Africa

(Mahtab *et al.,* 2008, Kramvis *et al.,* 2005). This genotype has not been detected outside Africa, except for a few rare cases mostly in individuals with an African background. Nevertheless, presence of this genotype has been detected in India (Singh *et al.,* 2009) and also in certain specific community in Colombia (Mulders *et al.,* 2004; Alvarado *et al.,* 2010).

HBV genotype G has been characterized in samples from USA, Mexico and France and appears primarily to be present as a coinfection with another HBV genotypes, most commonly genotype A. Genotypes F and H are found almost exclusively in Central and South America (Jutavijittum *et al.,* 2007). Recently, HBV genotype I and J were described in Northwestern China, Vietnam, Laos and Japan (Norder *et al.,* 2004; Tatematsu *et al.,* 2009; Arankalle *et al.,* 2010). However, genotype F is divided into 4 sub genotypes: F1-F4. Subgenotypes F1 and F2 have been further divided in F1a, F1b, F2a and F2b (Norder *et al.,* 2004; Devesa *et al.,* 2008; Korbanor *et al.,* 2010; Kramvis, 2014; Zhang *et al.,* 2016). Morever, intergenotype recombination which plays an important role in the evolutionary history of HBV has also been described (Zhang *et al.,* 2016). For instance, B/C recombinants have been reported in Southeast Asia and East Asia where it is also prevalent (Shi *et al.,* 2012). Other inter-genotype recombinants such as A/D, A/E, C/D and G/C recombinants have also been observed in different geographical regions (Yang *et al.,* 2006).

In Kenya, like other sub Saharan Africa countries, HBV genotypes A, D and E have been detected with genotype A being the most predominant (Mwangi *et al.,* 2008). However, information on the current circulating HBV genotypes still remain elusive in Kenya, even though the country is considered to be among the endemic countries for these viruses (Mwangi *et al.,* 2008). With ongoing generation of viral genetic diversity and increasing habits of human migration, the global HBV epidemic is becoming increasingly heterogeneous. As hepatitis infections become pandemic, progresses, an increasingly broad range of genetic diversity is being reported within the virus. The intermixing of diverse HBV subtypes around the globe is making it more complex in terms of management of HBV epidemic due to development of mutations by the virus that in turn confers drug resistance that are not captured by

reference HIV subtype B. This diversity is co-infection of HIV, HCV and HBV has increasingly become relevant in management(Modi *et al.,* 2007).Approximately 8-20% of the estimated 36 million HIV-infected patients worldwide are co-infected with HBV (Alter, 2006) with hepatic disease also on the increase (Day *et al.,* 2013).

2.8 Drug resistance in HBV

Human immune deficiency virus (HIV) adversely affects the natural history of HBV (Modi *et al.,* 2007) by accelerating progression of HBV related chronic liver disease, the risk for drug-related hepatotoxicity and hepatitis reactivation. The individuals require optimal control of viral replication in order to prevent the development of severe co-morbidities, such as liver cirrhosis and hepatocellular carcinoma (Lacombe *et al.,* 2010). The clinical impact of HBV coinfection in HIV-1–infected patients has progressively grown since the introduction of ART that has given the dramatic increase in survival to these individuals (Benhamou *et al.,* 1999). The decrease in AIDS-related mortality and morbidity attributable to effective combined antiretroviral therapy post 2004 has ushered a new era of liver-related disease associated with chronic hepatitis, which is now the second leading cause of death in HIV-infected patients. This has been witnessed in both developed and developing countries (Manosuthi *et al.,* 2006).

However, antiviral resistance has become an increasingly common problem during long-term treatment with nucleoside analogues (NA) including patients who receive sequential treatment with NA monotherapy (Keeffe *et al.,* 2008). When lamivudine-resistant HBV variants emerge, viral load and liver enzyme levels may increase, clinical hepatitis may occur, and HBV infection can be fatal in a minority of patients (Bessesen *et al.,* 1999). Thus, the clinical effectiveness of lamivudine monotherapy is limited by the frequent emergence of resistant HBV variants. Monotherapy with Lamivudine for HBV regiment is therefore not recommended (Wongprasit *et al.,* 2010). WHO recommends combination therapy of tenofovir plus lamivudine or emtricitabine in HBV/HIV-1–coinfected patients in order to prevent the emergence of resistant HBV variants (WHO, 2011). It further recommends that treatment

should be initiated regardless of the CD4 counts in HIV/HBV coinfections to prevent faster progression to liver cirrhosis and liver related death (Thio *et al.,* 2002).

Drug resistance testing is currently examined in all HIV individuals in Western countries before initiating antiretroviral therapy, based on a reported prevalence of transmission of drug-resistant HIV of around 10% (Trevin˜o *et al.,* 2009) and the demonstration of impaired treatment response when antiretroviral drugs are used empirically in patients with acquired drug-resistant trains (Fox *et al.,* 2006). Similar information for HBV infection is still scarce, but recent reports have emphasized that the rate of primary drug resistance mutations among drug-naive chronic hepatitis B-mono infected patients may be around 8% in Western countries (Ludwig *et al.,* 2008) and that patients infected with HBV resistant strains may be prone to subsequent treatment failure (Fung *et al.,* 2008). In almost all cases, lamivudine resistance mutations are the ones recognized. However, it should be noted that lamivudine-resistant HBV strains display cross-resistance to emtricitabine, telbivudine, and to a lesser extent entecavir (Thio *et al.,* 2007).

Little is known about the rate of transmission of HBV resistant strains among HIV patients in Kenya where treatment with lamivudine has been widely used for more than a decade (Trevin˜o *et al.,* 2009). Baseline drug resistance testing in HBV might be warranted in newly diagnosed HIV/HBV coinfected patients. Surveillance studies assessing the rate of primary HBV drug resistance in populations of HIV/HBV coinfected patients is therefore invertible. Moreover, a cost-benefit assessment of baseline HBV drug resistance testing in clinical practice is the implication this poses in selection of the first-line antiretroviral therapy in HIV infections (Trevin˜o *et al.,* 2009).

Antiviral resistance is one of the important factors that lead to treatment failure. In HBV where nucleoside analogues are used, emergence of drug resistance based on HBV polymerase are complex. However, eight codons are associated with primary resistance end up with predictable five major pathways.

These pathways may include;

i) The L-nucleoside pathway (rtM204V/I), whereby lamivudine, emtricitabine, telbivudine, and clevudine treatment select out the rtM204V/I. This pathway includes entecavir in lamivudine-experienced patients.

ii) The acyclic phosphonate pathway (rtN236T), in which adefovir and tenofovir treatment select out and/or consolidate the rtN236T HBV quasispecies (Angus *et al.*, 2003).

iii) Through a shared pathway (rtA181T/V), whereby treatment with either L-nucleosides or acyclic phosphonates can result in selection of HBV quasispecies with rtA181T/V. This pathway is seen in about 40% of adefovir failure and less than 5% of lamivudine failure.

iv) Naive entecavir resistance pathway (rtL180M + rtM204V with one of rtT184, S202, or M250 codon changes). In this pathway, three mutations are required to appear simultaneously accounting for the very low resistance profile of entecavir (Suzuki *et al.*, 2007).

v)Multidrug resistance pathway. Complex patterns and clusters of specific mutations in HBV polymerase associated with multidrug failure. A recent example includes rtA181T + rtI233V + rtN236T + rtM250L. It is important to note that the rtI233V and M250L substitutions in isolation do not confer significant drug resistance nor significantly reduce replication capacity in the absence of selection pressure, but appear to act to compensate for the replication defects associated with acquisition of multidrug resistance (Locarnini, 2008).

However, some of these broad clusters of compensatory mutations, especially those acquired during lamivudine therapy, are compromising future salvage of therapy options (Zhang *et al.*, 2016).

Nevertheless, in other genes of HBV, deletion mutations for instance in the PreS gene region and/or some point mutations in the major hydrophilic region (MHR) of S gene can lead to immune escape and occult HBV infection. It has also been shown that reverse transcriptase (RT) could also lead to an altered viral envelope due to the overlap between the envelope and polymerase (Zhang *et al.*, 2016).

Due to this effect, the mutation occurring at position A181T/V on RT region could cause a stop codon mutation (W172*), W172L and L173F mutations in the S region. In addition, occurrence of RT mutation at M204V/I position could also result into stop codon mutation at (I195M), (W196*) (W196S) and (W196L) positions in the S region; A1762T and G1764A mutations in the base core promoter (BCP) or G1896A mutation in Pre C, leading to decrease HBeAg expression or reduced replication fitness.(Zhang *et al.*, 2016).

Some mutations in the CTL epitope of HBV core gene have also been found to lead to reduced T cell immune escape. For the HBV X gene, occurrence of some point mutations or truncated mutants in this gene have also been found to lead to tumorigenesis or other end-stage liver disease (Zhang *et al.*, 2016).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

This study was conducted at Mama Lucy Kibaki hospital. This is a Ministry of health District hospital in Umoja II off Kangundo road, Umoja, in Embakasi sub County, Embakasi West Constituency in Nairobi County. The hospital is located along Spine Road in Umoja III estate and can be accessed by vehicles passing along Kangundo Road. This hospital has 112 bed capacity and serves a population close to 187,020 patients in 208 km^2. However due to ongoing decongestion plans of Kenyatta National hospital, Mama Lucy Kibaki hospital could be serving more than the above stated number of patients.

3.2 Approval of the study

This study involving human subjects was approved by Scientific and Ethical Review Committee of Kenyatta University. All approved procedures conformed to the international standards. (Aappendix i).

3.3 Study population

The study targeted both male and female participants. These individuals were HIV infected regardless of their treatment status attending comprehensive care clinic of Mama Lucy Kibaki hospital. The study was conducted between June and October 2015.

3.4 Study design

This was a cross sectional study.

3.5 Sample size calculation:

The sample size was determined according to Naing (2003)as shown in the formular below;

$$n = \frac{Z^2 P(1-P)}{D^2}$$

A prevalence of 41 % of HIV/HBV co-infections in South Africa was used (Lodenyo et *al.,* 2000).

Where**:**

n= Minimum sample size required

Z= 1.96 standard error

P= Postulated prevalence (41%).

D= 0.05 the inverse of 95% confidence limit (the allowable error)

$$n = \frac{1.96^2 \times 0.41\ (1-0.41)}{0.05^2} = 378 \text{ samples}$$

3.6 Inclusion criteria

- Infected with HIV and attending comprehensive care clinic in Mama Lucy Kibaki hospital regardless of their age and gender.
- Willing to participate in the study by giving consent/assent

3.7 Exclusion criteria

- Those who did not give an informed consent/assent

3.8 Sampling design

Study participants were recruited using a systematic sampling technique during the period between June and October 2015. They were arranged according to their hospital visits in ordering scheme where every third participant was recruited. However, the first participant was randomly selected from the allocated numbers based on the sample size. These participants were recruited until the target sample size was arrived at.

3.9 Demographic data

A structured questionnaire was administered to all patients who consented (appendix ii). Demographic data including age, ARVs status and antiretroviral therapy use were collected (Appendix iii).

3.10 Collection of blood samples

Sterile syringes and needles were used to collect, five-millilitre of venous blood sample in Ethylenediaminetetraacetic acid (EDTA) tubes. One-millimetre of blood was used for serological tests and three-millimetre for viral DNA extraction.

3.10.1 Serological profile markers

The five HBV serological markers (HBsAg, HBsAg, HBeAg, HBeAg and HBcAb) were determined using the one-step HBV-5 panel rapid diagnostic cassette according to the manufacturer's instructions (Healthaw Medical limited, Hangzhou, China). Briefly, 5 µL of the test plasma was placed into each of the five sample wells corresponding to the sero-markers. Hepatitis B virus sero-marker reactivities was recorded after 20 minutes of incubation (Webale *et al.*, 2015).

3.10.2 Extraction of viral DNA

The HBV DNA was extracted from blood by a column purification method according to the manufacturer's instructions (QIAamp™ MiniViral DNA Kit,

Qiagen, Valencia, CA, USA) . The extracted viral DNA was stored at -20°C awaiting further analysis.

3.10.3 Amplification of HBV-pol

The HBV-*pol* gene was amplified in a nested Polymerase Chain Reaction(nested PCR) using specific forward primer HBV HBPr1 (position: 2850-2868, 5'-GGGTCACCATATTCTTGGG-3') and Reverse primer HBPr135 (803-822, 5'-CAAAGACAAAAGAAAATTGG-3') (Stuyver *et al.,* 2000).Nested PCR using the products of the first PCR(as template)was performed with the following primers: forward primer, HBPr2 (position: 2867-2888, 5'-GAACAAGAGCTACAGCATGGG-3' and Reverse primer HBPr3 (position: 3226-3246, 5'-CCACTGCATGGCCTGAGGATG-3) (Stuyver *et al.,* 2000).

Briefly, 5.0 µl DNA was added to a reaction mix containing 12.5 µl Dream taq PCR Mix 2X (*Taq* polymerase, 5X buffer, dNTPS and $MgCL_2$), 0.5 µl of each Reverse and Forward primers, and 6.5 µl of Distilled water. The amplification was then carried out in a thermal cycler (MJ Research, Inc.) using the following conditions in the first PCR, one cycle at 95°C for 4 minutes, and 40 cycles of 95°C for 30 seconds, 46 °C for 30 seconds and 72°C for 2 minutes with a final extension of 72°C for 7 minutes. In the nested PCR, product of first PCR run was used as the DNA template for running the second PCR using the following conditions; one cycle at 95°C for 4 minutes, and 40 cycles of 95°C for 30 seconds, 50°C for 30 second and 72°C for 2 minutes with a final extension of 72°C for 7 minutes (Stuyver *et al.,* 2000).

3.10.4 Analysis of PCR products by gel electrophoresis

Once the 2^{nd} PCR was done, the amplicons were analyzed by gel electrophoresis. The PCR amplicons were loaded for visualization using UV transilluminator (UVP, San Gabriel, A, USA) after staining them with ethidium bromide (0.05%). The products were then allowed to separate for three quarters of the gel for 45minutes in electric field at 100 volts. In this study, an average molecular weight for reverse transcriptase gene(692bp) was expected (Figure 3.4).

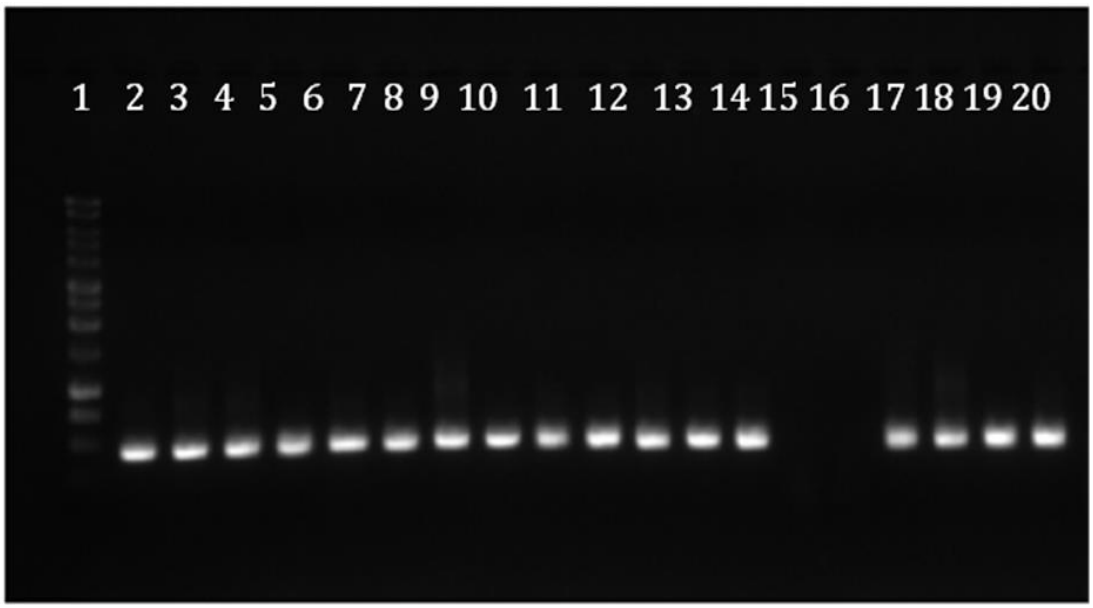

Figure 3.1: Agarose gel analysis of the PCR amplification

This is a 692 bp fragment of the HBV *pol* gene. Lane 1: 1kb DNA Ladder, lanes 2-14, patient samples. Lanes 15, 16 negative and 17-20 were positive controls.

3.10.5 Sequencing of PCR and amplicons purification

From the 2nd nested PCR products, direct sequencing was performed at Macrogen korea.Briefly the sequencing PCR was carried with total reaction mixture of 20 µl containing 3 µl of DNA, 5X sequence buffer, 2.0 µl Big Dye, 10.5 µl of distilled water, and 1.5 µl of primer. For each sample, two reactions were prepared using (5'-GAACAAGAGCTACAGCATGGG-3' and HBPr3 (5'-CCACTGCATGGCCTGAGGATG-3). The amplification was performed as follows; denaturation for 5 minutes at 96°C, and again for 10 seconds at 96°C, annealing at 50˚C for 5 seconds and final extension 60˚C 4 minutes for 25cycles. The products were then purified. Briefly, 2µl sodium acetate, 50µl of absolute ethanol, 20µl of sequencing PCR product and were mixed in a 200µl tube. This was vortexed and incubated at room temperature (RT) for 15 minutes in dark. The amplicons were centrifuged at 14000 revolutions per minute(rpm) for 30 minutes at RT. A five hundred (500ml) of 70% ethanol was added and centrifuge at 1500 rpm for 5 minutes

and supernatant discarded. The above two steps were repeated once and DNA pellet was air dried for minimum 45 minutes. After drying, 20µl of highly deionized (HiDi) formamide was added and heated at 95˚C for 3 minutes, chilled on ice for 3 minutes and finally transferred into sequencing tube and loaded on the automated ABI 310 genetic analyzer(Applied Biosystems, Foster City, CA) .

3.10.6 Analysis of DNA sequences

Generated sequences were assembled, edited and aligned with Bio Edit v7.0. Phylogenetic analysis was performed using Neighbour-Joining method, based on Kimura´s two-parameter distances, using Molecular Evolutionary Genetics Analysis (MEGA 5.1) (Tamura *et al.*, 2011). Analysis of drug resistance mutations was performed using the Drug Resistance Algorithm from the Stanford HBV database (Gnaneshan *et al.*, 2007).

CHAPTER FOUR

RESULTS

4.1 Study participants and their demographic characteristics

A total of four hundred patients consisting of 293 (73.3%) females and 107 (26.8%) males were enrolled with mean age of 33.4 years and standard deviation (SD) of 0.01.The females mean age was found to be 34.1years with SD of 0.49 and 31.7 years with SD of 2.89 for males. However, majority of the participants were aged between 30 and 49 years. Majority of the recruited participants were on first-line treatment for HIV infection 378(94.5%) with 22(5.5%) on second-line treatment for HIV infection. Most patients were on AZT/3TC/NVP therapy with mean duration treatment of 4.8 (0.01) years (Table 4.1).

Table 4.1: Demographic characteristics of the study participants attending HIV care clinic of Mama Lucy Kibaki Hospital, Nairobi

Gender	**All N (400)**	**Females n(293)**	**Males n(107)**
Mean Age (SD) in years	33.4 ±0.01	34.1 ±0.49	31.7 ±2.89
Mean Duration of treatment (years)	4.75 ±0.02	4.6 ±0.23	5.1 ±0.12
Regimen			
AZT/3TC/NVP	209	162	47
TDF/3TC/EFV	169	112	57
TDF/3TC/LPr	13	11	2
AZT/3TC/LPr	9	8	1
Age categories (years)			
<10	48	30	18
11-19.	41	31	10
20-29	29	19	10
30-39	110	80	30
40-49	104	70	34
50-59	50	49	1
60-69	9	7	2
70-79	9	7	2

KEY:

AZT: zidovudine; 3TC: lamivudine; NVP: nevirapine; TDF: tenofovir; LPV/r: lopinavir/ritonavir

Among the 400 participants that were screened, 111 (27.8%) were vaccinated. Those infected with HBV were at different stages of infection as follows; acute infection

were 12 (3%), on recovery were 19 (4.8%), chronic 10 (2.5%), asymptomatic or inactive 7 (1.8%) while those with occult HBV were 9 (2.3%). Majority of the co-infected patients were aged between 3 and 49 years. Overall, prevalence of HIV/HBV co-infections was 7.25% (29/400) (Table 4.2).

Table 4.2: Hepatitis B virus sero-reactivities, infection stages among HIV infected individuals visiting the HIV care clinics of Mama Lucy Kibaki Hospital, Nairobi, Kenya

Serological test	HBV immunization	Acute HBV	HBV recovery	HBV chronic	asymptomatic carrier	occult HBV	Non respondents
antiHBs	+	-	+	-	-	-	-
AntiHBc	-	+	+	+	+	+	-
AntiHBe	-	-	+	-	+	-	-
HBsAg	-	+	-	+	+	-	-
HBeAg	-	+	-	-	-	-	-
N= (400)	111	12	19	10	7	9	232
Prevalence (%)	27.8	3	4.8	2.5	1.8	2.3	58

KEY: + Positive - Negative

4.2 The HBV genetic diversity

Thirteen samples were successfully amplified and sequenced, with HBV-*pol* primers and analysed phylogenetically. The phylogenetic tree revealed a cluster of sequences with all being HBV genotype A. From the bootstrap analysis, the scores were over 84% hence confirming the reliability or significance of the phylogenetic tree analysis (Figure 4.1).

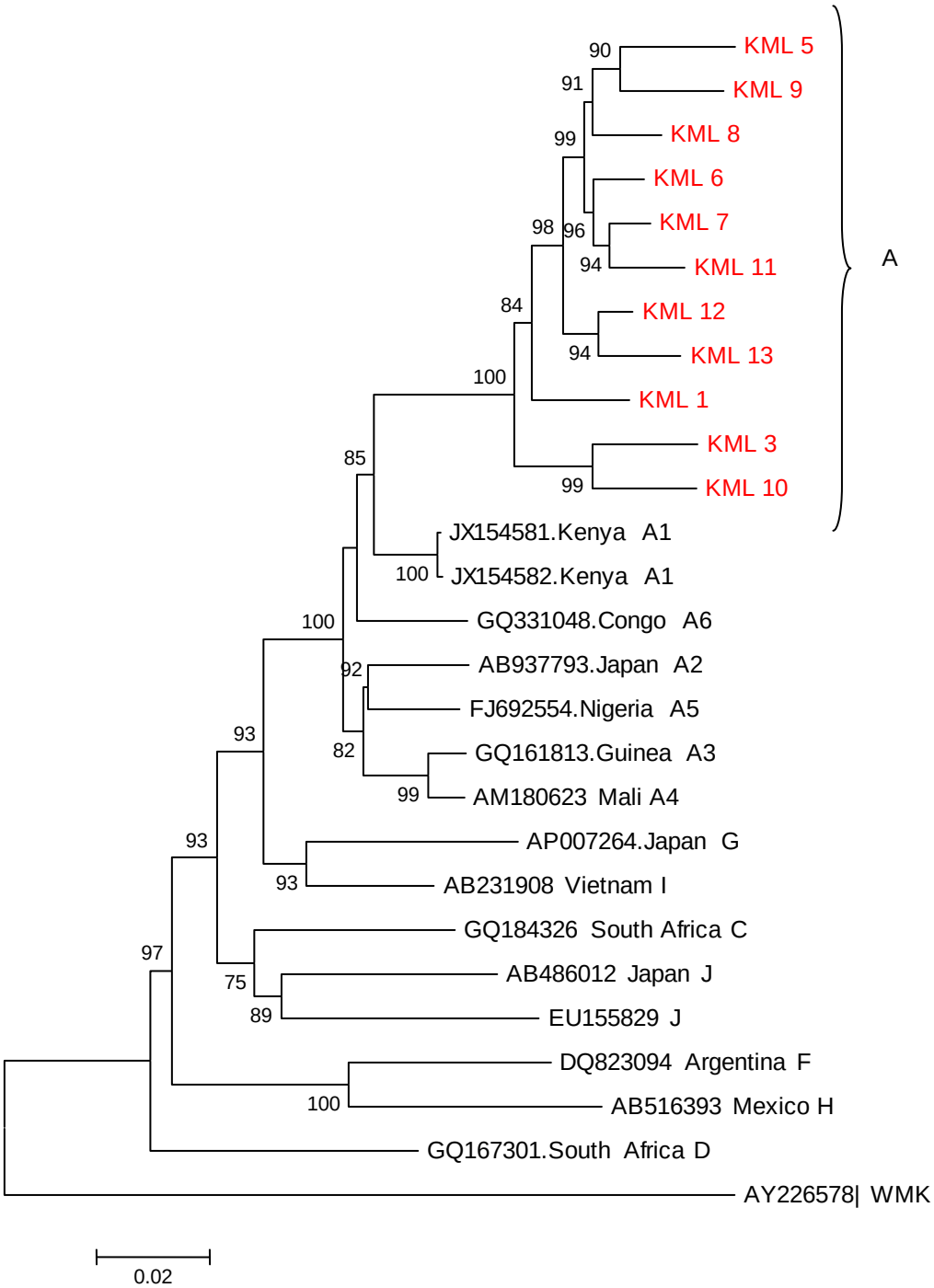

Figure 4.7: Phylogenetic tree of HBV-pol sequences from Nairobi, Kenya.

Neighbour-Joining method based on 1000 bootstrap replicates was used. Wooly monkey HBV (AY226578-WMHBV) was used as the out-group and bootstrap values above 70% are indicated. HBV isolates from study participants are indicated in red

4.3 The HBV drug resistance

Among the 13 subjects, six were infected with HBV strains harbouring primary/major and secondary/compensatory mutations. Triple Nucleoside analogues (NAs) associated mutations were detected at positions 173 (rtV173L), 180 (rtL180M) and 204 (rtM204). One sequence had multiple mutations at position 180 (rtL180M) and 204 (rtM204V) (table 4.3). In addition, other HBV mutations were also detected (Table 4.3)

Table 4.3: The patterns of HBV drug resistance mutations in this cohort of HIV infected individuals visiting HIV care clinic of Mama Lucy Kibaki Hospital, Nairobi.

Combined mutations patterns	n (13)	Frequency (%)	Drug associated
rtV173L, rtL180M, rtM204V	5	38.5	3TC, ETV and LdT t
rtL180M, rtM204V	1	7	3TC, ETV and LdT t
No major mutations	7	53.8	Susceptible

KEY:

3TC: lamivudine ETV: entecavir: LdTt: emtricitabine

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

In this study, the prevalence of HBV among HIV infected patients seeking health service at Mama Lucy Kibaki Hospital, Nairobi, Kenya was found to be 7.25% (HbsAg). In comparison with previous studies conducted in Kenya, it was found to be consistent with those obtained in Kenya by (Muriuki *et al.,* 2013) (6%), as well as in Malawi (5.7%), Rwanda (4.9%) and Nigeria (4.8%) (Pirillo *et al.,* 2007; Otegbayo *et al.,* 2008; Moore *et al.,* 2010). The observed prevalence was similar since the same targeted general population of HIV infected individuals in urban centres was used. However, these levels were also found to be higher than those previous obtained from an informal urban settlement of Viwandani and Korogocho in Nairobi (1.1%), (0.7%) (Kerubo *et al.,* 2015) who sampled from slums and those from Gambia (0.6%), Uganda (0.6%), Zimbabwe (0.8%), Corte d'voire (1.2%), Senegal (1.6%), and Zambia (2.2%) (Kallestrup *et al.,* 2003; Rouet *et al.,* 2004; Diop-Ndiaye *et al.,* 2008; Mboto *et al.,* 2010; Kapembwa *et al.,* 2011; Elzouki *et al.,* 2014). In this era of HIV infections which also share similar transmission routes with HBV(blood and body fluids), the HBV infection levels are also likely to increase. The increasing trend could be sustained with steady increase in engagement in high risk behaviours (sexual activities and drug users) in the city.

On the other hand, the HBV prevalence obtained in this study were low compared to those previously obtained in Kenya (Okoth *et al.,* 2006) (55.8%) and Malawi (20.4%) which were sampled amongst liver failure patients. The sampled patients were those with liver failure visiting liver clinic and whose majority consisted of individuals experiencing progressive liver disease due to HBV. Similarly, high rates HBV infections were also observed in Zambia (31.3%) among women attending antenatal services in Botswana (10.6%) and South Africa (22.9%) among the outpatient group in urban settings (Kasolo *et al.,* 2003; Nyirenda *et al.,* 2008;

Lukhwareni *et al.,* 2009). These differences in prevalence of HBV infection were associated with either the population sampled, or with a better access to free antiretroviral therapy that leads to prolonged life period hence a like hood of HBV infection (Muriuki *et al.,* 2013). This observation, suggests that HBV/HIV co-infections could be higher than expected especially for high risk populations (Muriuki *et al.,* 2013; Kibaya *et al.,* 2015).

The HIV-co-infections based on gender, were found to be significantly higher among male patients compared to their female counterparts. This could be associated with the risk of infection due to their sexual behaviour with multiple partners, drug use or alcohol consumption (Tessema *et al.,* 2010).

For the observed rates of asymptomatic carriers 7(1.8%), immune respondents 111 (27.8%) and occult Hepatitis 9 (2.3%), the findings from this study were slightly higher compared to those previously obtained in Kenya (Barth *et al.,* 2010; Kim *et al.,* 2011; Day *et al.,* 2013; Webale *et al.,* 2015). Those classified under chronic stage of infection, could be experiencing liver cirrhosis and in addition, have high risk developing hepatocellular carcinoma (Muriuki *et al.,* 2013). Due to the impact of HBV vaccination programme among children less than 14 years and willing adults, it was expected that high levels of vaccine type responses could have been more. The obtained levels of immune respondents could due to low immune response due to HIV infection. In addition, by virtual of HIV infection, low immune response could also have increased vulnerability to HBV infection. Nevertheless, Kenya as a country has never launched a campaign on HBV vaccination. An approach which if implemented, could have led to high numbers of HBV respondents. Even from those vaccinated, the Kayole residents may not have accessed high cost HBV vaccines. Confirmation of HBV infection in most situations has been relied upon for screening HBsAg. In this study, a five panel markers of HBV diagnosis was used. From the utility of the five HBV sero-markers, this study shows that there could be a possibility of accumulative high rates of HIV/HBV co-infections which could be at different .stages of the disease; which even previous studies conducted among the same populations that have conftheirmed (Webale *et al.,* 2015).

Phylogenetic analysis shows that the analyzed HBV was of sub Saharan origin as shown by the evolutionary clusters of isolates. All the 11 samples analyzed belonged to genotype A of HBV. . This suggests that there is local sex networks and circulation of the HBV genotype A in the area. Umoja and Kayole areas where the samples were collected, is occupied by residents of diverse age groups and sexual behaviours. It is also one of the Nairobi Eastland areas that are known to have youths who engage in prostitution, a factor that may not be ruled out in the present studied area. Our findings confirm that HBV genotype A is still the most common circulating genotype in this area like the rest of the country. This data agrees with previous studies that have been conducted in the other parts in Kenya like Nairobi, Kericho and Mombasa (Mwangi *et al.,* 2008; Kibaya *et al.,* 2015; Webale *et al.,* 2015; Ochwoto *et al.,* 2016). The predominance of HBV genotype A in the country suggests low rates of HBV transmission dynamics and recombination (Mwangi *et al.,* 2008; Webale *et al.,* 2015). From previous studies, genotype D and E have been detected (Mwangi *et al.,* 2009) among blood donors or intravenous drug users (Webale *et al.,* 2015) or sex commercial workers (Kibaya *et al.,* 2015). In this study, however, only genotype A was detected with low rates of HBV recombinants and this could be due to single sampling. In addition, the study participants were drawn from general population as compared to those from high risk population like intravenous drug users and sex commercial workers that have been studied previously.

The results from the present study have shown that only six of the 13 analyzed samples harboured drug resistant to HBV. These participants were on lamivudine containing ART. Thus, HBV infection was therefore active on lamivudine treatment for HIV. Previous studies in Kenya had reported lower prevalence of HBV lamivudine resistance in a longitudinal study that screened patients after twelve months of follow up (Kim *et al.,* 2011) and in Thailand (22.6%) (Wongprasit *et al.,* 2010). Compared to the present study, the participants had been on treatment for an average period of over four (4) years. The difference in drug treatment period may have led to the observed levels in HBV lamivudine resistance. However, lamivudine

resistance levels in the present study were lower compared to those obtained in China (89.4%) (Lei *et al.,* 2013). This study conducted in China analyzed chronically HBV infected individuals compared to our study that targeted patients visiting a comprehensive care clinic. The difference in the studied populations may have led to the variations in HBV lamivudine drug resistance.

HBV drug resistance mutation patterns among HIV/HBV co-infected participants in this study contributed to lamivudine resistance. The mutations that were detected not only caused lamivudine resistance but also a cross resistance to entecavir and emtricitabine. This was expected especially for drugs that are well known to have low genetic barrier hence develop the high rate of drug resistance. Higher cross-resistance rates have been reported in the previous studies (Wongprasit *et al.,* 2010). This could be explained by the differences in the treatment period and number of participants. Despite the patients being HBV drug naives, the observed drug resistance mutations against Entecavir and Emtricitabine could suggests a a possible transmitted viral strains, immunological and drug pressures on the virus leading to developed drug resistances.

This was across- sectional study that utilised HBc-IgM to classify HBV infection stages as either acute or chronic infections but not considering presence of HBc-IgG despite its persistence for several years after acute infection. Secondly, this study did not determine HBV viral load and CD4/CD8 counts that could have guided immune response and confirmation of occult HBV.

5.2 Conclusion

i. The following were identified Seroprofile in this study; chronically infected 10 (2.5%), occult 9 (2.3 %) and overall HBV/HIV co-infections prevalence (7.25%).
ii. Hepatitis B virus genotype A1 was the most predominant genotype among the patients visiting Mama Lucy Kibaki Hospital Kenya.

iii. Triple Nucleoside analogues (NAs) mutations 173 (rtV173L), 180 (rtL180M) and 204 (rtM204V) were detected in six participants.

5.3 Recommendations

i. Continuous surveillance for HBV infections among HIV infected individuals is necessary for early detection intervention and control measures.
ii. Regular surveillance of circulating HBV genotypes is necessary for analysis of their trends within population.
iii. There is need to clinically confirm if the participants failing in lamivudine treatment, could be put on high genetic barrier drug; like tenofovir to avoid the effects of lamivudine drug resistance and its cross resistance

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APPENDICES

Appendix i: Ethical approval

KENYATTA UNIVERSITY
ETHICS REVIEW COMMITTEE

Email: chairman.kuerc@ku.ac.ke
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Our Ref: KU/R/COMM/51/471

Date: 11th June 2015

Mabeya Nyatichi Sepha
Kenyatta University,
P.O Box 43844,Nairobi.

Dear Ms. Mabeya,

APPLICATION NUMBER PKU/341/E28- "SEROPROFILE, GENETIC DIVERSITY AND DRUG RESISTANCE OF HEPATITIS B VIRUS AMONG HIV INFECTED INDIVIDUALS VISITING MAMA LUCY HOSPITAL COMPREHENSIVE HIV CLINIC, NAIROBI, KENYA".

1. IDENTIFICATION OF PROTOCOL
The application before the committee is with a research topic, **"Seroprofile, genetic diversity and drug resistance of hepatitis B Virus among HIV Infected individuals visiting Mama Lucy Hospital comprehensive HIV clinic, Nairobi, Kenya"** received on 11th May 2015 and discussed on 12th May, 2015.

2. APPLICANT
Mabeya Nyatichi Sepha

3. SITE
Mama Lucy Hospital comprehensive HIV clinic, Nairobi, Kenya.

4. DECISION
The committee has considered the research protocol in accordance with the Kenyatta University Research Policy (section 7.2.1.3) and the Kenyatta University Ethics Review Committee Guidelines **AND APPROVED that the research may proceed for a period of ONE year from 11th June, 2015.**

5. ADVICE/CONDITIONS
i. Progress reports are submitted to the KU-ERC every six months and a full report is submitted at the end of the study.
ii. Serious and unexpected adverse events related to the conduct of the study are reported to this board immediately they occur.
iii. Notify the Kenyatta University Ethics Committee of any amendments to the protocol.
iv. Submit an electronic copy of the protocol to KUERC.

When replying, kindly quote the application number above.

If you accept the decision reached and advice and conditions given please sign in the space provided below and return to KU-ERC a copy of the letter.

PROF. NICHOLAS K. GIKONYO
CHAIRMAN: KENYATTA UNIVERSITY ETHICS REVIEW COMMITTEE

I SEPHA NYATICHI MABEYA accept the advice given and will fulfill the conditions therein.

Signature.....Dated this day.....16TH..... of...JUNE.. 2015.
cc. Vice-Chancellor.

Appendix ii: Informed Consent Form

Study title

Seroprofile, genetic diversity and drug resistance of hepatitis b virus among HIV infected individuals in Nairobi, Kenya

Purpose of the study

This is a research study designed to establish the diseases stage in HBV, genetic diversity and levels in drug resistance among HIV infected individuals attending HIV comphrensive clinics in Nairobi, Kenya

Inclusion criteria

- HIV infected individuals attending HIV comphrensive clinic.
- Those who had given out their informed consent will be included in this study.

Exclusion criteria

- HIV negative individuals.
- Those who had not given their consent, they will not be included in this study.

Benefits

You will not receive direct benefit from participating from in the study but viral susceptibility results will be released to you. However, information gained from the study will be used to develop more effective management services.

Subject's right to confidentiality

The results of study may be published, released to a funding agency or presented in a scholarly fashion. The confidentiality of the study subjects will be protected and they will not be identified in any away.

Risk to subject

There are no known major physical, psychological or social associated with participating in the study. However, the study subject may feel discomfort from blood draw and sometimes bruises forms, which go away in about 3-5 days.

Subject right to refuse to participate or withdraw

The study subject may refuse to participate or withdraw room the study at any time.

Signatures

The study has been discussed with me and all my questions have been answered. I understand that additional questions regarding the study should be directed to the investigators. I agree with terms above and acknowledge that I have accepted voluntarily.

…… **……**

Signature of participant **date**

…….. **…….**

Signatures of witness **date**

……. **……**

Signature of investigator **date**

Appendix iii: Questionnaire

Seroprofile, Genetic Diversity And Drug Resistance Of Hepatitis B Virus Among HIV-1 Infected Individuals In Nairobi, Kenya

Investigators:

i) Sepha N Mabeya
ii) Dr. Caroline Ngugi
iii) Dr. Raphael Lihana

Dear Sir or Madam,

Hello? My name is Sepha N Mabeya, a Master of science (Infectious diseases and Vaccinology) student at the Jomo Kenyatta University of Agriculture and Technology. I am conducting a research on seroprofile, HBV genetic diversity and drug resistance among HIV infected individuals in Nairobi. I would like you to take part in this study since your views are very important and will help in determine the diseases burden and levels of drug resistance in response to treatment. Questions will take less than ten minutes. It is up to take part of disagree. Feel free to answer the questions in any way you would like to answer. Answers that you will give will be treated with ultimate confidentiality. Many thanks in advance for your time and your efforts.

Number…… Date …… study site……

1. What is your gender?//Jinsia?

2. How old are you/Age /rika?

3. What is your residence?/Makao?

8. Are you on HAART treatment/Wanyua madawa ya virusi?

a) Yes/Ndio b) No/La

If yes/Ndio? When/lini?

9. Have you been vaccinated against HBV?

a) Yes
b) No

Thank you very much for the participation in this questionnaire!