HEPATITIS B VIRUS GENETIC DIVERSITY AND SURFACE ANTIGEN MUTATIONS AMONG VOLUNTARY BLOOD DONORS IN KENYA

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JOMO KENYATTA UNIVERSITY OF

AGRICULTURE AND TECHNOLOGY

2018

Hepatitis B Virus Genetic Diversity and Surface Antigen Mutations among Voluntary Blood Donors in Kenya

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A thesis submitted in partial fulfillment for the Degree of Master of Science in Medical Virology in the Jomo Kenyatta University of Agriculture and Technology

2018

DECLARATION

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

Signature …….....… Date …….....…….

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

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DEDICATION

This work is dedicated to Nyairo's family especially my wonderful father Mr. Alfred Momanyi Nyairo, my mother Mrs Trusillah Ongeri, siblings, my husband Vincent Sego, our sons Ryan and Alpha who gave me moral support and encouragement during my study.

ACKNOWLEDGEMENT

I am grateful to God almighty who has given me the grace and strength to enable the completion of this work. I would like to acknowledge my supervisors Prof. Zipporah Ng'ang'a and Dr. Samoel Khamadi for supervising and mentoring me through this process. Their commitment and support to this work was second to none. Much of the work presented in this thesis would not have been possible without the help of Dr. Raphael Lihana whose input and counsel contributed greatly to the success of this project. I would also like to acknowledge the head of Regional blood Transfusion services Dr. Margaret Oduor for her consent to collect and use samples from blood donors for this work.

I would like to express my gratitude to the KEMRI Production unit for their facilities and support during my research period. Special thanks are given to Dr. James Kimotho for his precious suggestions and support in my project. I would like to extend my gratitude and thanks to my family for their continuous support and constant faith in me.

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LIST OF ABBREVIATIONS AND ACRONYMS

'a' determinant	Alpha determinant
A	Adenine
Aa	Amino acid
ALT	Alanine transaminase
Anti -HBS	Antibodies to the HBV surface antigen
Anti-HBC	Antibodies to the HBV core antigen
ART	Antiviral therapy
BCP	Basic core promotor
bp	Base pair
C	Cytosine
cccDNA	Covalently closed circular Deoxyribonucleic acid
cDNA	Complimentary DNA
CHB	Chronic Hepatitis B
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
G	Guanine
HBcAg	Hepatitis B core Antigen
HBeAg	Hepatitis B envelope Antigen
HBIG	Hepatitis B immune globulin
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B Virus
HBx	Hepatitis B virus x protein
HCC	Hepatocellular Carcinoma
IFN-α	Interferon Alpha

IgM	Immunoglobulin M
Kb	Kilo base pair
KEMRI	Kenya Medical Research Institute
LFTs	Liver Function Tests
LHBS	Large Hepatitis B surface protein
MEGA	Molecular evolutionary Genetics Analysis
MHBS	Middle Hepatitis B surface protein
MHR	Major Hydrophilic Region
ml	Milliliter
mM	Millimolar
NAT	Nucleic acid testing
ng	Nanogram
nm	Nanometer
NTCP	Sodium taurocholate cotransporting polypeptide
OBI	Occult hepatitis B virus infection
OD	Optical density
ORFs	Open Reading Frames
PCR	Polymerase Chain Reaction
PEP	Post Exposure Prophylaxis
PrEP	Pre-exoposure Prophylaxis
RBTCs	Regional Blood Transfusion Centers
RNA	Ribonucleic Acid
RT	Room Temperature
S-gene	Surface Gene
SHBS	Small Hepatitis B surface protein
T	Thymine
TAE	Tris Acetate EDTA
TTIs	Transfusion Transmittable Infections

VEM	Vaccine Escape Mutations
WHO	World Health Organisation

ABBREVIATION AND AMBIGUITY CODES FOR AMINO ACIDS

Alanine	Ala	A
A**r**ginine	Arg	R
Asparagi**n**e	Asn	N
Aspartic Aci**d**	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Glycine	Gly	G
Glutamine	Gln	Q

ABSTRACT

Hepatitis B virus belongs to *Hepadnaviridae* family and replicates by reverse transcription of the viral RNA. Nucleotide variation within the virus has led to its classification into ten genotypes (A-J). The reverse transcription step within its life cycle is prone to introduction of errors and accounts for the current existence of mutants, recombinants, and quasi species of HBV. As a consequence, mutations may render blood donations false negative for hepatitis B surface antigen (HBsAg) upon serological testing. In Kenya, data on escape mutations of HBsAg is limited. This study aimed at determining HBV genetic diversity and surface antigen mutations among voluntary blood donors in Kenya. A total of 301 blood samples were collected from the Regional Blood Transfusion Centres (RBTCs) and tested for HBsAg using the Enzyme-Linked Immunosorbent Assay (ELISA) and nested PCR. The amplified products were sequenced, the generated HBV sequences analysed for S gene mutations and the genetic diversity of HBV was determined. All the statistical analyses were performed using SPSS version 19.0. Out of the 301 samples tested by PCR for the HBV DNA, two samples 2/301 (0.66 %) were identified were identified as occult HBV infection. The cases were confirmed by sequencing. All strains were genotype A, with A1 (94.20%) predominated, A2 (1.45%) and A3 (4.35%) sub genotypes. The amino acid substitutions Q101H, T118A, K122R, K133E, M133I, T143M, C149R, E164G and V184A were detected in ORF S with T143M and K122R as the most prevalent mutations. The present study revealed the predominance of genotype A, with A1, A2 and A3 sub genotypes among Kenyan voluntary blood donors. In addition, surface antigen mutations were detected. Different HBV genotypes have been associated with disease chronicity and development of Hepatocellular Carcinoma (HCC) and response to treatment. Surface antigen mutants evade the immune surveillance leading to false negative diagnosis for HBsAg in blood donations. This could lead to increased HBV transmission in the general population. Therefore, there is need for continuous surveillance of HBV genotypes and mutations with clinical significance in the population.

CHAPTER ONE

INTRODUCTION

1.1 Hepatitis B Virus Infection

Hepatitis B virus (HBV) infects the liver causing acute and chronic liver disease (Akbar *et al*., 2004; McMahon, 2005). Despite the presence of an effective prophylactic vaccine it is estimated that 2 billion people have been infected with HBV during their lifetime and over 350 million are chronic carriers of the virus and have an increased risk of developing liver cirrhosis and hepatocellular carcinoma (HCC). (WHO, 2012), with 65 million chronic carriers residing in Africa. The sub-Saharan region is highly endemic with Hepatitis B surface antigen (HBsAg) carrier rates of between 9-20 % (Kramvis & Kew, 2007). The prevalence of HBV chronic carriage ranges between 3% and 22% in blood donors where than 50% of blood donors and blood recipients have had a natural exposure to HBV (Allain *et al*., 2003). In addition 12.5% of patients who receive blood transfusion are at risk of post-transfusion hepatitis (Tessema *et al*., 2010).

Hepatitis B virus, a member of the *Hepadnaviridae* family, is a circular, partially double-stranded Deoxyribonucleic acid (DNA) virus with a genome of approximatively 3.2 kilobases in length. It contains four overlapping open reading frames (ORFs) PreS/S, PreC/C, P and X (Pourkarim *et al*., 2014).

Hepatitis B virus has considerable genetic variability related to high levels of virus production and absence of proofreading activity of the viral polymerase during the reverse transcription step of the replication cycle (Seeger *et al*., 2000).

This unique replication tactic accounts for the current existence of various genotypes, subgenotypes, mutants, recombinants, and even quasispecies of HBV. The virus has also been classified into 40 sub-genotypes and nine serotypes (*adw2, adw4, ayw1, ayw2, ayw3, ayw4, adrq+, adrq– and ayr* (Norder *et al.*, 2004; Schaefer, 2007). Its genotypes

and sub-genotypes have a distinct geographical distribution and are associated with ethnicity. These have a medical significance such as diagnostic escape, immune evasion and drug resistance (Song *et al*., 2006). Hepatitis B Virus has been classified into ten genotypes (A-J) based on >8% intergroup sequence divergence with different geographical and ethnic distribution (Tatematsu *et al*., 2009; Yu *et al*., 2010). Genotyping and HBsAg subtyping of isolates are useful tools to understand the epidemiology of HBV infection and accumulating data suggests that HBV genotypes and subgenotypes influence the clinical outcome of infection, including the risk of (Hepatocellular Carcinoma(HCC) (Pourkarim *et al.,* 2014). Reports have indicated that genotype C was associated with faster liver damage and higher risk of HCC compared to genotype B. Yu *et al*., 2005). Genotype D has been reported to be associated with severe liver disease compared to genotype A in some studies(Toan *et al*., 2006), while the opposite was reported by others, with genotypes A and F more associated with HCC development than genotyped (Livingston *et al.,* 2007). The influence of HBV genotypes on response to antiviral therapy has been also studied. Genotypes A and B have been associated with a better response to interferon-based therapies compared to genotypes C and D (Raimondi *et al.,* 2010).

In addition, the high genetic variability of HBV leads to selection of mutations in different viral genes under pressure of host immunity or antiviral treatment. Hepatitis B surface antigen detection is a primary target for diagnosis and immunoprophylaxis of HBV infection both in the routine virology laboratory and for blood donation screening (El-sherif *et al.,* 2007). The HBsAg, a major envelope protein of HBV is composed of 226 amino acids (El Chaar *et al*., 2010) and contains a major hydrophilic loop (MHR) of HBsAg at amino acid aa 100 to 170. Interestingly, the MHR contains a cluster of neutralizing B cell epitopes located between aa 124 to 147 called the alpha determinant (Zheng *et al*., 2004; Hu *et al*., 2009). The ‘a’ determinant is the main target for neutralizing antibodies used in active, passive immunisation and in diagnostic assays. Mutation within this region could alter the antigenicity of the protein, leading to failed

neutralization of HBsAg by anti-HBs antibody and may cause escaping from the host's immune system (Moradi *et al.,* 2012). In Kenya, little is known about the molecular characteristics of HBV strains. To date, few studies have reported the predominance of genotype A, sub genotype A1 (Mwangi *et al*., 2008; Ochwoto *et al*., 2013; Kwange *et al*., 2013).

1.2 Statement of the problem

Infection with Hepatitis B virus (HBV) causes acute and chronic liver disease (Akbar *et al*., 2004; McMahon, 2005). Globally, 2 billion people are infected with HBV, 65 million reside in Africa. An overall HBsAg prevalence of 8.8% has been reported in a rural population in Kenya (Mutuma *et al*., 2011) The high genetic variability of HBV leads to selection of mutations in different viral genes under pressure of host immunity or antiviral treatment (Ghany & Liang, 2007). Mutations evade the immune surveillance leading to false negative diagnosis during routine screening for HBsAg in blood donations therefore transmitting HBV to both vaccinated and unvaccinated populations (Moradi *et al.,* 2012). Studies focusing on HBsAg mutations are scarce in Kenya and the extent of mutations in the population is not well defined. Therefore, the main objective of this study was to determine Hepatitis B Virus genetic diversity and surface antigen mutations among voluntary blood donors in Kenya.

1.3 Justification of the study

In Kenya, the mainstay of donor screening is serology. However NAT is important since, besides narrowing the window period hence increasing safety of blood, its downstream applications provide information on genotypes as well as mutation profile of HBV**.** This study supports the potential use of molecular detection as an alternative when it has become widely available at lower cost for public health. Information obtained from the current study will apply on improving blood donation programs in

Kenya. Understanding the diversity of mutations is useful in designing a diagnostic assay or development of a test algorithm

1.4 Research Questions

1. What is the frequency of occult hepatitis B virus infection among voluntary blood donors in Kenya?
2. What is the prevalence of surface antigen mutations among voluntary blood donors in Kenya?
3. What is the Hepatitis B Virus genetic diversity among voluntary blood donors in Kenya?

1.5 Objectives

1.5.1 General Objective

To determine Hepatitis B Virus genetic diversity and surface antigen mutations among voluntary blood donors in Kenya.

1.5.2 Specific Objectives

1. To detect occult hepatitis B virus infection among voluntary blood donors in Kenya.
2. To identify HBV genotype and sub-genotype circulating among voluntary blood donors in Kenya.
3. To determine presence of surface antigen mutations among voluntary blood donors in Kenya

CHAPTER TWO

LITERATURE REVIEW

2.1 Hepatitis B Virus

Hepatitis B virus belongs to *Hepadnaviridae* family, genus *orthohepadnaviruses* (Schaefer, 2007). This genus is shared with the Chimpanzee hepatitis B virus, Gorilla hepatitis B virus, Orangutan hepatitis B virus and the Woodchuck hepatitis B virus (Bonvicino *et al.*, 2014). Humans are known to be the natural definitive hosts of human HBV, in whom it causes acute or chronic liver infection which may lead to various fatal complications (Bowyer *et al.*, 2011). Hepatitis B virus is a blood borne virus and is roughly 75-200 times more infectious than HIV (Bowyer *et al.*, 2011).

2.2 Hepatitis B Virus Structure

Hepatitis B infectious virus is 42 - 47 nm in diameter and circulates in blood in concentrations as high as 10^8 virions per milliliter (ml) (Liang, 2009). It is a circular double-stranded DNA virus, consisting of an inner nucleic acid, containing a core and an outer lipoprotein envelope, containing HBsAg protein (Figure 2.1). The virus secretes excess HBsAg into the serum of infected individuals, and this is the first serological marker to become detectable. It appears in the blood one to ten weeks after exposure and two to eight weeks before the onset of clinical hepatitis (William, 1999). The viral DNA is associated with DNA polymerase, an enzyme, which is important for the replication of the virus. Together they are surrounded by an inner coat of core protein (HBcAg). The HBcAg is found within the liver of infected individuals, but is insoluble and thus not detected in the blood (William, 1999). However the soluble component of HBcAg, called HBeAg, is secreted into the serum and cleared rapidly after acute infection but persists in the serum of patients with active viral replication (Liang, 2009).

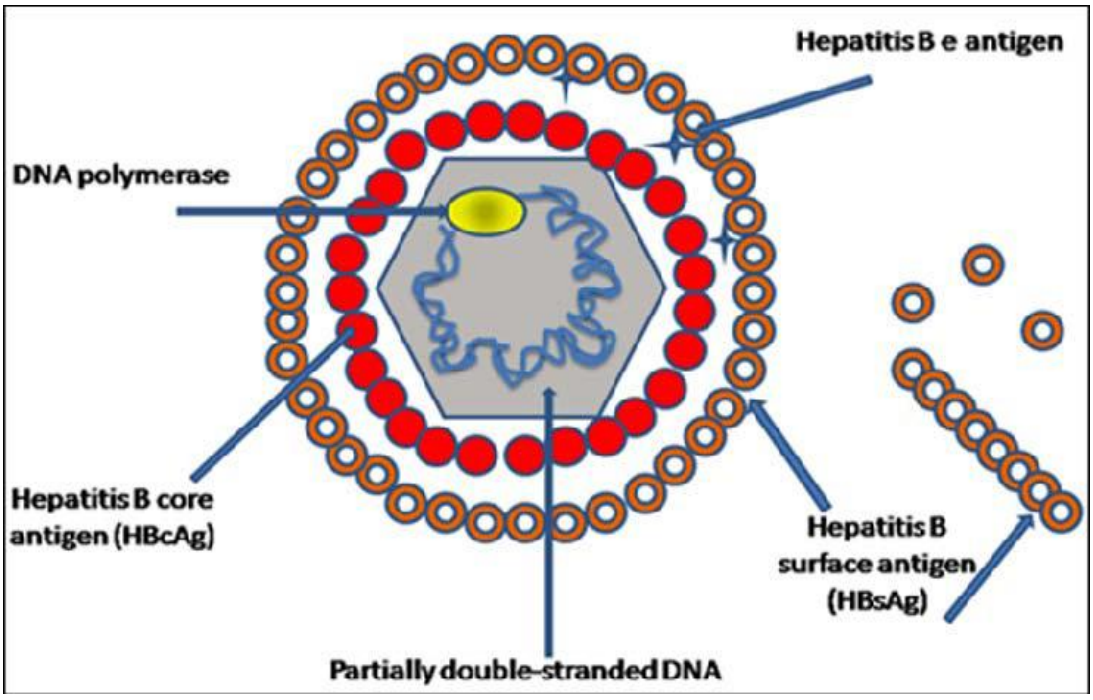

Figure 2.1: Structure of HBV particle and surface antigen (Dane *et al.,* 1970).

2.3 Hepatitis B Virus genome

The genome consists of a partially double-stranded circular DNA molecule of approximately 3200 nucleotides (nt) in length (Yano *et al*., 2015). This compact genome consists of four partially overlapping open reading frames (ORFs), which include the Precore/Core gene (PreC/C), which encodes the envelope protein(HBeAg) and the core protein (HBcAg), polymerase gene (P), which encodes the viral polymerase (reverse transcriptase, (PreS/Surface gene (PreS/S), which encodes: Large HBs, Middle HBs, Small HBs envelope proteins and finally the X gene, which encodes the small regulatory HBx (Liang, 2009; Shen *et al*., 2014). (Figure 2.2)

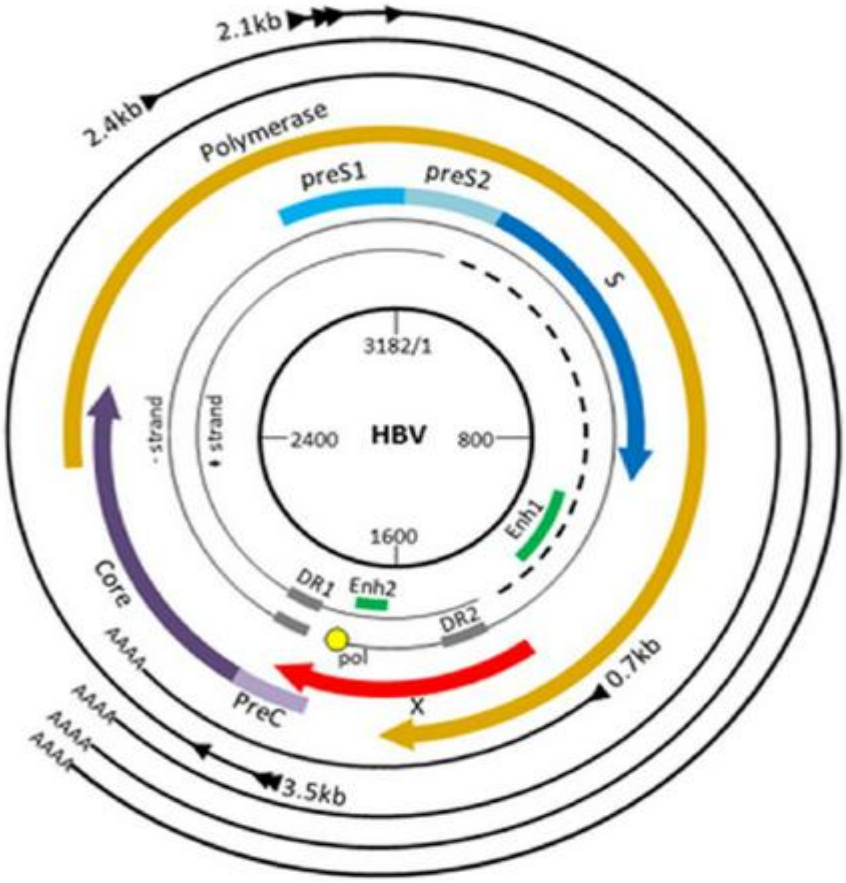

Figure 2.2: Genome organization of hepatitis B virus (Minor & Slagle (2014).)

HBV contains a small, partially double-stranded (~dsDNA) genome (see inner black circles) that consists of a full-length negative strand and an incomplete (dashed lines) positive strand. The genome contains four promoters, two enhancer regions (Enh1, Enh2), and two direct repeats (DR1, DR2). The four ORFs are depicted by the colored arrows. During virus replication, the ~dsDNA genome is repaired into covalently closed circular (ccc) DNA, which serves as the template for viral transcription. The four major transcripts, shown as thin black outer arrows, are described in the text. Note that the S ORF (blue) was the gene of interest.

2.4 Hepatitis B virus gene products

2.4.1 Surface Proteins

The HBV surface (S) ORF is composed of three different surface genes (preS1, preS2 and S), and encodes three different surface proteins: large (LHB), middle (MHB), and small (SHB) (Schadler & Hildt, 2009). The SHBs: The 226 amino acid S protein (HBsAg) (unglycosylated 24 kD; glycosylated 27 kD) (Gerlich *et al.*, 1992) is the most abundant protein of all three surface proteins and is encoded by the SHBs domain. The loop located between amino acids 124 - 147, forms the major antigenic determinant, known as the‘ a‘determinant, which is the initial target of the anti-HBs immune response during acute hepatitis (Moradi *et al*., 2012). Amino acid substitutions within this determinant, can lead to conformational changes affecting the binding of neutralising antibodies (Chongsrisawat *et al*., 2006).

The MHBs: The 281 amino acid MHBs protein (preS2/S), encoded by the MHBs domain, consists of the SHBs and a 55 amino acid preS2 (Ganem & Schneider, 2001). MHBs contains overlapping B and T cell epitopes and a binding site for neutralizing antibodies, located between amino acids 120 to 145 and the transactivation domain (Hildt *et al.*, 2002).

The LHBs carries a receptor recognition domain (encoded by LHBs domain 389 to 400 amino acids, which allows efficient receptor binding during hepatocyte membrane attachment (Wang *et al*., 2003). Middle HBs is another minor component on the surface of virions and subviral particles (10-20%) and is not required for viral morphogenesis (Wang *et al*., 2003). Hepatitis B surface antigen acts as a biomarker for the HBV-replication activity, and persistence of HBsAg for more than 6 months is generally considered the indicator for chronic hepatitis B (CHB) infection (Wang *et al*., 2003). The Hepatitis B surface antigen is the main target of protective humoral immune response induced by vaccination, and is also one major target for immune therapy

(Kondo *et al*., 2013). The S ORF also includes other important functional domains, such as essential binding sites for hepatocyte receptors, epitopes for T- or B-cells, major hydrophilic region (MHR) and the "a" determinant region, mutations in these domains are related to immune and vaccine escape (Moradi *et al.,* 2012).

2.5 Hepatitis B Virus replication cycle

Viral replication takes place via an RNA intermediate utilizing reverse transcriptase, which lacks proof reading activity (Yano *et al*., 2015). It also known to have a high error rate, the nucleotide exchange rate is 10^4 fold higher than that of typical DNA genomes estimated to be 0.1 and 0.7 per year (Xu *et al*., 2015). The initial stage of HBV infection involves the attachment of virus to the membrane of hepatocyte using a specific receptor recognition domain identified in the preS domain of surface protein. In addition sodium taurocholate cotransporting polypeptide (NTCP) a functional cellular receptor mediates HBV entry through binding of the preS domain peptide (Yan *et al*., 2012; Ni *et al*., 2014). Transcription starts from the core promoter to yield the 3.5 kb pregenomic RNA, which is packaged with polymerase into immature core particles, and then serves as a template for reverse transcription and negative strand DNA synthesis. The incomplete positive strand DNA is then synthesized. The mature core particles are packed into HBsAg and pre-S proteins in the endoplasmic reticulum (ER) then exported from the cell (Figure 2.3) (Pollicino & Raimondo, 2014).

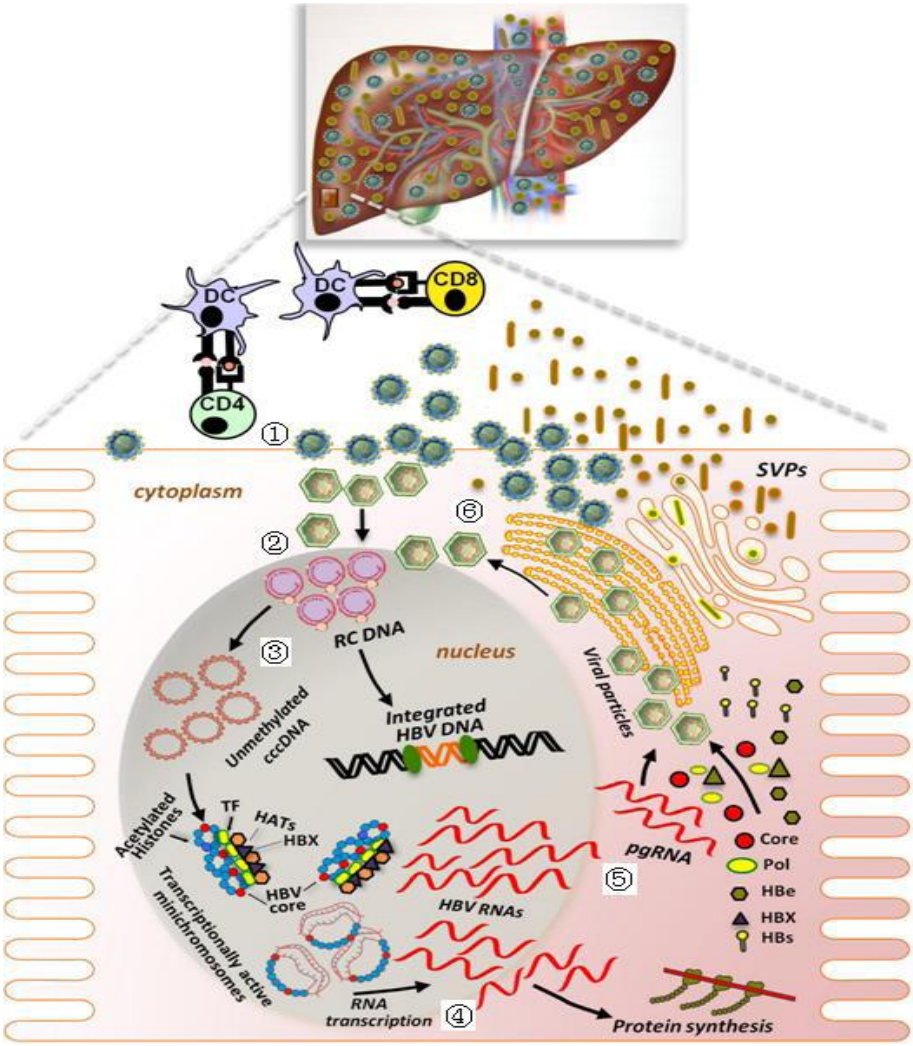

Figure 2.3: HBV infection cycle.

The 6 stages of HBV infection cycle: 1. viral attachment and entry; 2) transportation into the nucleus; 3) conversion of rcDNA to cccDNA; 4) transcription and translation; 5) synthesis of progeny viral DNA genomes; and 6) assembly of viral particles and secretion (Pollicino and Raimondo, 2014).

2.6 Hepatitis B virus Epidemiology, transmission and disease profile.

2.6.1 Epidemiology of HBV

2.6.1.1 Global prevalence of HBV

HBV infection is one of the most prevalent viral infections. More than two billion people who are currently alive have been infected, of whom 240 million are chronically infected. The global prevalence of chronic HBV infection is estimated to be 3.9% in males and 3.5% in females in 2005. HBV infection accounts for 30.3% of liver cirrhosis deaths and 45.4% of liver cancer deaths. Overall, 786,000 people were estimated to have died in 2010 from HBV-related liver diseases including acute or fulminant hepatitis, liver cirrhosis and liver cancer, which makes HBV infection as a 15^{th} ranked cause of death (Ott *et al.*, 2012).

The prevalence of HBV infection varies in different geographical locations and population groups across the world, the global distribution of HBV is shown is figure 2.4. Prevalence of chronic HBV infection is classified as high where prevalence is more than 8% such as Southeast Asia, China, sub-Saharan Africa and the Amazon Basin (Sugiyama *et al.*, 2007). Intermediate region where prevalence is 2%-7% including Eastern and Southern Europe, Middle East, Japan, and part of South America; and as low where prevalence is less than 2% in North America, Northern and Western Europe and Australia. In high endemic areas, 70%-90% of the population has a past or on-going serologic evidence of HBV infection and most infections were observed in infancy or childhood. In intermediate areas, 10%-60% of the population shows evidence of HBV infection and 2%-7% are chronic carriers. Many infections occur in adolescent and adults, but infection during infancy and children still contribute at high rate. In low HBV prevalence areas, 5%-7.5% of population has evidence of serologic HBV infection, of which 0.5%-2% are chronic carriers (Hou *et al.*, 2005). Hepatitis B surface antigen seroprevalence was 3.61%

worldwide where about 248 million individuals were HBsAg positive in 2010. Around half of the world populations reside in HBV endemic area 15%-25% die of cirrhosis and HCC Data from World Health Organization indicated a prevalence of 8.83% in the African Region, 0·81% in America ,Eastern Mediterranean Region 3.01%, European Region2.06%, South East Asian Region1.90% and Western Pacific 5.26% (Schweitzer & Hei, 2012).

2.6.1.2 Distribution of HBV infection in Africa

Hepatitis B surface antigen prevalence in the general population in the WHO African Region (8.83%), the prevalence of chronic HBV infection is high (>8%) Angola, Benin, Burkina Faso, Burundi, Cameroon, Cape Verde, Central African Republic, Congo, Cote d'Ivoire, Equatorial Guinea, Gabon, Gambia, Ghana, Guinea, Liberia, Malawi, Mali, Mauritania, Mozambique, Namibia, Niger, Nigeria, Senegal, Sierra Leone, South Sudan, Swaziland, Togo, Uganda, Zimbabwe. Intermediate areas with (2–8%) prevalence include: Algeria, Congo, Eritrea, Ethiopia, Kenya, Madagascar, Rwanda, South Africa, Tanzania, Zambia and a low prevalence region (<2%) is Seychelles (Schweitzer & Hei, 2012).

2.6.1.3 Hepatitis B Virus disease burden in Kenya

In a study carried out during the Kenya AIDS Indicator Survey to estimate the HBV infection prevalence showed that 28.0-35.3% had exposure to HBV where estimated prevalence of Chronic Hepatitis B Infection (CHBI) was 2.1% (Ly *et al.*, 2016). It is reported that in a rural population in Kenya there was an overall HBsAg prevalence of 8.8%, with the largest number (34%) of those positive being between 5 and 10 years of age. This suggested either mother to child transmission or early childhood exposure to hepatitis B virus (HBV) and 86.6%) were HCC cases (Mutuma *et al.*, 2011). A recent study among HIV co-infected patients reported HBV prevalence of 13.3% (Kerubo *et al*., 2015).

2.7 Transmission of Hepatitis B Virus

Hepatitis B virus is reported to be 100 times more infectious than the human immunodeficiency virus (HIV) and 10 times more infectious than HCV, making it highly transmissible (Gupta *et al*., 2008; Pande *et al*., 2011). HBV can be transmitted through contacts with body fluids from infected HBV patients. Blood is the most important route of HBV transmission, but other body fluids such as semen and saliva have been reported to be the source of transmission. (Seeger *et al.,* 2007). It is a highly stable virus, resistant to breakdown, can survive outside the body for at least 7 days (Lavanchy, 2004; Seeger *et al.,* 2007). Three major modes of transmission are documented, namely; the horizontal, sexual and vertical routes of transmission (Wasmuth, 2009). Horizontal transmission of HBV may occur through direct contact with infected blood and blood products, dialysis, sharing of needles among injection drug users, needle stick accident among healthcare professionals and intravenous drug abuse (Lavanchy, 2005; Wasmuth, 2009). Having multiple sexual partners and continuous unprotected sexual intercourse with HBsAg positive partners is the main cause of HBV infection in the adolescent and adult-age populations (Wasmuth, 2009). Chronically infected individuals do not immediately present with symptoms and as such are not aware of the HBV infection (Lavanchy, 2005). In the case of men who have sex with men (MSM), receptive anal intercourse is also a major determinant of acquisition of HBV infection (Freitas *et al*., 2014).

Vertical transmission or mother-to-child transmission (MTCT) occurs when a mother has an active infection during pregnancy or at birth. The risk of transmission is directly proportional to the viral replication rate in the mother. Pregnant women with no detectable HBV DNA are thus less likely to pass the infection on to their babies while the risk in mothers with high levels of viremia may be up to 90% (Wasmuth, 2009). Although screening for HBV infection in blood donors has contributed considerably to the reduction of transfusion transmitted HBV infection, HBV infection after blood transfusion is still a matter of concern.

2.8 Natural history of Hepatitis B Virus infection

The incubation period of HBV following successful infection typically is 8 to 12 weeks .At this stage, the clinical presentation will range from subclinical and asymptomatic carriage to acute self-limiting hepatitis or fulminant hepatitis to chronic hepatitis (Figure 2.6). Infection progression depends on the person's age, viral factors and the host immune response, which could lead to liver cirrhosis and 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 and may proceed to chronicity. However, in adults the infection is usually acute and self resolving (De Francis *et al.*, 2003).

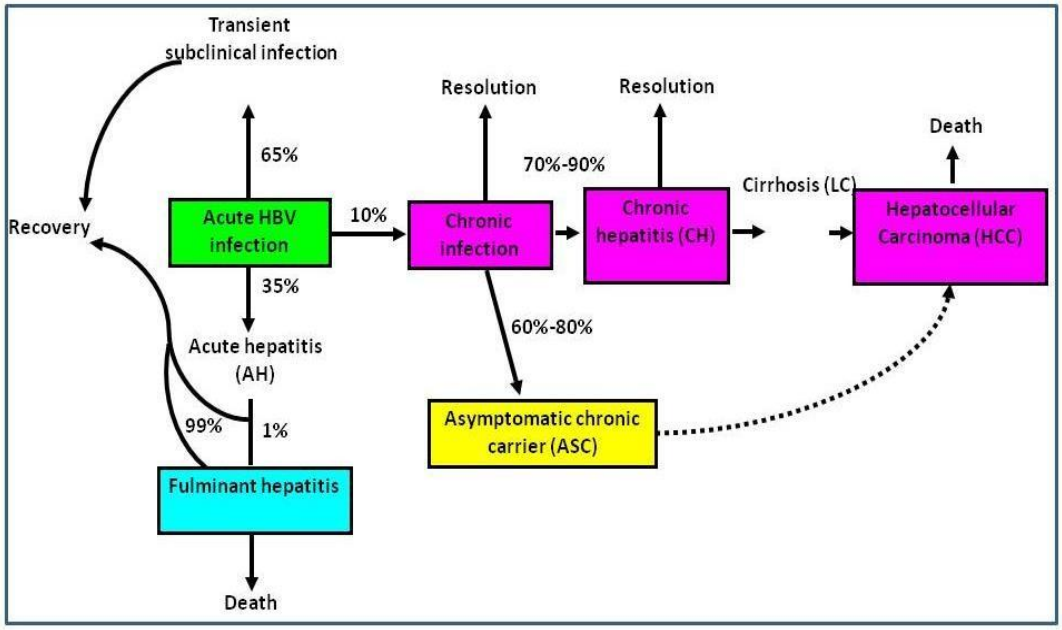

Figure 2.4: Natural history of Hepatitis B virus infection (Feitelson, 1992).

2.8.1 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 2.7). 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 and Williams, 1990), a state that is associated with high mortality.

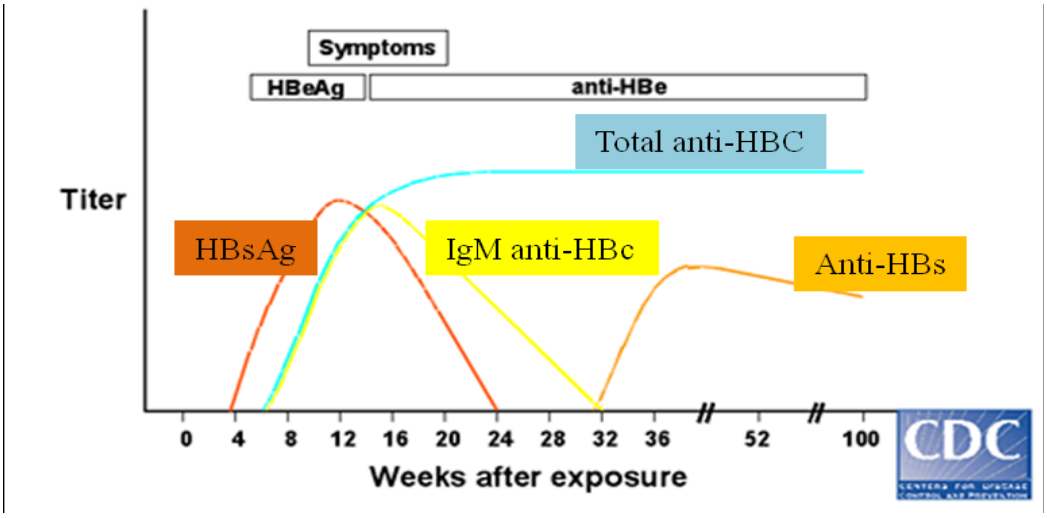

Figure 2.5: Markers of acute resolving HBV infection (Paar, 2008)

2.8.2 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 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 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:

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 with very high detectable antibodies. Alanine transaminase (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).

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 to 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).

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).

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).

2.8.3 Occult Hepatitis B infection (OBI)

Occult Hepatitis B infection **(**OBI) 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. When detectable, the amount of HBV DNA in the serum is usually below 200IU/ml (Ocana *et al*., 2011). The molecular basis of OBI is related to the long term persistence of viral cccDNA in the nuclei of hepatocytes. Individuals who are HBsAg negative, with serum HBV DNA levels < 200 IU/ml, are considered to be true OBI (Gibney *et al.,* 2008). Samples in which serum HBV DNA levels are similar to HBsAg positive overt infection, but are HBsAg-negative, have been termed false OBI or HBsAg covert infection (Bell *et al.*, 2012). OBI can be classified into: seronegative (negative for all serological markers), which accounts for approximately 20% of all OBI cases, and seropositive (anti-HBs and anti-HBcpositive), which accounts for 80% of OBI cases (Martinez et al 2015). The occurrence of OBI is worldwide and the distribution of OBI may reflect the general prevalence of HBV (Raimondo *et al.*, 2007). Essentially, OBI is found in four 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).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).

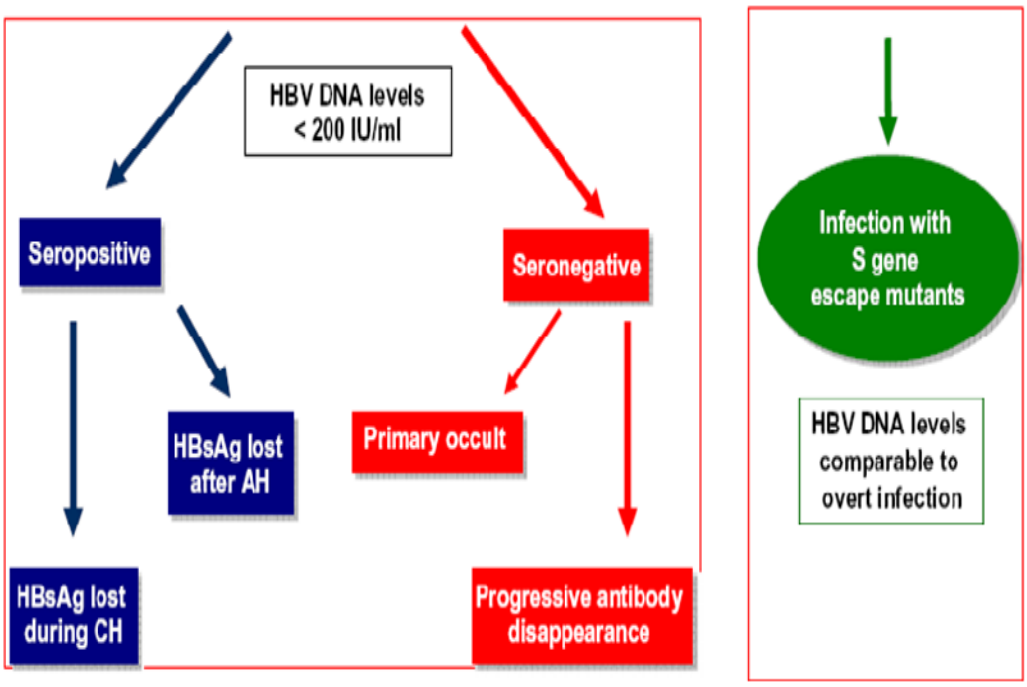

Figure 2.6: HBV profile in OBI and ‘‘false” OBI (Martinez *et al.*, 2015

2.8.4 Hepatitis B Genotypes and subtypes

A genotype is defined as the genetic constitution of an organism (Francois *et al*., 2001). Hepatitis B Virus genotype is due to the entire nucleotide sequence, and is more appropriate for investigation of geographic distribution and epidemiology. Hepatitis B virus has been classified into 10 genotypes (A-J) based on >8% intergroup sequence divergence with different geographical and ethnic distribution. For instance, Genotype A has been found mainly in North America, USA, Europe, India and Africa (Yu *et al*., 2010; Kramvis, 2014). In Africa, Genotype A is found in Southern, Eastern and Central Africa (Kramvis & Kew, 2007). Sub-genotypes have also been identified within genotypes A and D, subgenotype A1 has subsequently been found in Malawi (Sugauchi *et al.*, 2003), Somalia (Sánchez *et al.*, 2002), Zimbabwe (Gulube *et al.*, 2011), Uganda (Hannoun *et al.*, 2005) and Tanzania (Hasegawa *et al.*, 2006).

In Kenya the subgenotype A1 distribution has been reported. For instance, genotype A was found to be predominant followed by genotypes D and E among HBsAg positive blood donors (Mwangi *et al.*, 2008), another study sequenced the complete S gene reported subgenotypeA1, genotype D, subgenotype D1 (Ochwoto *et al.*, 2013). Genotypes B and C are prevalent in Southeast Asia and the Far East (Kramvis and Kew, 2007). Genotype D has been detected throughout the world and it is predominant in the Mediterranean, Middle East, South Asia and particularly in India (Yu *et al.*, 2010). Genotype E has also been detected in sub-Saharan Africa and West Africa (Suzuki *et al.*, 2005). Genotype G has a high prevalence in the United States, while genotype H is restricted to Central and South America, France, Colombia and Brazil (Weber, 2005). Recently, Genotype I was reported in Vietnam and Laos, while genotype J was identified in Japan (Yu *et al.*, 2010; Arankalle *et al.*, 2010). (Figure 2.4).

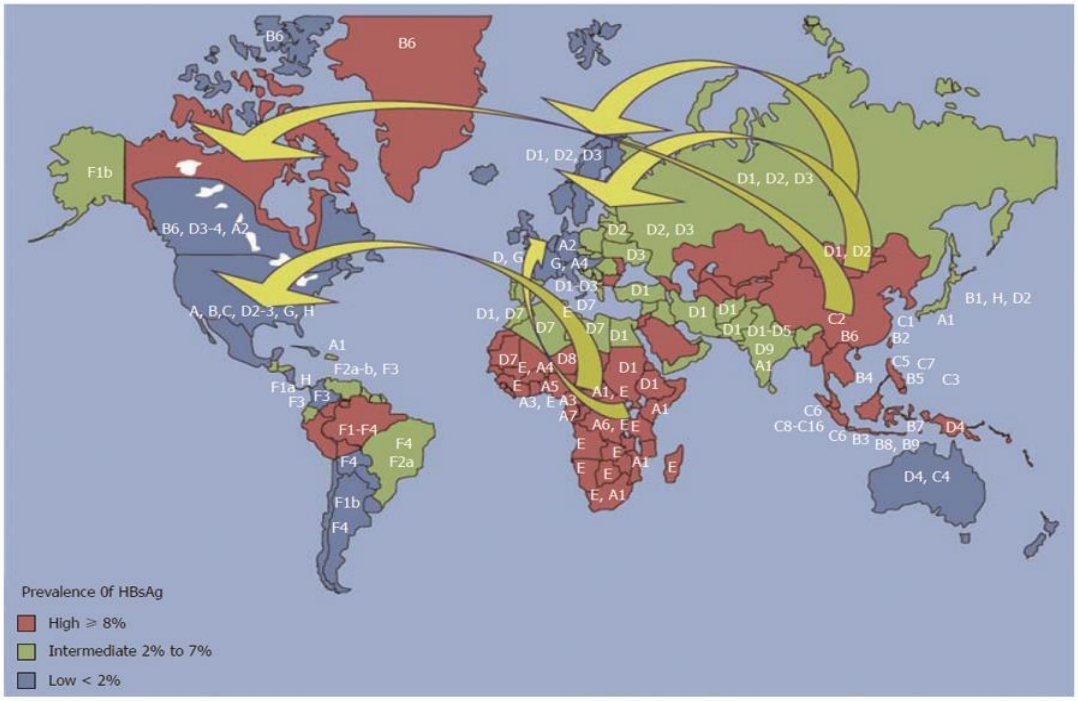

Figure 2.7: Geographic distribution of HBV genotypes and sub genotypes (Gerlich, 2013).

2.8.5 Clinical relevance of the HBV genotypes

The different HBV genotypes have been associated with some clinical features of HBV associated to liver disease, Hepatocellular Carcinoma (HCC), viral mutations and response to treatment (Kramvis & Kew, 2007). Genotype A is associated with a greater risk of progression to chronicity in adult acquired HBV infections (Kew *et al.*, 2005). In sub-Saharan Africa, subgenotype A1 is the predominant and has been associated with high rates of HCC, patients infected with subgenotype A1 have a higher rate of HBeAg negativity, low HBV DNA levels and develop HCC at a younger age compared to patients infected with other genotypes (Kew *et al.*, 2005). Data has indicated that genotype C is associated with faster liver damage and higher risk of HCC compared to genotype B (El-Serag *et al.*, 2012). Genotype D has been reported to be associated with severe liver disease compared to genotype A in some

studies, whereas genotypes A and F are more associated with HCC development than genotype D (Toan *et al.,* 2006). The influence of HBV genotypes on response to antiviral therapy has been also studied, genotypes A and B have been associated with a better response to interferon-based therapies compared to genotypes C and D (Raimondi *et al*., 2010).

Hepatitis B Genotype C has also been associated with a more severe liver disease and with a lower rate of response to interferon therapy (Croagh et *al*., 2015). The emergence of resistance to antiviral therapy with lamivudine seems to be independent of the genotype although it is related to the presence of HBeAg. HBV carriers infected with genotype C are more frequently positive for HBeAg (Echevarría *et al.,* 2006).

2.8.6 Hepatitis B virus mutations.

A mutation is defined as a random error occurring in the genome, which may lead to genetic variation in the organism (Paraskevis *et al.*, 2013) Because of the lack of proof reading activity by the HBV reverse transcriptase, error frequencies occur during replication. These result in HBV mixture of mutants and wild type in the entire genome (Datta, 2008). The mutations can occur either naturally or may be evoked during antiviral therapy where the mutation rate of HBV is relatively high with the nucleotide substitution rate per site per year estimated at 1.5×10^{-6} to 3.0×10^{-6} (Paraskevis *et al.*, 2013; Bouckaert *et al.*, 2013). The HBV genome encompasses four overlapping reading frames preS/S, preC/C, P and X, with the polymerase gene overlapping the envelope gene. As a result, mutations in the catalytic domain of the polymerase gene can affect the amino-acid sequence of the envelope protein (HBsAg) and vice versa (Pollicino *et al.,* 2014). Mutations in the HBsAg have several clinical effects, including: (i) reduced sensitivity to available diagnostic tests, (ii) a lack of immunity following vaccination with non-mutant HBV

variants (vaccine escaped mutant) and (iii) a failure of passive immunisation with HBV IgG (El Chaar *et al*., 2010).

2.8.7 Vaccine Escape Mutations

Hepatitis B Surface antigen contains major neutralising epitopes used in commercial hepatitis B vaccines (Francois *et al*., 2001). The benefit of the available anti-HBV vaccine in preventing new infection is evident worldwide and particularly in highly endemic areas (Torresi, 2008; Ni *et al*., 2012). The possible emergence of HBV genetic variants that are able to elude the control of the specific vaccine-induced anti-HBs antibodies have been reported, these escape mutants are a matter of concern when considering the efficacy of HBV vaccine in a given population (Ni *et al*., 2012). Regarding this, efficacy of HBV vaccine depends on HBV genotype prevalence in a given population. Administration of HBIG therapy has been implicated in the selection of HBV vaccine escape mutant. A study confirmed that first observation, reporting the identification of individuals who developed HBV infection in spite of having had vaccine induced circulating anti-HBs antibodies (Clements *et al.,* 2010). The most notable mutation responsible for HBV vaccine escape is a point mutation (G to A) at nucleotide position 587 of the S gene, consequently replacing glycine (G) with arginine (R) at amino acid residue 145 within the "a" determinant G145R (figure 2.5). Other common mutations involved in HBV vaccine escape include D144A, P142S, Q129H, I/T126N/A and M133L (Dindoost *et al*., 2012). For this reason, vaccine escape mutants are stable and thus may be transmitted to uninfected individuals. This creates a major concern as vaccination is currently the most effective preventive measure available (Weber, 2005). Mutant HBV may also remain stable over time, predominate over the wild type variant as a consequence of specific selective pressures such as the host immune response or prophylactic and sensitivity to antiviral drugs. Similar to their wild type virus, some HBV mutants are transmissible and as such have relevant medical and public health implications (Coleman, 2006; Rebecca, 2011).

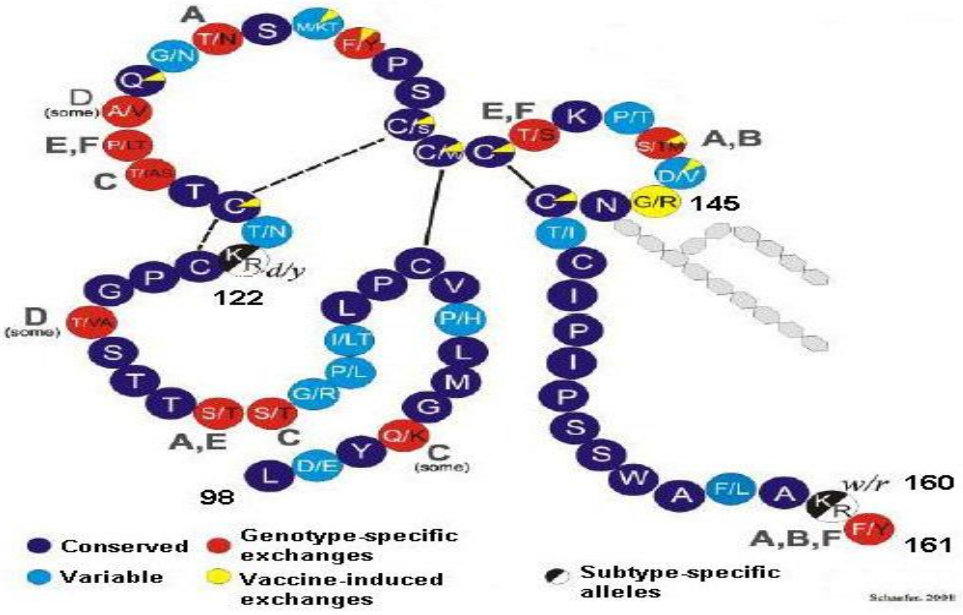

Figure 2.8: Genotype and vaccine induced specific exchanges in the "a" determinant of SHBs.

The "a" determinant consists of 2 loops: amino acid 124-137 and aa 138-147 or 149, held together by disulfide bridges represented by lines. The capital letters in black denote the genotypes of HBV (Schaefer, 2007).

2.8.8 Reduced sensitivity to available diagnostic tests

Routine screening of HBsAg among blood donors implemented in the early 1970s has significantly enhanced transfusion safety and has steadily reduced the incidence of transfusion-transmitted hepatitis B over the last four decades (Niederhauser *et al*., 2011). It was demonstrated that HBV transmission by blood components that are negative for HBsAg can still occur and HBV transmission remained the most frequent transfusion-transmitted viral infection (Alavian *et al*., 2012). Prevention and control is the most effective approach to assist in decreasing the spread of hepatitis B

infection. An increase in the number of infected individuals similarly creates a circumstance in which evolutionary progressions can lead to the emergence of new HBV isolates that are capable of evading detection, thereby entering the blood circulation (Roche *et al.,* 2010). NAT complements serological screening for blood donors and reduces the rate of transfusion-associated infection because it has the ability to detect the presence of infection by directly testing viral nucleic acid rather than antibodies. It is capable of detecting small amounts of DNA or RNA; this is beneficial during the window period and for occult hepatitis. Blood safety issues related to the safety of the blood or tissues of an HBsAg-negative donor and the risk of transmission are of important concern. There is a fear that escape mutants may be spread by blood transfusion, since HBV DNA detection by nucleic acid amplification technology (NAT) is not mandatory for blood donor screening in many countries and is not cost-effective (Fischinger *et al.,* 2010). However, in those areas where the prevalence of escape mutants is expected to be high, earlier studies clearly indicated that the transfusion of blood containing HBsAg has been associated with the development of post transfusion hepatitis B and that cases of HBV infection have been identified after transfusion of a HBsAg negative blood supply . Moreover, it is well known that mutants of HBsAg are able to cause infection and horizontal transmission despite the presence of anti-HBs (Bismas *et al*., 2013).

The most common mutation is G to R at 145 in the S gene (sG145R), which minimizes the binding of antibodies raised against S protein, to virions and subviral particles, and thus results in breakthrough infections. Another mutation at the same position sG145A, leads to diagnostic failure (Tong *et al.*, 2013). Therefore, there is a potential risk of false negative HBsAg results because commercially available monoclonal or polyclonal antibody may not detect the mutant HBV strains (Yokosuka and Arai, 2006). The failure of a diagnostic assay may be a threat to recipients of blood transfusions and organ transplants and therefore transfusion-associated hepatitis B signifies a global public health problem.

2.8.9 Hepatitis B Surface antigen mutation and hepatocellular carcinoma

Hepatitis B surface antigen mutants are asymptomatic and would not be detected by routine screening. There are no available guidelines provided for categorising those who should be screened for surface antigen mutants, such screening should be considered in blood donors, individuals with unexplained liver diseases and subjects who are HBsAg negative but anti-HBc and anti- HBs positive. Hepatocellular carcinoma accounts for 85-90% of liver cancers worldwide (McMahon *et al.,* 2009). Hepatitis B Surface antigen mutants have been shown to aid in the development of hepatocellular carcinoma through the capability of independent replication, infectivity in chimpanzees and participation in acute human hepatitis (Hunt *et al*., 2000). Point-mutations in the S promoter region as well as mutations in the start codon of LHBs and MHBs can also cause an unbalanced surface protein production that induces endoplasmic reticulum (ER) retention of surface proteins and subsequently triggers the ER stress signaling pathways. A study comparing mutations in preS/S gene showed that mutations at codons 4, 7 and 81 of the preS1 gene, codons 1 and 2 of the pre2 gene, and codon 68 in the surface gene were associated with HCC development (Chen *et al*., 2008). It is been reported that point-mutations at C2964A, C3116T and C7A were independent risk factors associated with HCC (Yin *et al*., 2010).

2.9 Diagnosis of hepatitis B Virus

2.9.1 Serology

Screening for serological markers, which are viral antigens and host antibodies helps establish a suspected HBV infection and distinguishes acute self-limiting infections from the different phases of chronic disease (Biswas *et al*., 2013). Hepatitis B surface antigen detection is a primary target for diagnosis and immunoprophylaxis of HBV infection both in the routine virology laboratory and for blood donation

screening (El-sherif *et al.,* 2007). It is also useful as a follow-up marker, since declining concentrations are observed in resolving hepatitis B (Biswas *et al*., 2013). Commercially available assays exist for quantitative and qualitative determinations of serological markers within clinical specimens (serum or plasma), based on the enzyme immunoassay (EIA) principle together with colorimetric or chemiluminescence signal measurement of results (Chevaliez & Pawlotsky, 2008). The specificity of HBsAg assays is over 99 % and sensitivity of HBsAg assays has continuously improved since the detection limit of licensed tests is under 0.5 ng/ml. However false negative results are observed due to the window period (WP), which is a delay between HBV infection and appearance of HBsAg, when the immune response is delayed or immune-complexes are present and low expression of HBsAg due to a low viral load enough for viral assembly. Another possible reason is the presence of S gene mutants and variants which are a particular concern for screening of blood donors since they result in false negatives for HBsAg serological testing (Larralde *et al.,* 2013). Studies have documented that some commercial ELISA kits could detect the wild-type but not the mutant viruses (Roosmalen *et al.,* 2006; Zhang *et al*., 2010**)** and the fear is that a failure to diagnose HBsAg will lead to diagnostic dilemmas and also to transfusion-associated hepatitis B infection.

2.9.2 Molecular Diagnosis Of Hepatitis B Virus Infection

Nucleic acid based tests for the direct detection of viral DNA are available for the diagnosis and management of HBV infection (Hatzakis *et al*., 2006). Quantitative viral load tests, genotyping assays, drug resistance mutation tests, and core promoter or precore mutation assays are also done.

Determining the presence of HBV DNA is a substitute to serological tests for HBeAg as it correlates with the presence of active HBV infection with high viral loads indicating infectivity. Thus molecular diagnostic methods provide supplementary

information regarding viral replication during the course of an HBV infection, especially in cases where serological profiles are atypical.

With the use of these diagnostic assays it is possible to detect new infections, determine protective vaccine efficacy, and monitor HBV-infected individuals on antiviral therapy. In spite of these assays there are now some new challenges for HBV disease. The HBV has the ability to change in response to antiviral therapy as well as vaccinations (Lefevre, 2014).

2.9.3 Prevention and treatment Of Hepatitis B Virus Infection

Early diagnosis, behavior modification and treatment of HBV infection are essential to improve the health outcomes. HBV infection can also be prevented by vaccination with recombinant HBsAg (Hou *et al*., 2005; Rebecca, 2011). Behaviour modification is aimed at reducing the risk of transmission of HBV infection involves the practice of risk-reduction measures such as safe sex and non-sharing of personal items (razors and syringes) as well as adherence to universal precautions in the health care setting (Franco *et al*., 2012).

In regions like Asia and Sub-Saharan Africa, passive immunoprophylaxis offers a better means of reducing the incidence of primary HBV infection as compared to behaviour modification. Immunoprophylaxis such as hepatitis B immunoglobulin (HBIG) is administered as a pre-emptive measure in neonates of HBsAg positive mothers and is also recommended for individuals following needle stick and sexual exposures or after liver transplantation (Hou *et al*., 2005). The vaccine may be administered as a monovalent formulation or in a fixed combination with other vaccines (diphtheria-tetanus-pertussis, *Heamophilus influenzae* type b, hepatitis A and inactivated polio vaccines). This is 3 or 4 doses to elicit production of anti-HBs (optimum protective titre ≥10 mIU/mL) and to prime immune memory cells (B and

Th cells) against the HBsAg component which confers long-lasting (~20 years) immunity to HBV infection (Zanetti *et al*., 2008).

Hepatitis B vaccination is also recommended for individuals at a high risk for HBV infection, such as health care workers, familial contacts of chronically infected individuals and persons with high-risk behaviours (Franco *et al*., 2012).

CHAPTER THREE

MATERIALS AND METHODS

3.1Study site

The study used samples collected from five Regional Blood Transfusion Centers (RBTCs) in Kenya: Nairobi, Nakuru, Kisumu, Mombasa, and Eldoret collected from June to September 2014.

3.2 Study design

This study was a laboratory based cross-sectional study.

3.3 Ethical Considerations

Approval to conduct the study was granted by KEMRI Scientific Steering committee and Ethical Review committee (SSC No. 2443- Appendix 3). Approval was also sought from the head of the RBTC. Since this was a laboratory based study that involved use of remnant samples, informed consent from the individuals was not required and there was no linking of the test results to the blood donors as the samples and plasma had unique laboratory codes.

3.4 Sample size determination

Sample size (N) was calculated manually. The average donor seroprevalence for HBV is estimated at 2.71% (KNBTS, 2009).The sample size was determined according to Naing (2003) as shown in the formula below;

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

The average donor seroprevalence for HBV is estimated at 2.71% (KNBTS, 2009).

Where**:**

n= Minimum sample size required

Z= 1.96 standard error

P= Postulated prevalence (2.71%).

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

$$n = \frac{1.96^2 \times 0.0271\ (1-0.0271)}{0.05^2} = 40 \text{ samples}$$

Therefore, considering the five Regional Blood Transfusion Centers the minimum sample size was:

n= 40 × 5 = 200 samples

3.5 Sample collection and processing

Whole blood samples were collected, packed, wrapped in a red biohazard bags and then transported through cold chain to Kenya Medical Research Institute (KEMRI), Hepatitis Laboratory. Briefly 3ml of whole blood was collected from each donor bag into blood collection tubes and labeled with unique codes for identification and then centrifuged at 2400 Revolutions Per Minute for 10 minutes. The plasma obtained was aliquoted into 1.8 ml tubes, placed in racks and stored at -80°C till use. Permission to use the blood bank samples was obtained from the head of the RBTCs (Appendix 4).

3.5.1 Hepatitis B Virus Serology

The samples were tested for HBsAg using *Hepanostika HBsAg Ultra* (BioMérieux, Lyon, France) kit as per the manufacturer's instructions. Briefly, 25µl of specimen diluent was added into the assigned wells and 100 µl of samples, three negative controls and one positive control was added. The samples were then incubated at 37°C for 60 minutes after which 50µl of conjugate solution was added into the wells. The wells were washed six times using Phosphate Buffered Solution (PBS). Addition of 100 µl TMB was followed and incubation for 15-30 minutes at room temperature in the dark. One hundred (100µl) of sulphuric acid was added to stop the reaction. Finally the plate OD was read at 450 and 620 nanometers within 15 minutes and the Cut off Value (COV) was calculated by adding the mean value of the negative controls to 0.040 (Kamat *et al*., 2014).

3.5.2 Extraction of viral DNA

Hepatitis B virus DNA was extracted from the plasma using the QIAamp DNA MiniKit (Qiagen, Hilden, Germany) -**Cat No./ID: 51304**. The extraction steps were performed according to the manufacturer's instruction as follows: twenty (20) µl Qiagen Proteinase K were pipetted into the bottom of a 1.5 ml microcentrifuge tube, 200 µl plasma was added to the microcentrifuge tube and 200 µl buffer AL were added to the sample, mixed by pulse-vortexing for 15 seconds. The mixture was incubated at 56ºC for 10 min followed by a short spin down, 200 µl ethanol (96-100 %) was added to the sample, mixed by vortexing and briefly centrifuged to remove drops from the inside of the tube lid. The mixture was transferred carefully to the QIAamp Mini spin column (in a 2ml collection tube) without wetting the rim. The cap was closed and the column in the collection tube was centrifuged at 8000 revolutions per minute (RPM) for 1 min. Thereafter, the QIAamp Mini spin column was placed in a clean 2ml collection tube, and the tube containing the filtrate discarded. Five hundred (500) µl Buffer AW1 were added to the QIAamp Mini spin

column without wetting the rim and centrifuged at 8000 rpm for 1 min. The filtrate was discarded. The QIAamp Mini spin column was replaced in a clean 2 ml collection tube, 500 µl Buffer AW2 was added. Filtrate was discarded and centrifuging step was repeated. Finally, the QIAamp Mini spin column was placed in a clean microcentrifuge tube, 60 µl elution buffer was added to the column and centrifuged at 14000 RPM for 3 min. The filtrate in this step contained the extracted DNA. The DNA was stored at -80°C (Tajik *et al.,* 2015).

3.5.3 Nested Polymerase chain reaction

Amplification of the HBV S gene by nested PCR was done using an ABI Thermocycler 9700 system (Applied Biosystems, USA) where first round PCR was performed using an outer primer set S1f (5'-TCCTGCTGGTGGCTCCAG-3'as sense primer and S1r (CGTTGACATACTTTCCAATCAA)-3' as anti-sense primer (Osiowy *et al.,* 2010). A master mix was prepared depending on the number of samples as shown in Table 3.1

Table 3.1: PCR master mix preparation

Reagent	Stock concentration	Concentration	Volume per tube (µL)
PCR Buffer with $Mgcl_2$	10X	1	2.5
dNTPS	2mM	1	2.5
$Mgcl_2$	25 mM	3	3
Primer Forward	10 µM	0.8	0.5
Primer Reverse	10 µM	0.8	0.5
Taq Polymerase	2.5U	2.5	1
PCR water	-		10
DNA extract	-		5
Total	-		25

The thermocycling conditions for first round PCR consisted of 94 °C for 5 min; followed by 40 cycles at 94 °C for 4 min; 94 °C for 45 seconds, 46 °C for 30 seconds, 72 °C for 2 min and final extension at 72 °C for 7 minutes. The nested PCR was done under the same conditions except that annealing was done at 50°C with 20µl master mix and 5µl of the first-round product using the primer S2f (5'-ACCCTGYRCCGAACATGGA-3'as sense primer and S2r(5'-CAACTCCCAATTACATARCCCA -3' as anti-sense primer (**Table 3.2**).

Table 3.2: PCR Primers for the full S- gene region. Primers used to amplify the full S gene region of HBV. R= A or G, Y= C or T according to IUPAC nucleotide code.

Primer Name (orientation)	Sequence (5'-3')	Nucleotide position	Expected fragment size region (bp)
S1r	CGTTGACATACTTTCCAATCAA	995-974	940
S1f	TCCTGCTGGTGGCTCCAG	55-72	
nested S2r	CAACTCCCAATTACATARCCCA	835-814	680
nested S2f	ACCCTGYRCCGAACATGGA	155-173	

3.5.4 Detection of amplified HBV S gene PCR products

The samples were subjected to nested PCR to amplify the HBV S gene. Detection of the amplified PCR products was performed using 2.0% agarose (SEakem LE® agarose; FMC BioProducts, Rockland, Marine, USA) gel in 1X TAE buffer (0.04M

Tris acetate, 0.001 M EDTA). The gel was prepared by dissolving 2 g of agarose gel into 100 mL of 1X TAE buffer by heating. This was cooled to about 50^{O}C. Briefly 2µl of the PCR product mixed with 2µl of blue 5x loading dye containing bromophenol blue, xylene cynol, and orange G (Promega, Madison, USA) were loaded into the wells on the gel. The loaded agarose gel was then subjected to 100 Volts of a unidirectional (from the anode to the cathode) electrical current for a duration of 30 minutes, while submerged in a 1X Tris-acetate-EDTA (TAE) buffer (Qiagen, Hilden, Germany).

The agarose gel was stained for 20 minutes with ethidium bromide (1µl in 100ml), Sigma-Aldrich® and the migrated sample DNA bands and 200bp DNA ladder (Promega, Madison, USA) in the agarose gel were visualized using ultraviolet transillumination and corresponded to positive controls (figure 3.2). A digital image of the gel was taken using a gel documentation system.

3.5.5 Sequencing of PCR and amplicons purification

The nested PCR products were sequenced, the sequencing PCR was carried with a total reaction mixture of 20 µl containing 3 µl of DNA, 5X sequencing 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 S2f 5'- ACCCTGYRCCGAACATGGA-3'as sense primer and S2r (5'- CAACTCCCAATTACATARCCCA -3' as anti-sense primer (Osiowy *et al.*, 2010).

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 for 4 minutes for 25cycles. The products were then purified. Briefly, 2µl sodium acetate, 50µl of absolute ethanol, 20µl of sequencing PCR product 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 centrifuged at 1500 rpm for 5 minutes and supernatant discarded.

The above two steps were repeated once and DNA pellet was air dried for 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).

3.5.6 Phylogenetic analysis of S gene sequences

The raw nucleotide sequences were assembled into single contig sequences using GENETYX version 9.1.0 (GENETYX Co., Tokyo, Japan) computer software. The quality of sequences was improved through trimming and editing where necessary. The assembled nucleotide sequences together with 22 downloaded reference strains of African, Asian and American origin from the GeneBank were exported to MEGA 6 software where a pairwise and multiple alignment was performed using MUSCLE(multiple sequence comparison by log-expectation) program in MEGA 6 (Edgar, 2004). Phylogenetic analysis was performed with the MEGA 6 software package using the p-distance algorithm for distance determination and the neighbor-joining method for tree drawing. The reliability of phylogenetic classification was evaluated by a 500-replication bootstrap test using the same software package, from the tree and by comparing the study sequences with the HBV references sequences, the sequences were assigned to specific genotypes and subgenotypes (Tamura *et al*., 2013), the bootstrap values above 50% were presented.

3.5.7 Sequence alignment and mutations detection

Mutations were determined by aligning the obtained sequences with known wild type sequences for any nucleotide variation using (MEGA6). The S-region nucleotide

sequences were translated into corresponding amino acids where amino acid differences including substitutions, insertion and deletion at each site were termed as mutations after comparison with the wild-type consensus sequences of respective genotype (Yin *et al.,* 2010). Mutations identified were further characterized to find out whether they are functionally essential or not. Each mutation was blasted using Pubmed searching machinery and Google searching machinery using the mutation and HBV as searching words (i.e. M133E HBV). Once a publication had identified and characterized the mutation, the publication was marked as a reference(Mayaphi *et al*., 2013).

3.6 Data Management

Raw data was coded and entered into excel spreadsheet. A back up of these data was done regularly to avoid any loss using flash disks. The records in the computer were password protected and could only be accessed by the principal investigator for confidentiality purposes. All the statistical analyses were performed using SPSS version 19.0.

CHAPTER FOUR

RESULTS

4.1 Detection of occult HBV among voluntary blood donors

A total of 301 samples were tested, of these 67(22.3%) were HBsAg positive and 69(22.9%) were HBV- DNA positive. Two samples (0.66 %) were positive for HBV DNA but HBsAg negative. A 780 base pair (bp) fragment was amplified (Table 4.1).

Table 4.1: Analysis of Hepatitis B Surface Antigen and HBV-DNA in 301 Blood donors.

HBsAg +ve (Hepatitis B surface Antigen positive), HBsAg -ve (Hepatitis B surface Antigen negative), HBV-DNA +ve(Hepatitis B DNA positive).

RBTCs	Total plasma collected	HBsAg +ve	HBsAg -ve	HBV-DNA +ve
Mombasa	72	23(31.9%)	49(68.1%)	25(34.7%)
Eldoret	60	7(11.7%)	53(83.3%)	7(11.7%)
Nairobi	58	17(29.3%)	41(70.7%)	17(29.3%)
Kisumu	56	11(19.6%)	45(80.4%)	11(19.6%)
Nakuru	55	9(16.4%)	46(83.6%)	9(16.6%)
Total	301	67(22.3%)	234	69(22.9%)

4.2 Detection Of HBV DNA

Figure 4.1 shows an example of an agarose gel documentation of S gene amplicons.

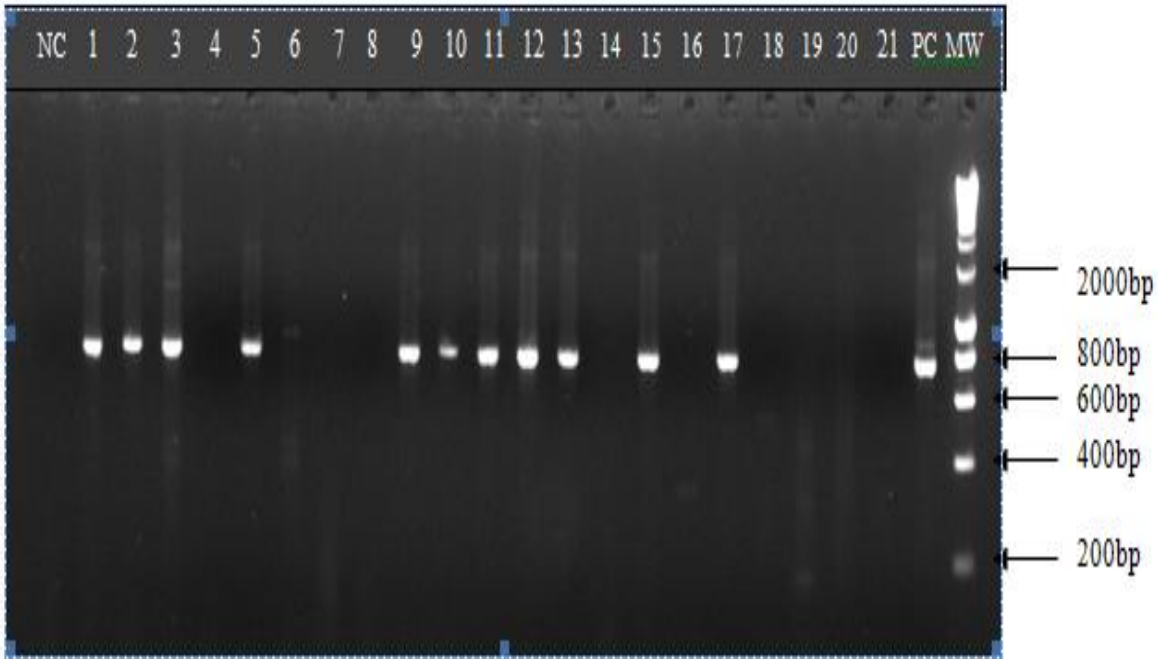

Figure 4.1: A 2.0% Agarose gel electrophoresis documentation of HBV S- gene amplicons.

NC=Negative Control, wells 1-21=study samples), PC=Positive Control and MW=200bp DNA Molecular weight marker.

4.3 Genotyping and phylogenetic analysis

Phylogenetic analysis of S gene sequences showed that HBV genotype A isolates clustered with sub-genotype A1 reference sequences from African A1 clade for HBsAg region analysis. Further genetic analysis of the A genotype revealed that HBV subgenotype A1 (94.20%) predominated, A2 (1.45%) and A3(4.35%) (Figure 4.2). From the bootstrap analysis, the bootstrap values above 50% were presented hence confirming the reliability or significance of the phylogenetic tree analysis (Figure 4.2). Isolates from all five blood donor regions clustered with sequences from neighboring Tanzania(0.02%), Uganda(13.46 %), Rwanda(7.69%), Somalia(17.30 %), Malawi, (13.46 %), Kenya(46.15%), and South Africa 3.84%)- African A1 clade). One sequence (0.02 %) in this study clustered with Asian A2 reference sequences from Japan based on HBsAg gene analysis. Three sequences (4.3%) formed a cluster with West African A3 sequences supported by 69 % bootstrapping for 500 replicates by neighbor-joining method phylogenetic analysis.

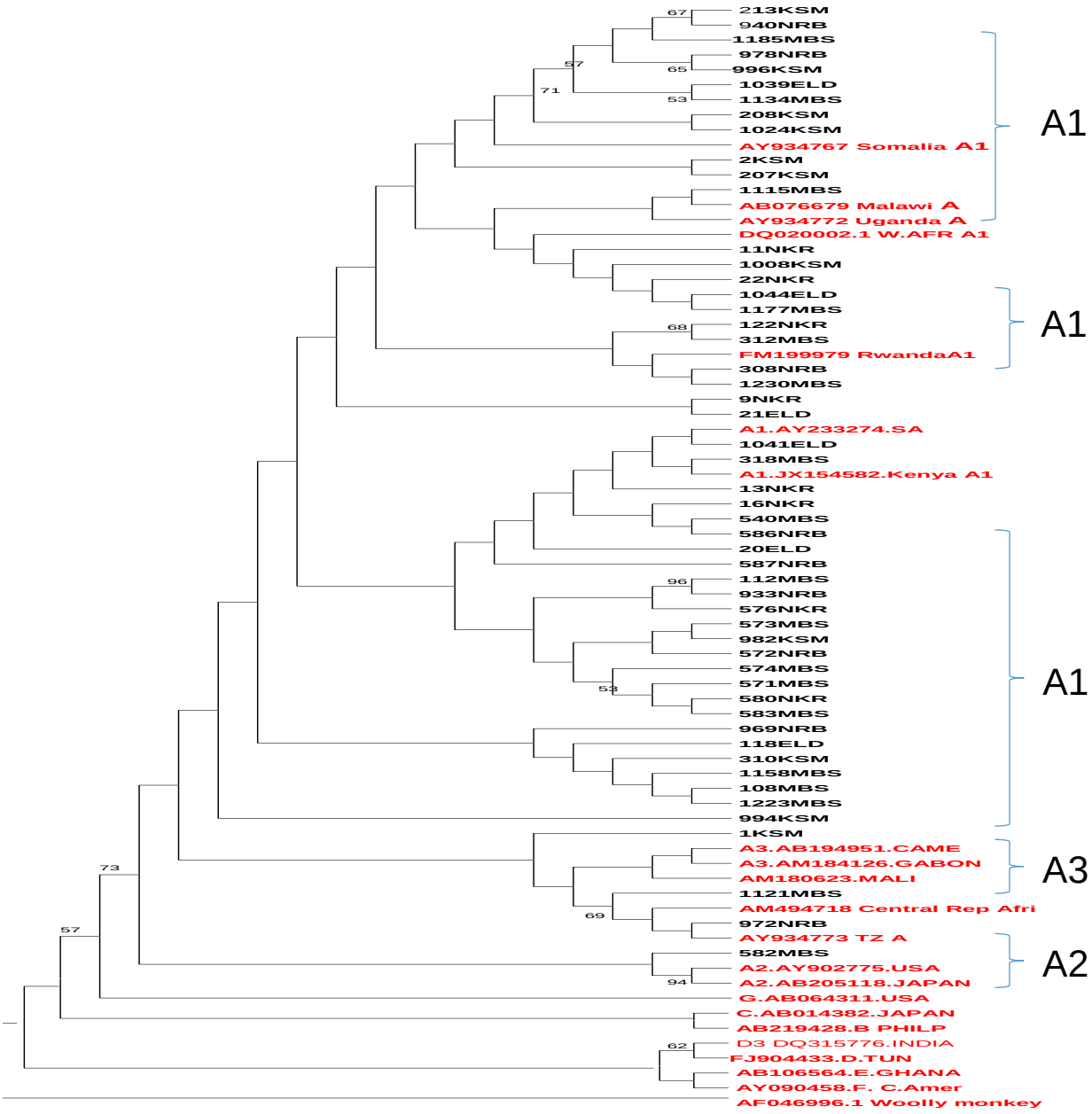

Figure 4.2: A phylogenetic tree of 52 sequences obtained from blood donors.

(Black Bold) with 22 reference HBV sequences, (Red bold) established using neighbour-joining. Bootstrap statistical analysis was performed using 500 replicates, indicated as percentages of occurrences on the nodes. The reference sequences are

labeled by their GenBank accession numbers and country of origin. The tree is rooted from the Woolley Monkey virus genome sequence.

Table 4.2: Distribution of A subgenotypes among voluntary blood bonors in Kenya
NRB-Nairobi, KSM-Kisumu, NKR-Nakuru, ELD-Eldoret, MBS-Mombasa. A1-subgenotype A1,A2- subgenotype, A3-subgenotype.

	A1	**A2**	**A3**
NRB	**9(17.31%)**	**1(0.02%)**	**0**
KSM	**11(21.15)**	**0**	**1(0.02%)**
NKR	**8(15.38)**	**0**	**0**
ELD	**6(11.53)**	**0**	**0**
MBS	**16(30.76)**	**0**	**1(0.02%)**

4.4Nucleotide and amino acid substitutions

For accurate mutation analysis, the generated sequences aligned with the consensus genotype A sequence (KT367718.1) using MEGA6 were visually inspected. Once an amino acid exchange was noted, the entire amino acid column was marked (Figure 4.3).

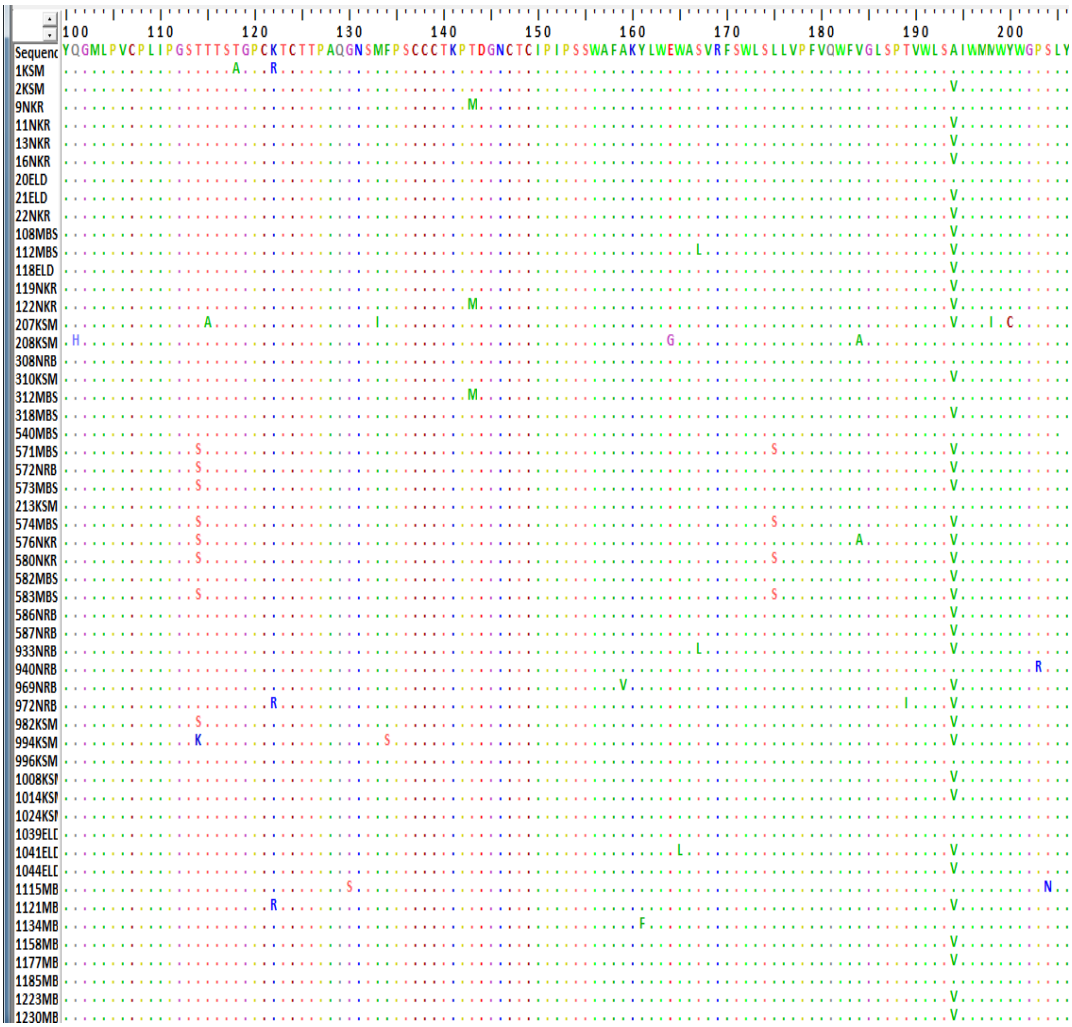

Figure 4.3: Amino acid alignment of the S gene from blood donor samples.

Analyses of amino acid variations within the HBV MHR (aa100 to aa170) compared to the reference sequence of genotype A (KT367718.1) retrieved from GenBank database. The amino acids were identified by single letter code. Dots represent identical amino acids with the reference sequence for genotype A shown on top of each sequence. Once the amino acid being checked was marked, the program was switched to the DNA mode to check the point mutation responsible for the amino acid exchange (Figure 4.3).

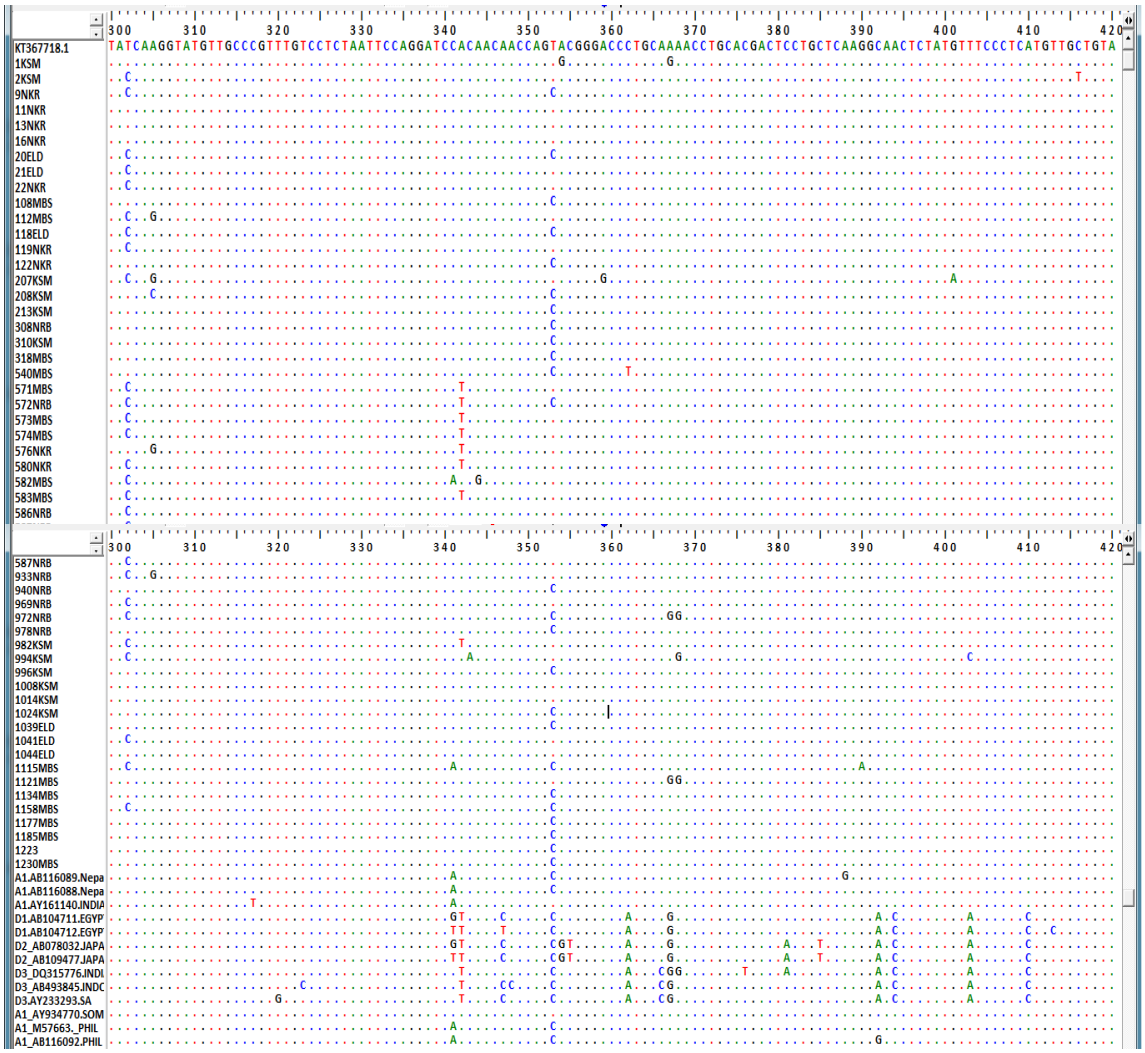

Figure 4.4: Alignment of the S-gene nucleotide sequences.

Alignment of the S-gene nucleotide sequences compared with a corresponding reference of genotype A isolates (KT367718.1). The dots indicate similar nucleotides. The red, blue, green and black colours represent the difference in the nucleotides.

All nucleotide variations detected for genotype A sequences are summarized in Table 4.3. Among the 79 nucleotide changes, 50 (63.3 %) were silent (no amino acid changing) and 29(36.7%) were missense (amino acid altering). Eleven of the 29 mutations (37.9%) were located within the “a” determinant, 63.6% (7/11) in the first

loop (positions 120-137) and 36.4% (4/11) in the second loop (positions 139-147). Eighteen of these 29 mutations had only one amino acid change in the studied region and 10 had multiple mutations (figure 4.2).

Table 4.2: Nucleotide variations detected within genotype A sequences.

Sample Code	Missense Mutation	Amino Acid Change
1KSM	A354G,A367G	T118A,K122R
2KSM	C416T	A194V
9NKR	_	T143M
20ELD	_	_
21ELD	_	_
22ELD	_	_
108MBS	_	_
112MBS	_	S167L
118ELD	_	_
119NKR	_	_
122NKR		T143M
207KSM	_	M133I,E164G
208KSM	G306C	Q101H,V184A
213KSM	_	_
308KSM	_	_
310KSM	_	_
318KSM	_	_
540MBS	_	_
571MBS	_	_
572NRB	_	_
573MBS	_	_
574MBS	_	_

576NKR	_	V184A
580NKR	_	_
582MBS	_	_
583MBS	_	_
586NKR	_	_
587NRB	_	_
933NRB	_	S167L
940NRB	_	N146K,C149R,P204R
969NRB	_	A159V
972NRB	__	K122R,T189I
978NRB	_	S193L
982KSM	_	T114S
994KSM	_	T114K,M134S
996KSM	_	_
1024ELD	_	_
1039ELD	-	_
1041ELD	-	W165L
1115MBS	_	G130S, S209N
1121MBS		K122R
1134MBS		Y161F
1158MBS	_	_
1177MBS		T143M
1185MBS	_	_
1223MBS	_	_
1230MBS	_	_

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECCOMENDATIONS

5.1 Discussion

5.1.1 Detection of occult HBV among voluntary blood donors

The present study showed the prevalence of HBV among blood donors to be 69/301 (22.9%). This is high considering the criteria for selection of blood donors excludes high risk groups. Since the donors are expected to be healthy individuals, this finding indicates there is a probable high exposure of HBV in the population or the screening criteria are not stringent. Normally the criterion for donor selection involves a questionnaire that identifies previous or current exposure through blood transfusion, TTIs or traditional practices IDUS. In addition to screening, serology is done to rule out positive cases. The high prevalence as reported in the current study is not attributable to occult infection. This study reports a low frequency of 0.6% occult hepatitis B infection among blood donors. Results in the literature, reported among blood donors in other studies indicate low frequency of occult infections. A prevalence of 0.5% occult infections has been reported (Allain *et al.,* 2003; Arraes *et al*., 2003; Antar *et al*., 2010; Chang *et al*., 2014). Regardless of this low frequency of occult infections, these findings could be an indication of potential HBV transmission from blood and blood products

In a study in Colombia, a prevalence of 1.98% was reported as occult HBV infection (Rios-Ocampo *et al*., 2014). A similar study among blood donors in Brazil observed 2·7%) (Moresco *et al*., 2014). On the other side, a high frequency of OBI was described in a study in China where twenty-eight samples from (HBsAg) negative plasmas were confirmed HBsAg negative and DNA positive($HBsAg^{-}/DNA^{+}$), of which 22 were classified as OBIs and 6 as window period infections (Zheng *et al*., 2011).

Hepatitis B surface antigen negative but DNA positive results indicate a probable Occult infection, window period infection or escape mutations (Brojer *et al*., 2006) ;Candotti, 2009 ; Said, 2011). In Kenya, HBV screening in blood banks relies only on the detection of HBsAg. The screening for HBV by the NAT technique is highly sensitive and specific for viral nucleic acids. Nucleic acid testing detects the virus earlier than the ELISA screening method. NAT also adds the benefit of resolving occult donations. In this regard, HBV DNA detection could be a useful tool for reducing the transmission of HBV via blood products.

It is documented that HBV transmission can be spread from HBsAg negative and anti-HBc (occult) (Hudu *et al*, 2014). The implication of 0.6% occult infection in the present study implies that the blood could have been transfused thereby transmitting HBV. Discrepancy in serology and NAT testing has been reported in other studies (Yuki *et al*., 2003; Chen *et al*., 2014; Elbahrawy *et al*., 2015). This findings, though rare seems to be the case even in our settings.

5.1.2Genotyping and Phylogenetic analysis

Hepatitis B virus genotype A was the major genotype among blood donors in Kenya. Sub-genotypes A1, A2 and A3 were identified. This finding suggests that the majority of infections in Kenya could be of sub-type A and its variants. There is growing evidence that HBV genotype influences the course and outcome of the disease. Different studies have shown that genotype A1 is associated with high rates of HCC in sub-Saharan Africa, this association of genotype A with advance disease outcome is probably a course to worry since this is the main genotype in Kenya. A blood donor population is considered a 'healthy' population, presence of genotype A could therefore indicate a long standing transmission circulating in the population without notice. This could consequently lead to development of advanced disease over time. Early detection is critical to limit the HCC disease pathology. Since testing for hepatitis is not routinely done in Kenya, less the genotyping, the population could be exposed to a potential

chronicity of infections. Therefore, testing and genotyping would be important in HBV programs especially in the implementation of diagnostic measures in the population. This genotype has also been shown to be the most prevalent among patients with acute hepatitis B (Lyra *et al.,* 2005).

Thus, the present study suggests that most people in Kenya may develop chronic HBV infections and high viral resistance to treatment. Most genotype A strains belong to sub-genotype A1 however, in the present study 1 isolate (figure 4.2) clustered closely with A2 subgenotype from USA. This suggests that subgenotype A2 have a low prevalence in Kenya. One isolate clustered closely with sub-genotype A3 reference from Tanzania, this finding probably reflecting the effect of the coastal migratory route. Twenty four isolates clustered closely with genotype A1 from Kenya.

The findings could suggest that there is local sex networks and circulation of the HBV genotype A in the major towns in Kenya where the samples were collected. These towns consist of occupants of diverse age groups and sexual behavior. It is also noted that voluntary blood donors are recruited among youths and university students. Findings this study 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).

Sub-genotype A1 and A2, observed in the present study have been reported in previous studies (Mwangi *et al*., 2008; Kwange *et al*., 2013; Ochwoto *et al.*, 2013). The finding of HBV sub-genotype A3 in the present study possibly suggests a diverse HBV genotype A in Kenya. Thus the mapping of genotypes is continuously improving, probably explaining the diversity of genotypes in Kenya over time.

Genotypes also contribute to the outcome of treatment with interferons and pegylated interferons. HBV genotyping that was previously regarded as a research tool has been suggested as an important element to guide the choice of therapy by several investigators and national professional associations (Cooksley, 2010). Recent data from multivariate analysis showed that HBV genotype was a strong predictor for sustained virological response in peginterferon treated-patients (Chan *et al*., 2003). Genotype information of chronic HBV infections enables practicing physicians to identify those patients at risk of disease progression and to determine the appropriate and optimal anti-viral therapy (Tanwar & Dusheiko, 2012).

5.1.3Nucleotide and amino acid substitutions

In the present study, regarding variations in the Major Hydrophilic Region of HBsAg, the results indicated a prevalence of 36.7% (29) mutations among this blood donor population. It is important to note that studies on mutations among blood donors are rare, making this study one among the few. A higher prevalence of 46.6% mutation rate was reported in China among patients (Shi *et al.*, 2012).

Comparable findings to the present study reported 39% of mutations among children receiving HBIG injection at birth and full course of plasma derived vaccine (Hsu *et al*., 2009). Other studies have reported a lower prevalence; including 14.8% in Argentina (Pineiro *et al.*,2008) ; 17.2% in Iran (Mohebbi *et al*., 2008); 22.6% among chronic patients (Lazarevic *et al.*,2010). A prevalence of 5.5 % for hepatitis B-escape mutations among blood donors has also been reported in Malaysia (Hudu *et al*., 2015).

These findings highlight a concern about long term success of vaccination and testing programs since breakthrough infections caused by emergence of surface mutants have occurred in immunized infants and blood donor population (Hudu *et al.,* 2015). Results from the current study have clinical implications in blood safety as well as immunization programs. The two samples that were sero-negative for HBsAg but positive for the

corresponding HBV- DNA were observed to have mutations; T143M and M133I which are associated with diagnostic failure. Mutations in MHR also influence the antigenicity and can impair virion secretion consequently leading to HBsAg detection failure in OBI individuals. Similar findings have been reported in Indonesian and Spain blood donors that showed these mutations were associated with altering antigenic properties of variant HBsAg, leading to problems in diagnostic assays, escape to vaccine and HBIg therapy (Le *et al.,* 2010). These mutations could possibly be escaping detection by serology and can infect both vaccinated and unvaccinated population, thus putting the whole population at risk. Additionally, the present study found escape mutations T118A and Q101H that have been associated with diagnostic failure, C149R a vaccine escape mutation and M133I HBIg therapy escape mutation which have also been reported in other studies (Brojer *et al.,* 2006; Hsu *et al.,* 2010).

In Blood safety today, infection status is important for blood recipient and donor as well. For the recipient to eliminate any risk of infection whether it's false negative, window period, occult or due to presence of mutations. Since mutations alter antigenic epitope in the 'a' determinant region of HBV, this is likely to reduce sensitivity of detection thereby transfusion of unsafe blood. Transfused patients are also likely to become source infections to either health workers or the general population upon recovery from disease. Mutations are also attributed to Occult HBV infection representing a potential transmission source of HBV via blood transfusion or organ transplantation. In addition, occult HBV infection has been associated with cryptogenic chronic hepatitis and hepatocellular carcinoma. In Kenya, demand for blood is high due to anemia caused by infections e.g. malaria or due to accidents. As a result unsafe transfusions increase the risk of Transmission Transmissible Infections (TTIs). On the other hand, false test result has an implication on the blood donor. Usually, donors are notified of test results after donated blood has been screened in blood transfusion centers. Result communicated to the donors should therefore reflect the true status of TTIs. This then forms the basis of follow-up testing or referral for management of TTIs.

Mutations altering test results therefore affects any donor directed interventions since a number of intervention exists ranging from counseling, confirmatory testing, care and management including ART and adherence. Such donors especially the 'repeat donors' can also continue to donate if not excluded. In both cases, whether recipient or donor, presence of mutations can result to complication of infections in un-resolved infections including HCC, interference with effectiveness of vaccination. Despite the success of vaccination and ART, chronic HBV infections remain a major problem since many chronically infected patients are unaware of their disease (Cohen *et al.*, 2011, Richter *et al.*, 2012; Hahne *et al*., 2012).

Based on the number of sequences obtained in the present study, several important findings were observed concerning the mutation patterns. In total, 22 mutations were detected in the 52 samples analyzed. These mutations were distributed across the sequenced genome fragment including 11 of 22 in 'a 'determinant, 4 of 11 in the first and7 of 11 in the second loop respectively. Since these samples were collected from across different sites in Kenya, this probably demonstrates an underlying genotypic mutation dynamics of HBV occurrence in the population.

Hepatitis B vaccine is an effective means of preventing HBV infection, producing protective levels of antibodies in up to 95% of recipients (Weber, 2005). Antibodies elicited by the hepatitis B vaccine specifically target the "a" determinant of the surface antigen which was targeted in this study. The success of vaccination programs has been challenged by discovery of mutant HBV showing amino acid substitution in HBsAg which may lead to evasion of vaccine induced immunity. Such vaccine escape mutants were detected in this study (C149R). Infection of immunized individuals with a vaccine escape mutant (VEM) is therefore possible, This also suggest possibility of contact with HBV infection with mutant strains subsequent to vaccination (Huang *et al*., 2009; Xu *et al.*,2010).

In adolescents or adults who are infected with HBV, 1-3% become chronically infected; whereas up to 90% infected neonates develop chronicity. World Health Organization aims to control HBV worldwide by integrating hepatitis B vaccination for all infants and some high-risk adolescents into national programs. Presence of mutants would be an impediment to success of such a program. Consistent with this WHO goal, and based on the findings of present study, it is important to explore vaccine derived mutations in blood transfusion settings (Lazarevic, 2014).

In Pre-exposure Prophylaxis and Post Exposure Prophylaxis, mutations in HBV ‘a ‘determinant have been shown to affect hepatitis B immunoglobulin (HBIg) therapy and prophylaxis. The major target of antibodies that are induced by the vaccine and HBIg is against the group-specific “a” determinant of hepatitis B surface antigen (HBsAg), commonly described as a major B-cell epitope, comprising residues 124-147 (Azam *et al*., 2013). In the current study, mutations within this residual were detected indicating an underlying problem in the donor population. It is important to note that prevention of HBV infections involves; screening for HBV; implementation of vaccination program and administration of immunoglobulin pre-or -post exposure to contaminated blood during delivery, organ transplant or work place exposures. Depending on the extent of mutations in our population, in an event of exposure, individuals requiring these interventions may not therefore, effectively benefit. Indeed, the rate of perinatal transmission of HBV is 20% to 90% in absence of immunoglobulin (Patton *et al*., 2014). Screening pregnant women for HBV, administering HBV vaccine, and administering hepatitis B immune globulin (HBIG) at birth for newborns of infected mothers (Zahra *et al*.,2016) has been shown to be effective ways of preventing perinatal transmission that could result in markedly reduced prevalence of HBV infection.

Fundamentally, the major concern is that these “a” determinant variants may go undetected by conventional HBsAg screening, and for some mutants, vertical, horizontal transmissions and infection of the vaccinated population occur which poses a substantial risk to the community.

5.2 Conclusions

The prevalence of Hepatitis B surface antigen was (22.3%) while the prevalence of HBV DNA was (22.9%) among voluntary blood donors.

This study has shown that HBV subgenotypes in Kenya are diverse with A1, A2 and A3 subgenotypes circulating among blood donors in Kenya.

The prevalence of Hepatitis B surface antigen escape mutations was 36.7 % with T143M and K122R being the most prevalent mutations.

5.3 Study limitations

1. The donor plasma used in the study lacked clinical information. Therefore, the pattern of substitutions /mutations could not be linked to clinical profiles of the donors.
2. The purposive sampling method used in this study led to sampling bias which would have been minimized had a larger sample size been used in the study.
3. Due to financial and time constraints on this study however, a larger sample size could not be employed.

5.4 Recommendations

More studies are needed to understand the magnitude of occult Hepatitis B and to develop a national strategy on blood donor screening policy among voluntary blood donors.

There is need for continuous surveillance of circulating HBV genotypes for analysis of their trends within the population.

Furthermore, HBV genetic profiles are needed as a further step in optimizing vaccines, diagnostic tools and control measures according to the predominant escape mutations.

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APPENDICES

Appendix 1: Reference sequences accession numbers from the National Center for Biotechnology Information (NCBI).

ACCESSION #	SUBTYPE	COUNTRY	YEAR
AB076679	A1	Malawi	2002
AY934772	AI	Uganda	2005
AM494718	A	Central Rep Africa	2007
FM199979	A1	Rwanda	2009
AY934767	A1	Somalia	2005
AB219428	B	Phillipines	2006
FJ904433	D	Tunisia	2009
AB106564	E	Ghana	2003
AY090458	F	C.America	2002
JX154582	A1	Kenya	2013
AB205118	A2	Japan	2005
AB194951	A3	Cameroon	2007
AM184126	A3	Gabon	2006
AY233274	A1	South Africa	2004
AB014382	C	Japan	2006
DQ020002.1	A1	W. Africa	2005
AF046996.1		Woolly Monkey	1998

Appendix 2: General Generated Sequences

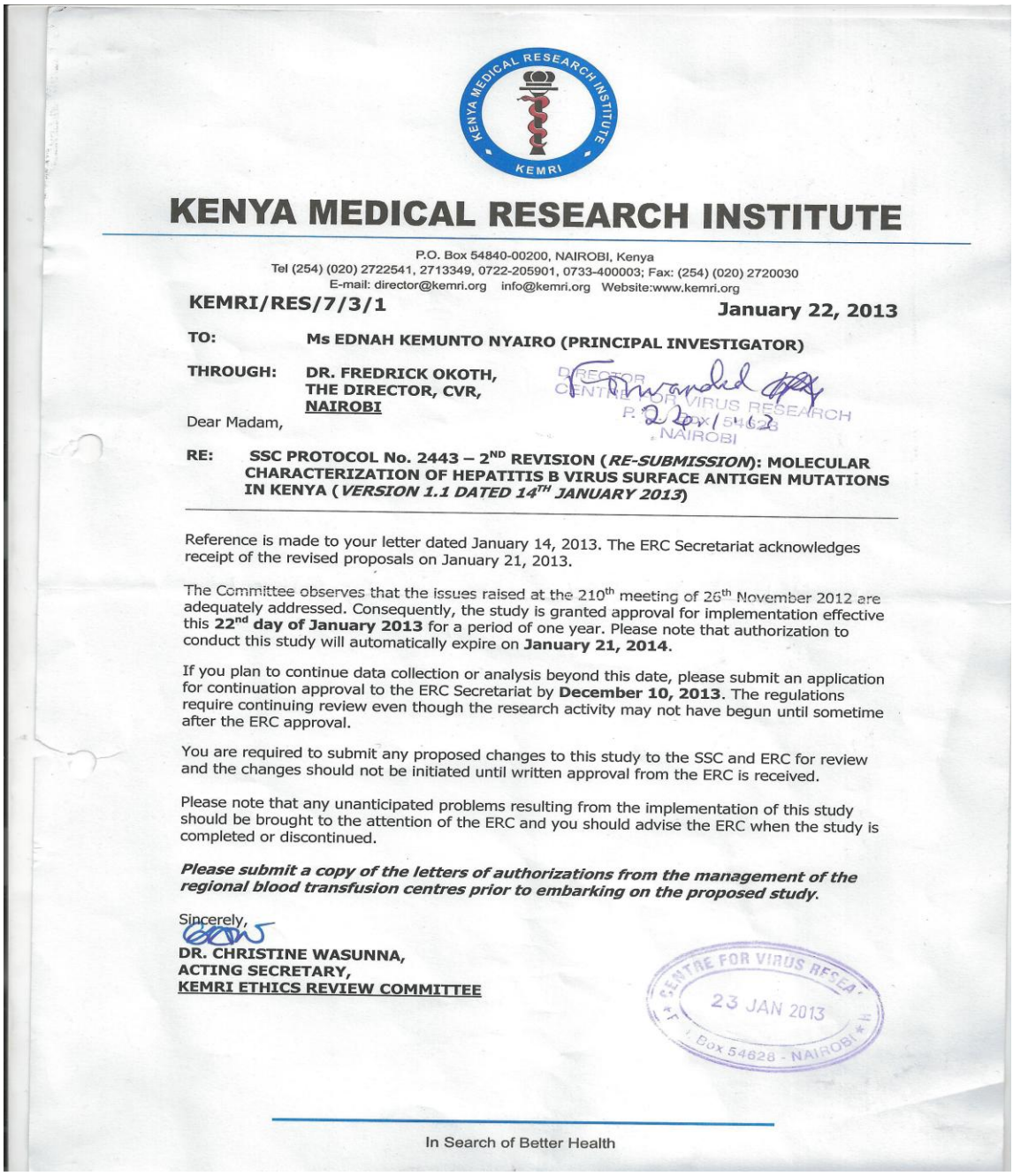
The generated sequences were deposited in the Genbank and assigned the following accession numbers.

GenBank: (KR816101-KR816152).

Sequence Name	GenBank Sequence ID
1KSM	KR816101
2KSM	KR816102
9NKR	KR816103
11NKR	KR816104
13NKR	KR816105
16NKR	KR816106
20ELD	KR816107
21ELD	KR816108
22NKR	KR816109
108MBS	KR816110
112MBS	KR816111
119NKR	KR816112
122NKR	KR816113
207KSM	KR816114
208KSM	KR816115
213KSM	KR816116
308NRB	KR816117
310KSM	KR816118
312MBS	KR816119
318MBS	KR816120
540MBS	KR816121
571MBS	KR816122
572NRB	KR816123
573MBS	KR816124
574MBS	KR816125
576NKR	KR816126
580NRK	KR816127

582MBS	KR816128
583MBS	KR816129
586NRB	KR816130
587NRB	KR816131
933NRB	KR816132
940NRB	KR816133
969NRB	KR816134
972NRB	KR816135
978NRB	KR816136
982KSM	KR816137
994KSM	KR816138
996KSM	KR816139
1008KSM	KR816140
1014KSM	KR816141
1024KSM	KR816142
1039ELD	KR816143
1041ELD	KR816144
1044ELD	KR816145
1115MBS	KR816146
1121MBS	KR816147
1134MBS	KR816148
1158MBS	KR816149
1177MBS	KR816150
1185MBS	KR816151
1223MBS	KR816152
1230MBS	KR816153

Appendix 3: Ethical Approval Letter



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KEMRI/RES/7/3/1 **January 22, 2013**

TO: **Ms EDNAH KEMUNTO NYAIRO (PRINCIPAL INVESTIGATOR)**

THROUGH: **DR. FREDRICK OKOTH,**
THE DIRECTOR, CVR,
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P. O. Box 54628
NAIROBI

Dear Madam,

RE: **SSC PROTOCOL No. 2443 – 2ND REVISION (*RE-SUBMISSION*): MOLECULAR CHARACTERIZATION OF HEPATITIS B VIRUS SURFACE ANTIGEN MUTATIONS IN KENYA (*VERSION 1.1 DATED 14TH JANUARY 2013*)**

Reference is made to your letter dated January 14, 2013. The ERC Secretariat acknowledges receipt of the revised proposals on January 21, 2013.

The Committee observes that the issues raised at the 210th meeting of 26th November 2012 are adequately addressed. Consequently, the study is granted approval for implementation effective this **22nd day of January 2013** for a period of one year. Please note that authorization to conduct this study will automatically expire on **January 21, 2014**.

If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to the ERC Secretariat by **December 10, 2013**. The regulations require continuing review even though the research activity may not have begun until sometime after the ERC approval.

You are required to submit any proposed changes to this study to the SSC and ERC for review and the changes should not be initiated until written approval from the ERC is received.

Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of the ERC and you should advise the ERC when the study is completed or discontinued.

Please submit a copy of the letters of authorizations from the management of the regional blood transfusion centres prior to embarking on the proposed study.

Sincerely,

DR. CHRISTINE WASUNNA,
ACTING SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

CENTRE FOR VIRUS RESEARCH
23 JAN 2013
P. O. Box 54628 - NAIROBI

Appendix 4: Permission Letter from Regional Blood Transfusion Services.

MINISTRY OF MEDICAL SERVICES

Telephone: 020-2723569
FAX: 020-2723569

KENYA NATIONAL BLOOD
TRANSFUSION SERVICE (K.N.B.T.S)
P. O. BOX 29804-00202
NAIROBI

Ref. No: INST/7/20/Vol. I

16 MARCH 2013

All Regional Blood Transfusion Centres

Attn: Technical Directors

RE: PERMISSION TO ALLOW KEMRI PRODUCTION DEPARTMENT TO COLLECT ALL HBV POSITIVE SAMPLES

In line with operational research to improve blood safety, I have given KEMRI-production department authority to collect blood samples of units that test HBV positive for confirmation of the same and genotyping.

The results will be shared with your centres at the end of the study.

This letter also serves as an introduction of Ms Ednah k. nyairo, who will be the lead person from KEMRI in this work. Please work with her the modalities of sample collection.

Sincerely,

DR. MARGARET ODUOR
Head, KNBTS

I'm big hearted
I give blood

IT'S SAFE AND IT SAVES

Appendix 5: Publication in Peer Reviewed Journal, Arising from this Work

Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.6, No.14, 2016

Mutations in the "a" Determinant Region of Hepatitis B Virus Genotype A among Voluntary Kenyan Blood Donors

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Missiani Ochwoto[3] Keith Talaam[3] Joseph Mwangi[3,1] Raphael Lwembe[3]
1.Institute of Tropical Medicine and Infectious Diseases P.O BOX 54840-00200, Nairobi, Kenya
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3.Kenya Medical Research Institute P.O BOX 54840-00200, Nairobi, Kenya

Abstract
Occurrence of mutations within the major antigenic alpha determinant region of hepatitis B surface antigen (HBsAg can alter HBV antigenicity resulting in failures in diagnosis, vaccine and hepatitis B immunoglobulin therapy. This study aimed at detection of mutations in the "a" determinant region of HBV surface antigen among voluntary blood donors in Kenya. This was a cross sectional study involving serology and molecular techniques. This study involved analysis of samples from blood transfusion centers. A total of 301 blood samples from donor blood were collected for the study. Sero-status for HBsAg was determined using Enzyme-Linked Immunosorbent Assay (ELISA). A fragment of the *S* gene including the "a" determinant was amplified by PCR from the HBsAg positive samples and sequenced for mutation analysis. Mutations and phylogenetic analyses were performed using Mega 6 software, Bioedit software and GENETYX® software version 9.1.0. Out of the 301 samples tested 69/301 (22.9%) were Polymerase Chain Reaction (PCR) positive including 2/69(2.9%) were sero-negative for HBsAg. All isolates were genotype A, sub-genotype A1. A total of 29 mutations were observed of which 37.9% were located within the "a" determinant. Mutations T143M and K122R were the most frequent in this study. Escape mutations associated with diagnostic failure, vaccine and immunoglobulin therapy escape were also identified. These findings are important for policies related to vaccine implementation and therapeutic and diagnostic guidelines.
Keywords: Escape mutants, genotype, hepatitis B virus, antigenic determinant, surface antigen

1. Introduction

Hepatitis B virus infection (HBV) is a major public health concern especially in Africa, Middle East and Asia (Akbar *et al*., 2004, McMahon, 2005). More than 4 billion people have had contact with HBV worldwide (Ying-Hui, 2012). Of these, about 360 million people are chronic carriers of HBV that can cause death from liver cirrhosis and hepatocellular carcinoma (HCC) (Lavanchy, 2004; WHO, 2015).

Though HBV is a DNA virus, it replicates via reverse transcriptase (polymerase enzyme) which has no proof-reading activity leading to the generation of mutations occurring naturally during infections (Kramvis 2014).

Hepatitis B surface antigen (HBsAg) is a primary target for diagnosis and immunoprophylaxis of HBV infection (El-sherif *et al*., 2007). The HBsAg which is a 226 amino acid sequence contains a highly conformational, hydrophilic domain known as the "a" determinant located at positions between aa124-aa147 within the Major Hydrophilic Region (MHR; aa110-aa165 (Hu *et al*., 2009). The dominant epitopes of "a" determinant are the targets of neutralizing antibodies used in active, passive immunization and in diagnostic assays. (Kay *et al*., 2007). Mutations in this region can reduce the binding affinity between the HBsAg and antibody to the HBsAg resulting in weak reactivity of serological assays. Although, mutations have been described in all the four open reading frames (ORFs) of the HBV, S escape mutants is the most worrying since it may be spread by blood transfusion. HBV DNA detection by nucleic acid amplification technology (NAT) is not mandatory for blood donor screening in many countries, especially in those areas where the prevalence of S escape mutants is projected to be high (Fischinger *et al*., 2010). It is imperative that the prevalence and types of variants of the "a" determinant found in the population be monitored, because this will affect policy decisions relating to vaccine and diagnostic reagents design (Zuckerman, 2000; Francois *et al*., 2001).To date cases of blood donations with diagnostic escape mutations not detected by HBsAg screening assay have been reported (Levicnic, 2004). Therefore, we aimed at detection of mutations in the "a" determinant region within the S gene among voluntary blood donors in Kenya.

2. Methods

This study was cross-sectional where 301 plasma samples were collected from blood transfusion centers in Kenya. The samples were then transported to Kenya Medical Research Institute (KEMRI) HBV laboratory through cold chain and stored at -80°C till use. The presence of HBsAg was tested using Hepanostika *HBsAg Ultra* (BioMérieux, Lyon, France) kit as per the manufacturer's instructions.