MOLECULAR CHARACTERIZATION OF ACUTE AND CHRONIC HEPATITIS B VIRUS AMONG PATIENTS ATTENDING SELECTED HOSPITALS IN KENYA

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DECLARATION

I, **Ochwoto Missiani (Reg. No.H80/50145/2015),** do hereby declare that this thesis is my original work and that it has not been presented for award of a degree in any other university.

Signature ……..……..…... Date…….

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Dedication:

This thesis is dedicated to the many Hepatitis B infected patients I met during my research; their unconditional support was not in vain.

I also dedicated the thesis to my entire family for their continuous support both financially and morally, more so to my wife and my son and daughters, God Bless you.

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List of Abbreviations and Acronyms

Abbreviation or Acronym	Meaning
µl……..……...	Micro litre
^{0}c.....	Degree Celsius
Ab…….……	Antibody
Ag……..……..	antigen
ALP……..	Alanine Phosphatase
ALT……..	Alanine Transaminase
Anti-HBc…….....	Anti-hepatitis B core
Anti-HBe…….	Anti-hepatitis B envelop
Anti-HBs……...	Anti-hepatitis B surface
Arg.....	Arginine (CGC
AST…….	Aspartate Aminotransferase
BCP/PC.....	basal core promoter/precore
Bp.....	Base pair
CH.....	Chronic Hepatitis
CHB.....	Chronic Hepatitis B
CI ……..……	Confidence Interval
CVR……..…….	Center for Virus Research
DNA……..…….	Deoxyribonucleic Acid
cccDNA……	closed covalent circular Deoxyribonucleic Acid
dNTP.....	deoxyribonucleotide triphosphate.
EIA.....	enzyme immunoassay
ELISA……..……..	Enzyme Linked Immunosorbent Assay
ERC……..……..	Ethical Review Committee
HBeAb…..…….	Hepatitis Be Antibody
HBeAg….…….	Hepatitis Be Antigen
HBs Ab…….……..	Hepatitis B surface Antibody
HBs Ag…….	Hepatitis B surface Antigen
HBV/A	Hepatitis B vrus Genotype A
HBV/D	Hepatitis B vrus Genotype D
HBV/E.....	Hepatitis B vrus Genotype E
HBV……..….	Hepatitis BVirus
HBV-DNA…….	Hepatitis B Virus -Deoxyribonucleic Acid
HCC…….….	Hepatocellular Carcinoma
HCV.....	Hepatitis C virus
IARC.....	International Agency for Research on Cancer
IgG antibody.....	Immunoglobulins class G
IgM antibody.....	Immunoglobulins ClassM
IU/L…….	International Units per Liter
KEMRI ……..	Kenya Medical Research Institute
KNH…….	Kenyatta National Hospital
LFTs……..	Liver function tests

Abbreviation or Acronym	Meaning
MEGA.....	Molecular Evolutionary Genetics Analysis
$MgCl_2$.....	Magnesium Chrolride
min.....	Time in minutes
MTRH.....	Moi Teaching and Referral Hospital
NCBI.....	National Center for Biotechnology Information
ng.....	Nano gram
nm…….	Nanometer
NNPH.....	New Nyanza Provincial Hospital
nt.....	Nucleotide
OR.....	Odd ratio
ORFs…….	open reading frames
PC.....	Pre-core
PCR.....	Polymerase Chain Reaction
PHAC……	Public Health Agency of Canada
Pol…….	Polymerase
pro	Proline amino acid (CCC)
RNA …….....	Ribonucleic Acid
rt	Reverse transcriptase
SAS.....	Statistical Analysis System
sec	Time in Seconds
SERU ……	Scientific Ethical Review Unit
SPSS…….....	Statistical Package for Social Scientists
SSC…….....	Scientific Steering Committee
TBE.....	Tris/Borate/EDTA,
TMB…….....	Tetramethylbenzidine
USA.....	United States of America
USD…….....	United States Dollar
γ	Gamma
χ^2 test.....	Chi-square test
>……	More than
<……	Less than

ABSTRACT

Background: Hepatitis B Virus accounts for majority of liver related deaths in the world with 2 billion persons infected worldwide. It's primarily transmitted through contact with body fluids of the infected person. The virus is categorized into ten (A-J) geographically distributed genotypes. The infection starts as acute infection and may lead to chronic infection and liver cancer. The virus has four phases; the immune-tolerant phase, immune-clearance phase, Immune control Phase and Immune escape Phase. What exactly influences HBV natural disease development from acute state to chronic state and to liver cancer is not so clear, however, the progression may depends on changes on the viral genome and the host's immunity clonal selection mechanism. The changes occur through genome mutations for the host and virus. These genome changes can be detected early before the onset of chronic infection.

Objectives: The aim of the study was to characterize and compare hepatitis B Virus and Human TP53 tumor suppressor gene responsible for acute and chronic viral hepatitis among patients attending selected clinics in Nairobi, Kisumu and Eldoret. The study also established a consensus nucleotide sequences from the sequences generated, to be used in development of a kit that may detect and/or predict Hepatitis B Viruses likely to develop chronic infection.

Methodology: Blood samples were taken for serological and molecular analysis from patients suspected as having viral hepatitis and Liver cancer in the selected clinics in Nairobi, Kisumu and Eldoret. The samples were transported to KEMRI where they were screened for Hepatitis B surface antigen (HBsAg), antibody to the Hepatitis B core protein (HBcAb), anti-HIV and anti-HCV using commercially available kits. Viral DNA of BCP/PC, polymerase and Surface regions was extracted, amplified and sequenced from all HBsAg positive. Similarly human TP53 exon 4 and 7 was amplified and sequenced. Antibodies to HBV core protein (HBcAb) IgM positive and HBsAg were considered as acute infection whereas HBV core protein (HBcAb) IgG positive and HBsAg positive were Chronic infection. Statistical data analysis of factors will be performed using SPSS, Univariate descriptive analyses using proportions for categorical variables and measures of central tendency for continuous variables was done. The sequences were aligned and mutation detected using Bioedit software. The Phylogenetic trees drawn using Mega 7 software.

Results and Discussion

A total of 389 samples from patients with jaundice were collected from the three selected sites; of which Nairobi (KN) and its environs had 285 , Eldoret (ELD) and Kisumu had 28 and 76 samples respectively. The mean age (±SD) of patients was 39.8 years (±14.1) for Nairobi, 35.2 years

(±11.9) for Kisumu, 32.1 years (±10.4) for Eldoret. The prevalence of Hepatitis B virus was found in 50.6 % using HBsAg and only 2.3% had probable acute infection with IgM antibody to the core protein positive.. 132 samples were successfully sequenced. Among HBV DNA positive specimens HBV genotype A was the most prevalent (90.0 %) then genotype D (9.7 %) and E (0.7%). Analysis of HBV/D full genome isolate had close similarity to subgenotype D6, subgenotype D4 and HBV D/E recombinant. Two isolated recombinant sequences had greater than 4 % nucleotide divergence from other previously known HBV D/E recombinants. Among 61 HBsAg negative randomly selected samples, Occult hepatitis B (OBI) was determined to be 32.2% and was significantly associated with a HBc positive status and a lower mean a HBs titre in jaundiced patients. A1762T/G1764A and G1896A pre core and core mutations were common among Hepatitis B chronic patients. Two known drug resistance mutations (A194T and V191I) were detected in sequence from two chronic patients; one genotype D and the other genotype A.

Among TP53 positive samples it was observed that at Codon 72, Homozygous Proline (P) amino acid was the most common (54.5%), as compared to homozygous Arg/Arg (R) (12.1%,) which was the least common. Other patients had the heterozygous Arg/Pro (33.3%) indicating that the patients had both the Proline and arginine in either the forward or reverse strand. The homozygous state was observed in the extreme ends with homozygous Arg/Arg being common among those patients without HCC and homozygous Pro/Pro being common among with HCC At Codon 249, patients who had serine mutation (Arginine changing to Serine) were 24.2% of which 75.0% of them had HCC and the remaining 25.0% had only hepatitis without HCC. All pre-core and core mutations A1762T/G1764A (33.3%) and G1896A (83.3%) were among HCC with TP53 Codon 249 mutation. Generally, the study did not observe any association between patients with HCC and either codon 7 or 249 polymorphisms (Fisher test=3.5 and p=0.12).

Conclusions: From the results, Hepatitis B virus is prevalent among jaundice patients, seeking health care at Kenyan hospitals. The prevalence observed in the study was higher than the national Hepatitis B prevalence. There are new recombinant strains of Hepatitis B virus among the selected Kenyan population that very little information is known about them. Similarly, OBI was determined to be highly prevalent among jaundiced patients in Kenya presenting for medical care (32.2%) with mutations T116N and T118A observed within the HBsAg major hydrophilic region. Kenyan population have variant Codon 72 and Codon 249 polymorphisms, however there was no association between HCC and the codons polymorphisms.

Recommendation: A number of mutations and viral molecular diversity have been observed in this study, we recommend that HBV Diversity in the country should be considered when designing interventions for HBV.

1.0 INTRODUCTION

1.1 Introduction

Hepatitis is the inflammation of the liver, which is characterized by onset of jaundice. Viruses, among other causes, are the major cause of acute, sporadic or chronic hepatitis. There are a number of viruses that cause hepatitis among them those designated Hepatitis A-E (Perrillo 2010). Of all the Hepatitis the most prevalent and worldwide spread is Hepatitis B virus (HBV). HBV accounts for 2 billion infections, and 257 million persons had chronic hepatitis B by the year 2015. HVB accounted for 1.34 million deaths in 2015 from cirrhosis, liver failure, and hepatocellular carcinoma (Jemal *et al.,* 2010, WHO, 2017). Majority of HBV infections are in Sub-saharan Africa and Kenya is categorized among countries with a prevalence >8% (WHO, 2017).

1.2 Hepatitis B virus Infections

The main mode of HBV transmission is through contact with fluids of infected people during sexual intercourse with a person infected, sharing infected needles or/and syringes, blood transfusion of unscreened blood or blood components, body tissues and organs. Infants can be infected perinatally from an infected woman to her infant during pregnancy (WHO 2008). The incubation period is 78 days after infection and the person develops general symptoms that include nausea, having abdominal pain on the upper part of the abdomen, feeling fatigue, malaise, and having jaundice.

1.2.1 HBV Natural progression

HBV infections lead to a variety of liver diseases ranging from acute infection (if the infection lasts for less than six months), to chronic infection. The chronic infection ranges from HBV carriers, then chronic liver disease (cirrhosis, fibrosis and cancer) (Ahn *et al.,* 2003). More than 95% of new born and half (50%) of children infected with HBV progress to chronic hepatitis infection. The proportion changes significantly when an adult is infected with Hepatitis B, where only less than 5% of those infected with hepatitis B virus progress to chronic infection (Okoth *et al.,* 1996).

The natural progression of hepatitis B from acute to development of Hepatocellular Carcinomar can be categorized in phases. The naming of these phases has been changing over time. The early studies use three phases namely “immune tolerant phase”, the “immune active phase” and

"inactive" hepatitis B phase whereas Most recent studies use four phases (Croagh and Lubel, 2014). The stages are characterized by presence or absence of Hepatitis e Antigen positive (HBeAg+) (Phase 1 and 2) or Hepatitis e Antigen negative (HBeAg-). They include:

> **(a)The immune-tolerant phase is** defined by Hepatitis B surface antigen positive (HBsAg+), hepatitis B e antigen positive (HBeAg+), normal hepatic enzyme (transaminases) levels and high levels of serum viremia (HBV DNA concentrations). Biopsies in immune tolerant patients are generally benign, without signs of significant inflammation or fibrosis (Tran, 2011). Most people in poor resource setting countries infected with HBV and are at Immune tolerant phase do not know their status.
>
> **(b) The immune-clearance phase:** This is a period of immune mediated liver damage as evidenced by necro-inflammation on liver biopsy and varying degrees of fibrosis. It is categorized by presence of HBeAg, absence of HBeAb, intermittently abnormal hepatic enzyme levels (ALT) and falling serum HBV DNA concentrations as compared to immune tolerance phase.
>
> (c) **Immune control Phase:** It is characterized by absence of HBeAg and presence of hepatitis B e antibodies (HBeAb) that are sufficient to clear residual levels of circulating HBeAg, the Hepatitic enzymes are fairly normal and a marked decline in serum viremia (Kennedy *et al.,* 2017)
>
> **(d)The Immune escape Phase is** defined by sero-conversion from HBeAg to hepatitis B e antibody (HBeAb), normal hepatic enzyme levels and low or non-detectable serum HBV DNA concentrations (Kennedy *et al.,* 2017). The liver histology shows moderate severe to moderate inflammation. Previously it was referred as "HBeAg negative carrier stage" (Seeger *et al.,* 2000, Lok *et al*., 2006). The liver histology shows moderate severe to moderate inflammation.

HBV Acute infection comprise of phase 1and 2. It is self-limiting and occurs when the surface antigen in the serum is eliminated and within six months the alanine aminotransferase (ALT) levels goes back to normal (Noordeen F. 2015). After infection, about 5% of adult and >90% of children infected with HBV are able to clear the virus in their body and blood leading to develop protective neutralizing antibodies (anti-HBs)(Ahn *et al.,* 2003). Sometimes, the clearance of the virus within six months may be unusual due to slow development of immune antibodies and long viral incubation period (Noordeen F. 2015), but the immune response has to clear the virus. It has been noted that of persons infected with HBV at anytime retain a residual viral genome in their liver cells in the form of closed covalent circular or cccDNA and in during the immunosuppression

condition at include during HIV infection, liver transplant process, dialysis processes and HCV infection the virus may be reactivated (Blackberg *et al.,* 2000, Noordeen F. 2015).

Chronic HBV infection is defined as when an infected person is not able to clear HBsAg from the blood within six months (Noordeen F. 2015). After infection, a typical chronic infection will follow all the four phases described above. After phase 3 (Immune control Phase), very few infected persons will clear HBsAg and develop anti-HBs. The Immune control Phase may take months to many years to transform and develop HCC. The immune system constantly attempts to control the replication of the virus, this results to development of hepatitis clinical signs commonly referred as "Hepatitis sub-clinical or symptomatic flares." The flare may lead to progressive fibrosis, cirrhosis, and ultimately hepatocellular carcinoma (HCC) or liver cancer (Song *et al.,* 2003).

1.3 Chronic Hepatitis (CH) and Hepatocellular Carcinoma (HCC)

What exactly influences HBV natural disease progression from acute state to chronic state and to HCC is not so clear. However various factors have been postulated to influence the disease manifestation and increase its progression to chronic infections (Figure 1.1). These factors include; viral factors (genotype and molecular variation), host factors (age, sex, immune response and genetics) and environmental factors (lifestyle, exposure to aflatoxin, alcohol, diabetes, and smoking) (Ahn *et al.,* 2003, Croagh and Lubel, 2014) (Figure 1.1). The factors may work independently or in interaction with the other factors.

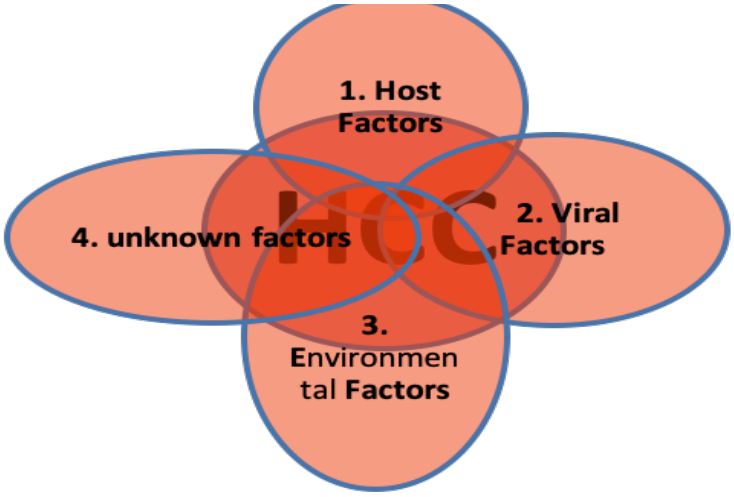


Figure 1. 1*Factors postulated to influence the HBV natural disease progression to chronic hepatitis*

Viral molecular characterization of acute and Chronic HBV display that the virus develops mutations that enable it escape the host immune response. These mutations encode changes in different parts of the viral proteins during the selective pressure from the host immune response (Colagrossi *et al.,* 2018). A number of mutations have been documented in the four overlapping open reading frames (ORFs) namely;

(a) The Surface (S) ORF that codes for Hepatitis B surface Antigen (HBsAg) which is the earliest antigen to be released during infection and responsible for viral detection or screening and viral vaccination.

(b) polymerase (Pol) ORF that codes for Polymerase gene which is targeted during viral treatment

(c) X ORF that codes for the X genes that codes for Transcriptional proteins or regulatory genes and

(d) Core ORF that codes for Hepatitis B core antigen (HBcAg) that is located in the liver and Hepatitis B envelop Antigen (HBeAg) responsible for the viral replication activity (Colagrossi *et al.,* 2018).

This study characterized the acute and chronic HBV virus to establish the viral molecular variations that may affect the acute and the chronic disease progression.

The most active host factor that prevents the final stage of chronic HBV (Hepatocellular Carcinoma (HCC) or Cancer of the liver) is TP53 tumor suppressor gene. TP53 regulates the cell cycle and control the formation of tumor cells (Lane, 1992 and Toufektchan, *et al.,* 2018). Human TP53 tumor suppressor gene is one of the most reserved genes and any molecular diversity affects its function resulting in cancer. Viruses are known to cause mutations in tumor suppressor gene, such viruses include HBV, According to Yan et al 2015, there is a relationship between the HBV mutants and P53, mutations of HBV core promoter may regulate TP53 activities, preventing or promoting cellular transformation and proliferation (Yan *et al.,* 2015). This study characterized Human TP53 tumor suppressor gene of acute and chronic HBV patients to identify the mutations associated with the infections.

1.4 Problem Statement

Chronic Hepatitis B (CHB) and Hepatocellular Carcinoma (HCC) remain one of the major public health problems worldwide (WHO, 2017), despite of the existence of a stable vaccine for HBV. HCC is the sixth most common cancer in the world and fourth among Male (Mohandas 2004). Chronic HBV accounts for majority of liver related infections and deaths in the world (Chang *et al.,* 2009). A report from National Cancer registry reported that 75% of HCC in Kenya was due to HBV infection (Mutuma *et al.,* 2011). What exactly influences HBV natural disease progression from acute state to chronic state and to HCC is not so clear to date. Studies have shown that acquired HBV genotypes and mutations (alteration) may accelerate its pathogenesis (Chang *et al.,* 2009). In Kenya, HBV infection is prevalent but there is limited data on viral mutations, the type of the genotypes that exist in the country and their role in development of CHB, HCC and general disease burden. Similarly, HBV affect TP53, there is no study in Kenya that has ever associated the two.

1.5 Justification

In the current HBV prevalence in Kenya, there is a need to establish the prevalence and role of HBV mutations and genotypes among acute and chronic patients. Equally, the roles of human P53 mutations among the chronic Hepatitis B need to be established in country. Such roles with assist in disease intervention geared towards control management and care. This study will determine the, prevalence, distribution and molecular diversity of HBV in Kenya and reveal the association between the viral factors and host p53 mutations of infected patients and hence their roles in causing HCC.

1.6 Research Questions

1. Which genotype(s) and mutations of HBV infections are in circulation in Kenya?
2. What are the predominant mutations for TP53 in circulation in Kenya and their association with HBV infections?
3. Is the high prevalent of HBV HCC in the country due to exposure to combined Host and viral factors that collectively change the viral genome at various regions before resulting to HBV HCC?

1.7.0 Main Objective

To characterize and compare hepatitis B Virus and Human P53 gene responsible for acute and chronic viral hepatitis among patients attending selected clinics in Nairobi, Kisumu and Eldoret

1.7.1 Specific Objectives

1. To characterize hepatitis B viruses responsible for acute and chronic viral hepatitis among patients attending selected clinics in Nairobi, Kisumu and Eldoret
2. To characterize human Host P53gene among the patients attending selected hospitals in Kenya
3. To determine the association of Host (P53gene) mutation on the viral genome of the acute and HCC patients

2.0 LITERATURE REVIEW

HBV accounts for 2 billion infections, and it is estimated that 257 million people had chronic HBV infection by 2015. It accounts for 1.34 million deaths from liver failure, cirrhosis, and HCC in 2015 (WHO 2017). After HBV infection >90% of adults and <5% of the children mount immunity to clear the virus and develop protective antibodies, whereas, the remain proportion progress to chronic infection. This literature review will explore two factors namely: viral and Host factors that have been postulated to influence the progression from acute to chronic HBV infection.

2.1Influence of Viral factors in HBV progression

2.1.1 HBV viral genome

HBV is a prototype member of *Hepadnaviridae* and the only one in the family that infects humans (Kramvis *et al.,* 2008). It is the smallest of the DNA virus consisting 3200 nucleotides, partially double stranded with reverse transcribing ability. The viral genome of HBV is circular and has four ORFs that are overlapping. They include: the surface region (preS1, preS2, S) which codes for HBsAg, the polymerase (Pol) region that codes for Polymerase (reverse transcriptase RT protein), X genes that codes for Transcriptional proteins or regulatory genes and core (C) region (pre-C and C) that codes for HBcAg and HBeAg, (Kramvis *et al.,*2005).The core ORF is divided into a 29 amino acid for the pre-core region and 181 amino acid for the core region. The two frames are separated by in-frame initiating ATG codons (Chauhan *et al.,*2006). HBV core promoter directs the transcription of 3500bp mRNA which is a pregenomic RNA and a pre-core mRNA that are later translated to form core protein and polymerase that are needed during the replication of the virus (Figure 1.2). (Revill *et al.,* 2010) On the other hand, the pre-core region encodes the pre-core protein that produce HBeAg, which is released into the bloodstream of infected patients (Tacke *et al.,* 2004). Naturally, the presence of HBeAg is an indicator of active proliferation of the virus or cessation of viral replication in the liver (Ahn *et al.,* 2003, Okamoto *et al.,* 1994),

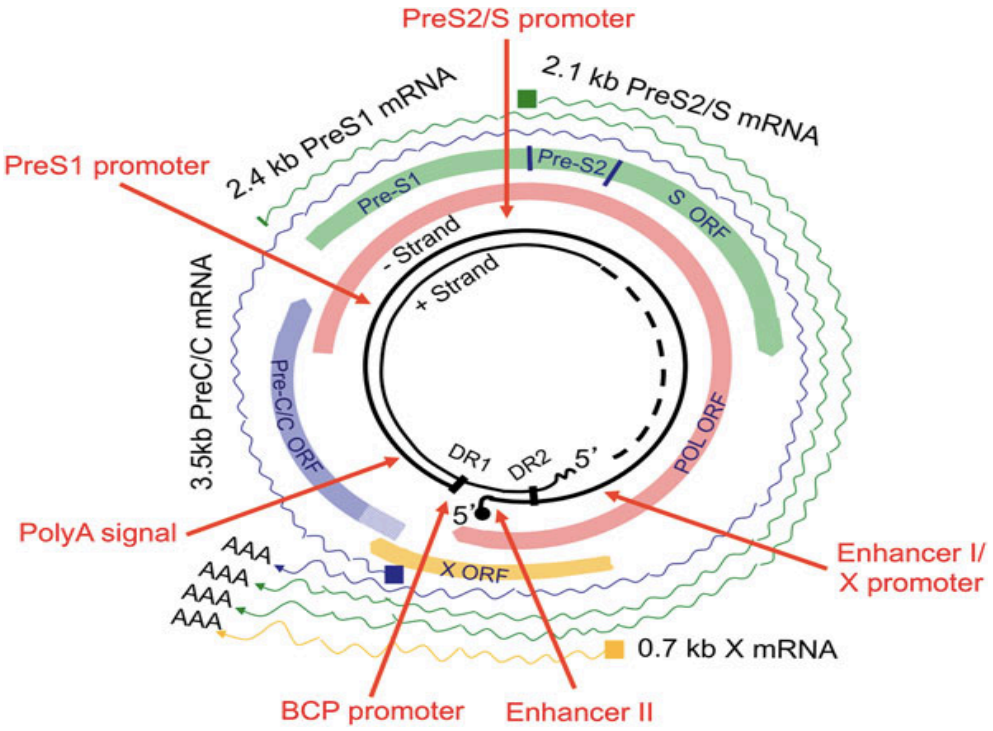
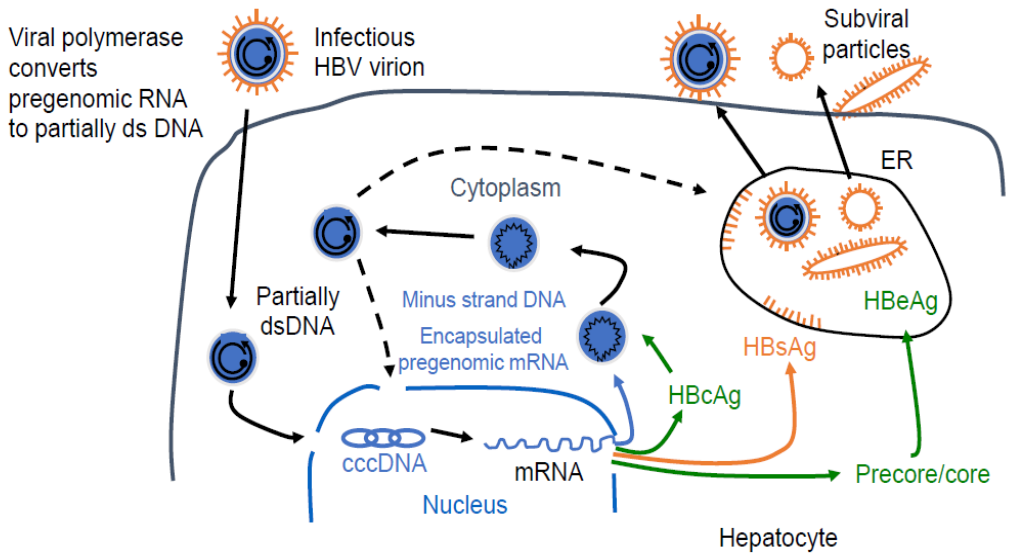


Figure 1. 2 HBV Genome showing the overlapping open reading frames of Surface gene (S ORF) shown in Green colour, the polymerase gene (POL ORF) shown in a red colour, Core gene (Pre-C/C ORF) shown in a purple colour and the X gene (X ORF) shown in a yellow colour.
Adopted from Hu J. 2016.

2.1.2 HBV Life cycle

Hepatitis B Virus makes its new progeny using reverse transcription during its replication process. After infection, the virus attaches to the hepatocytes of the infected person and gain entry through endocytosis. The virus uncoats itself and the Nucleic (DNA) material is transferred the liver cells nucleus. The viral core protein then dissociates in the nucleus using the host DNA polymerases. This is then changed to covalently closed circular DNA (cccDNA) which then serves as a template for transcription. After additional processing the four viral transcripts form progeny virions which are released (Figure 1.3).



Adapted from Lai CL *et al.,* 2000, J.Med.Virol.61;367-373

Figure 1. 3 *HBV lifecycle in the hepatocytes and the antigens formed and released at each stage of the lifecycle.*

2.1.3 HBV mutations and chronic infections

Like in Human immunodeficiency Virus (HIV), HBV has a viral reverse transcriptase that lacks a proof-reading capacity therefore, as it transcribe its DNA genome a number of 'errors' are made in the subsequent progeny viruses (Fung *et al*., 2009). The HBV nucleotide substitution rate, per site per year, has been estimated to be 1.4×10^{-5} to 5×10^{-5}, which is 10^4 times higher than any other DNA viruses (Kramvis *et al.,* 2005). There are two categories of errors that emerge from lack of proof reading; mutations that do not lead to significant change in the structure or function of the virus and mutation that lead to any significant change in the virus structure or function may be as a consequence of specific selection pressure within the host population of quasi-species (Francois *et al.,* 2001 and Fung *et al*., 2009). HBV Mutation can occur at all viral genes, however they tend to cluster into mutational patterns in the BCPs and the Pre-core regions (Gunther *et al.,* 1999).

2.1.4 HBV BCPs and the Pre-core regions Mutations

Mutation in Pre-core or BCP will affect the expression of HBeAg either at transcriptional, translational or at post-translational level (Fung *et al.,* 2006, Kramvis and Kew, 2007). As a result, there will be HBeAg negative results in a serological diagnosis with or without expected decrease

in virus replications, seen in studies with reduced replication (Fung *et al.,* 2009, Hadziyannis *et al.,* 2003 and Chen *et al.,* 2000) and others with increased replication (Buckwold *et al.,* 1996, Tong *et al.,* 2007, Pang *et al.,* 2004). Interference of HBeAg expression among mutants will therefore impair these functions leading to chronicity, liver cirrhosis, and/or HCC.

There are various mutations on the pre-core and BCPs that have been associated with the expression of HBeAg and to chronic HBV infection. The most common pre-core mutation is a guanosine (G) to adenine (A) change at nucleotide 1896 (G1896A), which converts a tryptophan amino acid (TGG) to a TAG (UAG) stop codon. This leads to premature termination of the translation of the pre-core region at codon 28, thus preventing the production of HBeAg (Carman *et al*., 1989, Okamoto et al., 1994). Mutation G1896A is common in genotypes B, C, D, E and G where the nucleotide at position 1858 of encapsidated HBV is a Thymine (T) but not in genotype A where nucleotide 1858 is a Cytosine (C) (Revill *et al.,* 2010, Kramvis and Kew 1999). In many cases, a second pre-core G to A mutation at nucleotide 1899 is also found associated with G1896A, but its significance remains unknown (Okamoto *et al.,* 1994).The other mutation that has attracted much attention is double A1762T(A→T); changing lysine to methionine amino acid and G1764A (G→A; changing valine to isoleucine amino acid) nucleotide exchange, which results in a substantial decrease in HBeAg expression but enhanced viral genome replication. Studies from infected transgenic animals, shows that this mutation causes reduced HBeAg secretion (Kramvis and Kew 1999). More often than not, this mutation has been associated with HCC (Baptista *et al.,* 1999, Liu *et al.,* 2008, Chauhan *et al.,* 2006).

The sequence around the Pre-core initiation codon is conserved in all the known genotypes as 5'-AGCACCAUGC-3' (nucleotide 1808-1817). This sequence conforms to the optimal context for translation initiation, the so-called Kozak sequence (Kozak, 1999), however, double or triple mutations have been described at nucleotide 1809, 1811, and 1812 of HBV genome. This position corresponds to -5, -3 and -2 of the pre-core translation initiation codon (Baptista *et al.,* 1999 and Ahn *et al.,* 2003). Initially, it was considered a missense mutation changing 1809 G to T; alanine to serine) and 1812 C to T; proline to serine (Kramvis and Kew 1999), however, these mutations alter the 'Kozak' sequence of the pre-core ORF stable traits and affect HBeAg expression, besides; the co-existance of 1762T, 1764A and 1809-1812 mutations reduces expression of HBeAg in an additive manner (Ahn *et al.,* 2003, Chen *et al.,* 2008).

Other mutations in BCP and pre-core include; T1753V, nt.1766, and 8nt deletion at 1762-1780. They have been described both in asymptomatic and Chronic HBV patient (Kramvis and Kew 1999) however their role and association with HBeAg is yet to be established.

In summary, During HBV infection, BCP Mutation leads to abolition of HBeAg expression and are associated with alter the immunologic phase. This change leads to increased severity of the liver disease in adults with chronic Hep B infection (Locarnini *et al* 2003).

In Kenya a study conducted on 58 HBV positive patients by Ochwoto *et al.,* 2013, ten (17.2%) patients had 1762T/1764A mutations, that slows down the expression of hepatitis e antigen, during transcription. Another mutation known as “Kozak mutations” (1809nt to 1812nt), known to affect the expression of HBeAg at translational level, was observed in 8.6% (5/58) patients (Ochwoto *et al.,* 2013). To assess the effect of the mutation, Six HBeAg-negative isolates had precore initiation codon mutations, which completely abolish HBeAg expression at translational level.

2.1.5 HBsAg Mutations

The HBsAg is coded by the S-gene and it contains amino acid of Major Hydrophilic Region (MHR) that starts at positions 100-160. These amino acids are highly conformational and hydrophilic. Within this part is a special region from amino acid 124 to 147 known as "a" determinant that represents the immunodominant region of HBsAg (Locarnini *et al.,* 2019*)*as shown in figure 2.1

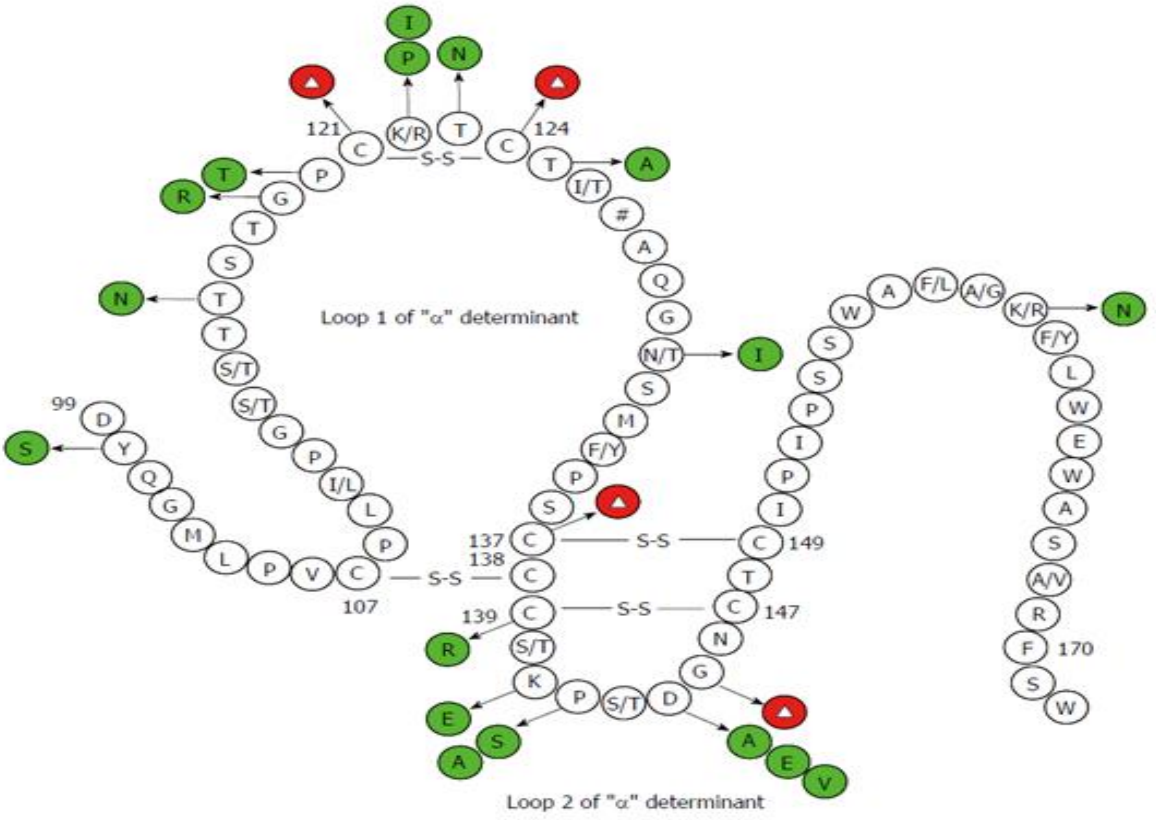


Figure 2.1 ***Amino acids in the "a" determinant region of the HBsAg gene. The green and Red marked amino acids represent the common Hot spots mutations***
(Adopted from Zhu et al., 2016)

Many diagnostic assays and the reagents that are used for testing HBV virus target the "a" determinant region of HBsA. Alteration of amino acids in the "a" determinant region may lead to reduced levels of HBsAg protein expression and antigenicity and (Kann and Gerlich, 2005*)*. Individuals with such mutations will serologically test negative, but positive for HBV DNA, their blood remain infectious if transfused (Raimondo *et al.,* 2007). Hepatitis B variants are naturally occurring, and may have been selected over a long period, whereas hepatitis B mutants are variants that have evolved over a shorter period of time (Locarnini, 2003).

The "a" determinant region is also targeted towards conformational epitopes of HBV Neutralizing antibodies of the vaccine. Virus antigenicity is lost when the cysteine residue at amino acid position 124, 137, 138, 139, 147 or 149 is substituted with serine *(Figure 2.1),* suggesting that these amino acids are critical for expression of the full antigenicity (Locarnini, 2003).

A study by Hemert *et al*. 2008 reported a unique RNA splicing event (deleting nucleotides 2986-202) that completely removes surface protein gene expression without affecting the other genes

like the polymerase, the core or X-protein and their related functions (Hemert *et al*. 2008). In Kenya we have limited data on HBsAg mutations and their role in vaccination and diagnostic (Kwange *et al.,* 2013).

2.1.6 HBV genotypes and chronic infection

There are ten HBV genotypes that are also referred to as viral genomic deviations. The genotypes are alphabetically named as A-J, each genotype have sub-genotype groupings, that are based on >4.0 % but <7.5 % full genome divergence. Both HBV genotypes and sub-genotypes exhibit a distinct global geographic distribution pattern (Norder *et al.,* 2004). Subgenotype A1 is predominant in Southern, Central and Eastern Africa (Miyakawa *et al.,* 2003, Kramvis and Kew 2007, Hübschen *et al.,* 2009, Ochwoto *et al*., 2013 and Kwange *et al.,* 2013). Quasi sub-genotype HBV/A3, previously HBV/A3, A4, and A5 (Norder *et al.,* 2004), is found in West Africa and Haiti (Olinger *et al.,* 2006 and Andernach *et al.,* 2009).

Genotype A is the most predominant genotype that is found in almost all continents (Norder *et al.,* 2004). On the other hand Genotype B is mostly found in Europe, Asia and America (Olinger *et al.,* 2006). Genotype D is common in Mediterranean region, in South America, in Asia and Europe (Zehender e*t al.,* 2014). There are six sub-genotypes of Genotype D designated as HBV/D1 to HBV/D6. These subgenotypes have several other recombinants that are found mainly in Asia and Africa (Yousif M and Kramvis A 2013, and Ghosh *et al.,* 2013). Genotype E is mostly found in central, West and Northern Africa, it has low genetic diversity with no known subtypes, however this genotype is often associated with a number of recombinant especially with HBV/D and HBV/A (Mahgoub *et al*., 2011 and Hübschen *et al.,* 2009). Kenya is a country geographical located at a junction of HBV genotypes A to the south and west, genotype D to the northern and E to the Northern and Western (Ochwoto *et al.,* 2013 and Kwange *et al.,* 2013 and Mwangi *et al.,*2008).

2.1.7 Genotypes and chronic infection

HBV follow up studies conducted have shown that between genotype B and C, genotype B is more frequent in acute hepatitis as compared to genotype C, and within genotype C, sub-genotype C2 is often associated to an increased risk of HCC (Cao, 2009). Genotype A is more aggressive and leads to more chronic hepatitis than genotype D (Mayerat *et al.,* 1999). When two or more genotypes exist in an individual it leads to increase in viral load and extreme infection outcome that often leads to chronic infections. In East Africa, HBV genotype D and A are the most common

with genotype A being predominant among liver patients and blood donors (Mwangi *et al.,* 2008, Kwange *et al.,* 2013 and Ochwoto *et al.,* 2013).

2.2 Host factors that influence HBV progression

There are four host factors that influence HBV progression; sex (gender),age, immune response and genetics.

2.2.1 Gender and HBV progression

Gender plays a significant role in HBV transmission, infection and progression with males being more infected with compared to females (Dounias *et al.,* 2005; Harania *et al.,*2008; Hyams *et al.,* 1989 and Miller *et al.,* 1998). In s study conducted by Wen et al, 2004 showed that among children, HBV-positive boys are significantly more likely to harbour circulating viral DNA, indicative of active replication and they develop hepatitis related cancers more than girls of same age (Wen *et al.,* 2004). Various reasons have been given for higher HBV prevalence and disease progression in male than female, that include, their lifestyle such as drinking habits, polygamy or multiple sexual partners among males (Kirk*et al.,* 2004). However, if this explanation was to apply, we could be experiencing similar trend in other sexually transmitted diseases (STDs) such as HIV. Some researchers have focused on male sex hormone; androgens and oestrogens. The findings show strong evidence for the difference in gender and HBV infection. For instance, Theve and her group (Theve *et al.,* 2008) found out that male sex hormone influence hepatitis B virus in male young mice. Recently another research showed that apolipoprotein A-1 isoforms in male are responsible for the high prevalence in male (Yang *et al.,*2009).

2.2.2 Age and HBV progression

HBV progression to chronic infection among children is quite high, over 95% develop HCC and among adults over 5% develop chronic. After infection the disease progresses slowly among adults and may take up to 30 years before development of HCC

2.3 P53 Tumor Suppressor Gene

TP53 is one of the most active host factors that prevent the final stage of chronic HBV HCC or Cancer of the liver. TP53 regulates the cell cycle and control the formation of tumor cells (Lane, 1992 and Toufektchan, *et al.,* 2018). The precursor p53 tumor suppressor protein regulates the cell formation activities; including cell transcription, repairing of DNA, stability of the genome, controlling the cell cycle, and programing the cell death (cell apoptosis) (Harris 1996) and hence

functions as tumor suppressor. In most cases P53 has been referred as "the guardian of the genome" based on its role (Toufektchan, *et al.,* 2018). Cancer is defined as uncontrollable cell multiplication and therefore it is very important for cells in multicellular organisms to suppress the cancers.

2.3.1 Location of the human TP53 gene

The TP53 gene is located on chromosome 17 (short arm, 17p13) (figure 2.1(a), a region that is frequently deleted in human cancer. The gene has 10 introns and 11 exons (Figure 2.1(b) coding for a 2.2 Kb mRNA that generate multiple transcripts as a result of alternative promoter usage and alternative splicing. The translation of the gene begins in exon 2. In addition, multiple p53 isoforms result from the use of alternative translational start codons in the various mRNAs.

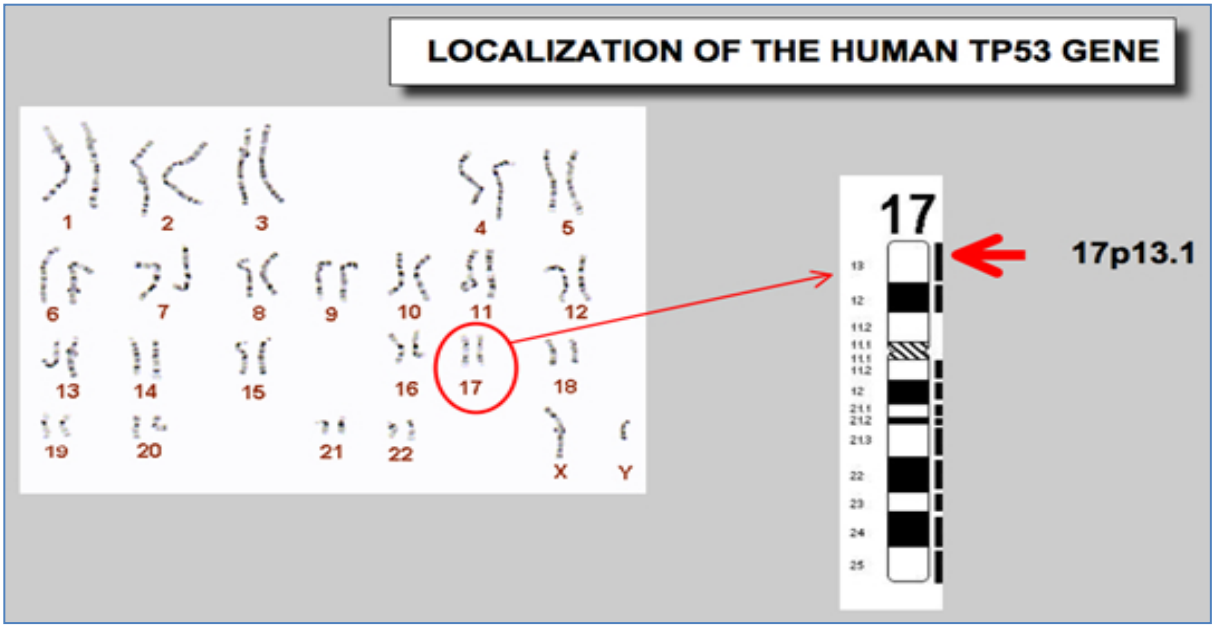


***Figure 2.2**: **(a)**Location of the P53 Human Tumor Suppression Gene in chromosome 17*

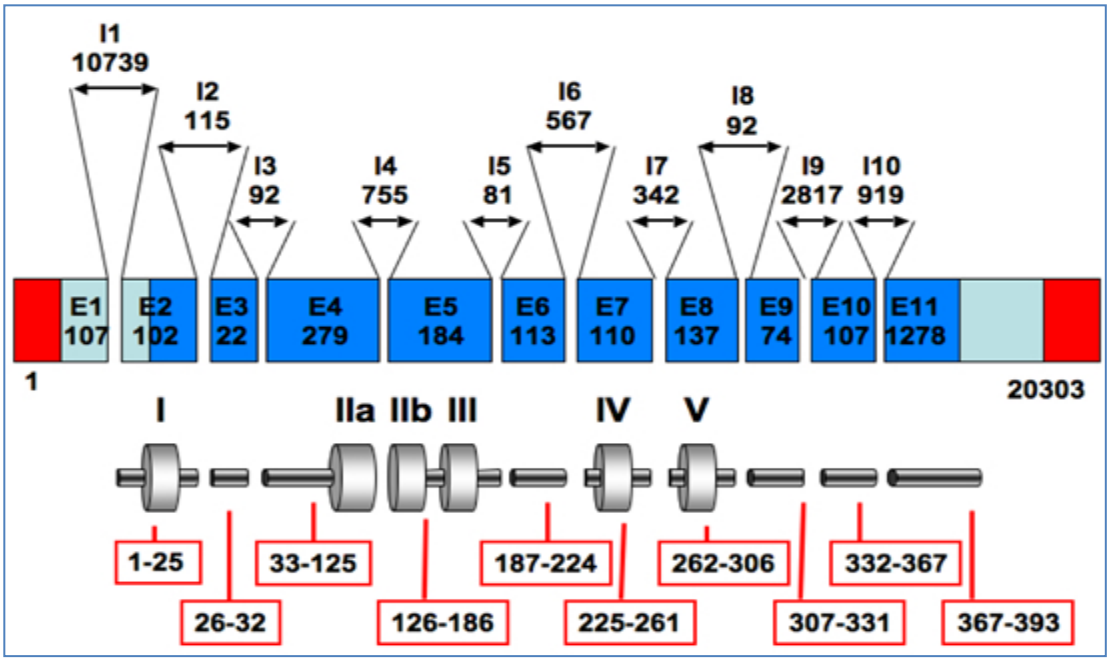


Figure 2.3: (b): linear structure of the human p53 gene
The introns are in black and the 11 exons in blue. The gene is coding for a 2.2 Kb mRNA. Translation begin in exon 2. Sizes of exons and introns are shown in bp. *(Adapted from the P53 Website http://p53.free.fr/p53_info/p53_gene.html accessed on January 7th 2019)*

2.3.2 Functions of P53 in cancers

Cancer is basically defined as uncontrolled proliferation of genetically transformed cells that cannot adequately respond to the regulatory mechanisms of the body. There are a number of known regulatory mechanisms that include; TP53 Tumour suppressor gene, **retinoblastoma Protein (RB)** encoded by the RB1 gene located on **c**hromosome 13,more specifically, 13q14.1-q14.2., Adenomatous polyposis Coli (APC),Von Hippel–Lindau (VHL) (Lane 1992 and Aylon *et al*., 2011). The most studied and common regulatory mechanism is TP53 Tumour suppressor gene that plays the key role in maintaining the genetic homogeinity of somatic cells through slowing down cell proliferation, repairing DNA mistakes and programming cell death or apoptosis (Lane 1992, Lavine *et al.,* 1994, Sigal *et al.,* 2000, and Aylon *et al.,* 2011). In addition, functional p53 protein (TP53) is involved in the initiation of antitumor cytotoxic-T-cell activity against all carcinoma cells (Zambetti GP 2007 and Levine 2009) is referred to as "the guardian of the genome" (Lane, 1992, Toufektchan and Toledo, 2018).) Most studies have shown that, the p53 tumour suppressor-signaling pathway is inactivated in most human cancers. The inactivation depends on how the

pathway is targeted during tumour genesis. This will result in partial or full malfunctioning tumour suppression activity of p53 (Zambetti GP 2007).

Malfunctioning of tumour suppressor genes leads to uncontrolled cell growth, which can lead to cancer (Sigal *et al.,* 2000). Similarly, inherited abnormalities of tumour suppressor genes have been found in some family cancer syndromes, causing those cancers to run in these families (Olivier *et al.,* 2003 and Arcand *et al.,* 2015). DNA Alteration of tumour suppressor genes is the most common cause of TP53 malfunction. A single gene mutation can affect the conformation of the protein or stop the protein from being made at all or cause a gene to be turned on and make more of the protein than usual (Petitjean *et al.,* 2007).

2.3.3 Alterations of TP53

A functional p53 exists as a homo-tetramer and a defective TP53 could allow abnormal cells to proliferate, resulting in cancer. Studies have shown that most human cancers (>50%) are associated with one or more TP53 genetic alterations. These alteration, leads to the presence of both wild-type and mutant allele of TP53 existing within an individual (Caron de Fromentel *et al.,* 1992, Harris, *et al.,*1994). Majority of the TP53 genetic alterations occur in the core domain, a region that contains the sequences that are specific for DNA binding activity of the TP53 protein (residues 102-292) (*see figure 2.4*), and they result in loss of DNA binding (Bei L. and Zhu W. 2006, Cho *et al.,*2008).

There are two different ways that these alterations causing TP53 malfunctioning act to cause malignancy: First, the mutant TP53 protein may have acquired a new tumour -promoting activity which is independent of wild-type TP53 that leads to increased number of tumours, this is refereed *as the gain-of-functions of TP53 cancer mutants* (Song H and Xu Y. 2007). A number of evidences have been documented hypothesizing the gain of function of the p53 mutant stems and the most appealing is the transactivation mechanism of the mutant p53 protein (Sigal A and Rotter V. 2000). Transactivation of multidrug resistant site has been considered a promising candidate as the mechanism for mutant p53 anti-apoptotic function. This fact is supported by the evidence that mutant p53 confers resistance against drugs such as etoposide and doxorubicin (Sigal A and Rotter V. 2000). However, it is still unclear which gene(s) trans-activated by mutant p53 that are responsible for the gain of function (Sigal A and Rotter V. 2000).

Secondly, in a situation where both mutant and wild type exist in an individual, the mutant TP53 protein may act in a dominant negative fashion, ultimately leading to loss of heterozygosity and thus a further growth advantage for the malignant cells (Brachmann *et al.,* 1996).

2.3.4 Structure of p53 gene and hot spot mutations

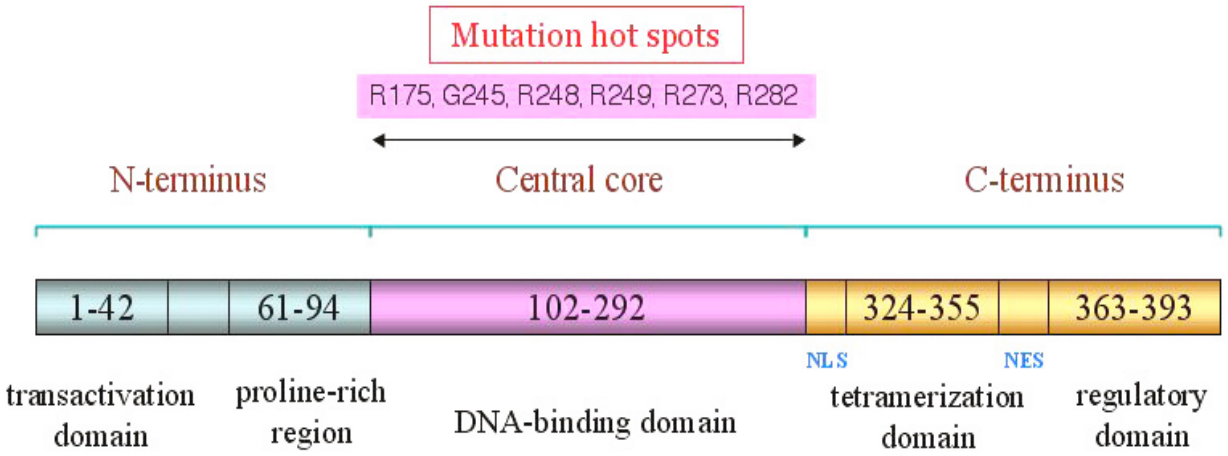
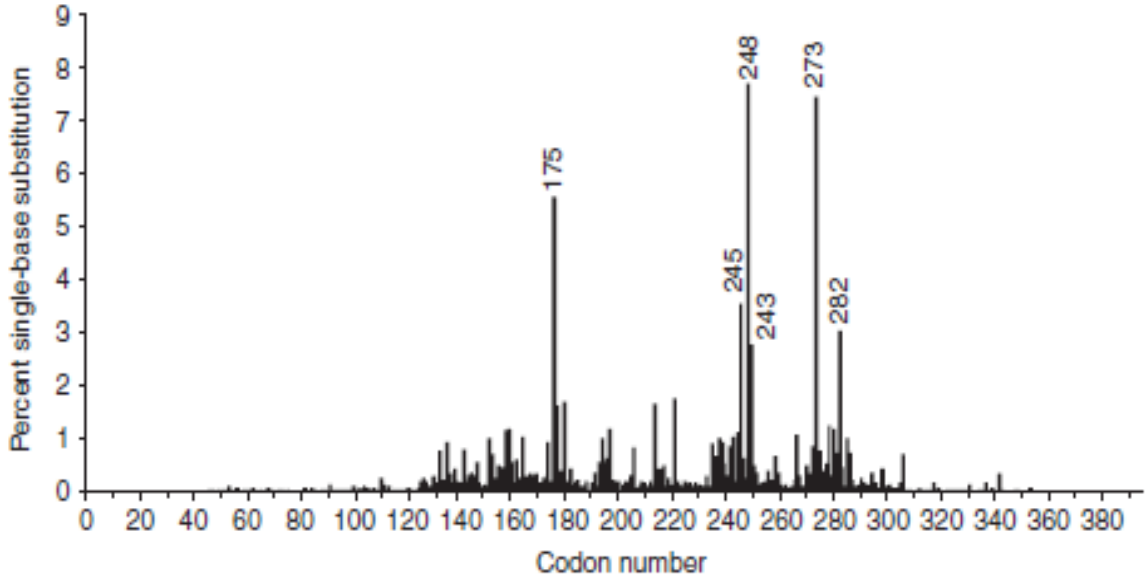


Figure 2.4:*Schematic representation of the p53 structure showing the 393 aminoacids and the three structural domains*

(Adapted from Bei L. and Zhu W. 2006 accessed on January 7th 2019)

Hot spot mutations that have been identified in various human cancers include (but not limited to) the Arg175, Gly245, Arg248, Arg249, Arg273, and Arg282. The proportion of occurrence of each mutation (figure 2.4) shows that Arg175, Arg248 and Arg273 are the most prevalent. Among liver cancer with HBV the most common hotspot are in exon 4, codon 72 and in exon 7, codon 249.

For proper understanding of the TP53 gene polymorphism and its association with the HCC, this study investigated the most common hot spot in exon 4 and 7.



Data from the IARC TP53 Database (Petitjean et al., 2007) accessed on January 7th 2019

Figure 2.5: Histogram displaying the position of somatic point mutations in the coding sequence of the TP53 gene.

2.3.5 Alteration of P53 gene and cancer progression

Among the roles of the p53 include the ability to initiate stop the cell cycle, repair DNA, induce apoptosis and antitumor cytotoxic-T-cell activity against carcinoma cells (Aylon *et al.,* 2011). These roles are affected in case there is an alteration during the transcription of p53 that is encoded by the human gene TP53. The alterations affect master regulators of various signaling pathways involved in tumour suppression (Levine 2009 and Lane 1992). Indeed, the alteration of p53 occurs in almost every type of cancer (Figure 2.5). Hepatocellular carcinomas ranked number ten in TP 53 mutations with a prevalence of 31.9%. Top in the list is ovary, Colorectum and Esophagus (Figure 2.5).

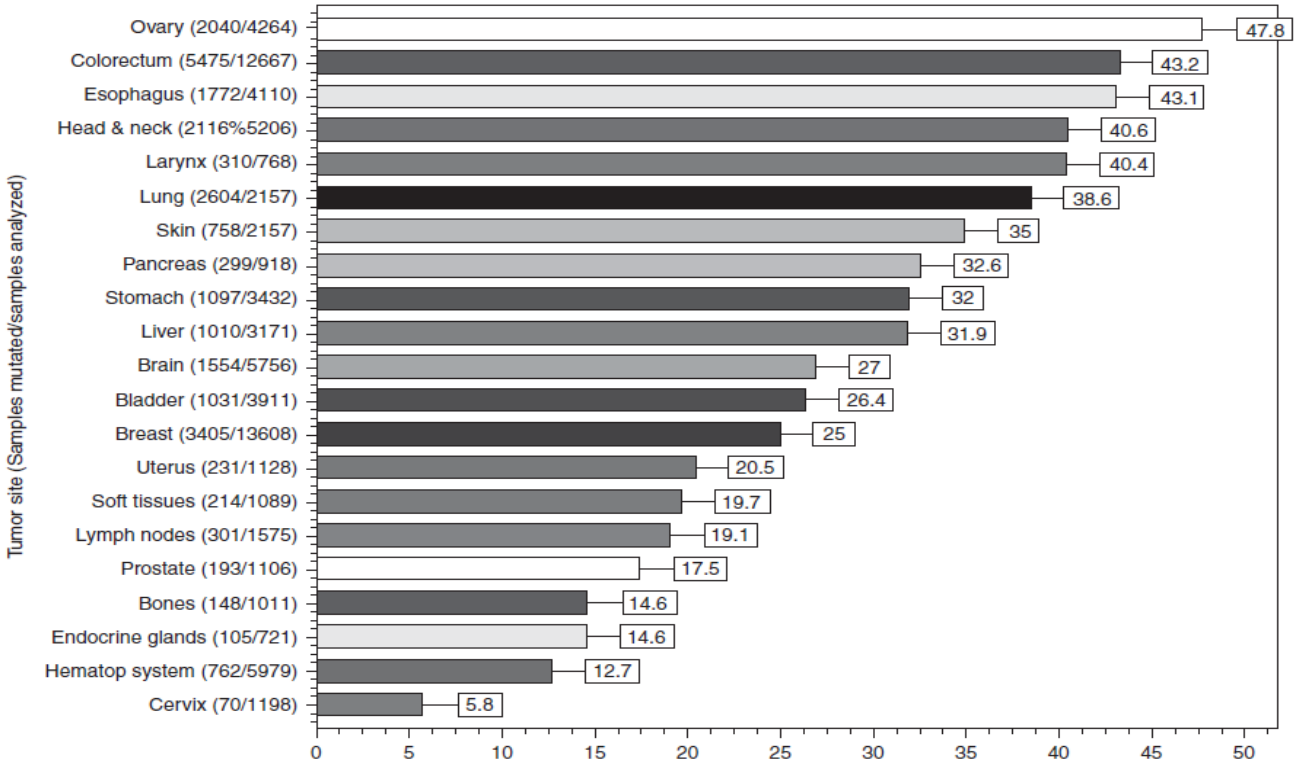


Figure 2.6: Occurance of altered p53 tumour suppressor gene in various tumours (Adapted from Cold Spring Harb Perspect Biol 2010; 2:a001008) accessed on January 7th 2019

2.3.6 When does the TP53 mutation occur in the HCC stages?

The question on when the TP53 mutations occur during the different stages of malignancy transformation raises the possibility that the mutated p53 may contribute differently to various steps of cancer progression (Rivlin *et al.,* 2011). There is no consensus reached to answer if the mutations are involved directly at the beginning of cancer formation or at the more advanced stage of the cancer. The time at which the mutation occur may result to acceleration of the HCC malignant transformation.

2.3.7 Serum circulation of the mutated P53

The TP53 is found in all body cells for effective functioning of the gene. Bood plasma is the most effective mode of transportation for both the mutant and wild type TP53 gene in an individual.

3.0 METHODOLOGY

3.1 Study Sites

This study was conducted in selected hospitals of three major towns in Kenya: Nairobi, Eldoret, and Kisumu. In Nairobi, the Kenyatta National Hospital (KNH) was selected, because it is a national and referral hospital in Kenya. In Kisumu New Nyanza Provincial Hospital (NNPH) currently called Ojaramogi Oginga Odinga Memorial Hospital was selected based on them being the largest provincial hospitals in South-western Kenya. Kisumu city is located at the shore of Lake Victoria, western-Kenya and it is the entry point to four other countries that border the lake; Uganda to the north, Tanzania to the south, Rwanda and Burundi to the west. In Eldoret town, Moi Teaching and Referral Hospital (MTRH) was selected based on it being a referral hospital and it serving the larger Rift valley and western region.

3.2 Study Population

The study recruited all consenting patients presenting with jaundice or hepatocellular carcinoma in these four selected hospitals, the patients filled the questionnaire (*Appendix 2:Patient Questionnaire*).

The population of these towns is cosmopolitan with Nairobi having the largest population. Eldoret town is an agricultural town located North-western Kenya. Within its environs, we have wheat, corn, livestock, tea farmers, and pastoralist. Population of Kisumu city is largely known for fishing activities.

3.3 Study design

This study was a cross sectional study where blood samples from the jaundiced or HCC patients attending each hospital was collected. Depending on the disease status after six months or Immunoglobulin laboratory test the patients were categorized into acute and chronic hepatitis B. For those patients in hospital wards, a blood sample was collected and the HCC stage was obtained from their hospital records. All collected blood samples were then transported to KEMRI for laboratory processes that included serology and molecular analysis.

3.4.1 Inclusion criteria

The study recruited all patients attending the selected hospitals:

- Presenting with jaundice or HCC or patients with known history of viral hepatitis in the last six months,
- The patients willing to fill the consent form and a self administered questionnaire
- And a patient who was willing to give a blood sample for both serological and molecular analysis.

3.4.2 Exclusion criteria

The exclusion criteria were:

- Patients Presenting without jaundice or patients without known history of viral hepatitis in the last six months,
- Patients not willing to fill the consent form and/or fill the self-administered questionnaire were not included
- And patients who could not give a blood sample for both serological and molecular analysis.

3.5 Sampling procedure

Census sampling approach was used where all patients attending the selected clinic in the three towns were selected as long as they meet the inclusion criteria outlined above and were willing to give blood specimen for laboratory screening.

3.6 Sample size determination

The minimum number of sample was calculated using the formula by Fisher *et al.,* 1998: $\mathbf{n=Z^2pq/d^2}$

Where;

$Z^2=1.96^2$,

p=0.08 (p= prevalence; 8% for HBV),

q = 0.92 (1-p),

$d^{2=}0.05^2$

$n = 1.96^2 \text{ X } (0.08 \text{ X } 0.92) / (0.05)^2 = 108 \approx$ **110 samples**

The minimum samples that was required for this study was**110** per study site**.** Therefore, this study included a minimum of 366 patients from all the three urban locations; Nairobi, Eldoret and Kisumu.

3.7 Data Collection Methods

The following methods of data collection methods were employed;

a.) **Questionnaire**; was used to collected the patients' socio-demographic and socio-economic data and infection onset date (*appendix 2*).

b.) Every patient was required to give about 7mL blood sample for serological analysis of HBV.

c.) The blood sample was collected from the patients who met the criteria

3.8.0 Blood Sample Collection, Transportation and Storage

About 7mL of blood samples was collected from the patient through vein puncture method by authorized personnel at the study site. The samples were stored at 4^0C at the collecting site before transported to Kenya Medical Research Institute (KEMRI) within 24 hours. Upon arrival, serum was separated from the red blood cells through centrifugation at 2500 revolutions per minute (r.p.m) for 10 minutes. The serum was then aliquoted into two vials; one vial was kept at 4^oC for serological analysis and the other vial was kept at -80^0C for molecular analysis. The hepatitis positive blood samples were transported to the University of Manitoba- Canada for molecular analysis and sequencing. Later nucleic acid of HCC positive and HBV positive samples were extracted, amplified and the PCR amplicons for TP53 were send to Macrogen Inc for Sequencing.

3.9.0 Objective 1: To characterize hepatitis B viruses responsible for acute and chronic viral hepatitis

3.9.1Laboratory Serological investigations and confirmation

Regardless of the hepatitis B marker, EIA has almost similar protocol. Two of the three EIA tests were done in a fully automated machine Hepatitis B surface antigen (HBsAg), antibodies to core (Anti-HBc) whereas test for Antibodies to HDV was manual. All tests were done based on the manufacturer's manual. Briefly in all the EIA tests; all reagents were allowed to reach the room

temperature before use and gently mixed prior to use. Required volume of standards and samples are added to each well and covered and then incubated at the correct temperature conditions. After washing the wells using the wash buffer and a squirt wash bottle, detection antibodies for the specific test are added. The plate is well covered and incubated at room temperature for 1hr before it is washed as above.. After washing the plate HRP conjugate is added to each well, covered and then incubated for a half an hour at room temperature. The plate is washed as above and a chromogenic substrate to each well followed by incubation at room temperature in the dark for 30min. 100µL of stop solution to each well and the optical density of each well is read at 450nm and 550nm using EIA reader. For results interpretation the cut of value (CoV) was calculated from the negative and positive controls and results interpreted as reactive or non-reactive.

3.9.3 HBV Nucleic Acid extraction

DNA was extracted from all HBsAg positive samples and 67 HBsAg negative randomly selected samples using the QIA-amp® DNA blood extraction kit (Qiagen Inc., Toronto, Canada) as per the manufacturer's manual (Appendix 3). Briefly; 20µl of proteinase K and 200µl of the sample were mixed in a microcentrifuge tube. Then 200µl of AL buffer was added and mixed thoroughly to form a homogeneous solution and then incubated for 15min to allow the cells to lyse. Absolute ethanol (200µl) was then added to each microcentifuge tube to concentrate the nucleic acid before the content was transferred to the spin columns. After centrifugation at 8000rmp, for 1min and the tube containing the filtrate was replaced with unused collection tube. 200µl of wash buffer 1 was added to the spin colomn and centrifuged as above, collection tube was discarded. The step was repeated using wash buffer 2. The extract was eluted in 60µl nuclease free water (Ambion®, Life Technologies, Burlington, Ontario, Canada).

3.9.4 HBV Nucleic Amplification

Five microliters (5µl) of the extracted viral DNA was amplified in a nested PCR using two sets of primers targeting the HBsAg (S1 and S2) and BCP/PC region (EP1-4) as shown in Table 2.1. In both cases a total volume of the master mix was 50µl per tube. Each tube contained 10µl of 10X buffers, 2.5UAmplitaq Gold polymerase (Life Technologies) 20µM forward and reverse primers, and 1.25mM dNTPs. The regions were amplified at 94^{o}C for 10minutes for initial denaturation, 94^{o}C for 30 seconds for denaturation, 55^{o}C for 30sec for annealing and 72^{o}C for 40sec for extension. The PCR was set for 40 cycles before a final extension of 72^{o}C for 5minutes. After the 1st PCR the amplicons were taken for gel electrophoresis using 2% agarose gel. Five microliters

(5µl) of all 1st PCR negative amplicon were subjected to the second stage of amplification that was similar to the 1st PCR using a different set of S2 and C2 primers for HBsAg and BCP/PC, respectively (table 2.1). Briefly; a PCR master mix of 50µl per tube was prepared and the 2nd PCR profile were similar to the one used in1st PCR. The nested amplicon were viewed on 2% agarose gel electrophoresis and all the positive BCP and HBsAg (for both 1st and 2nd amplicons) were gel-purified and send for sequencing, Kowalec *et al.,* 2013.

Table 2.1: Primers used for amplification

PrimerName	Sequence (5'-3')
S1 (LLr)[1]	CGTTGACATACTTTCCAATCAA
S1 (LLf)[1]	TCCTGCTGGTGGCTCCAG
S2 (nLLr)[1]	CAACTCCCAATTACATARCCCA
S2 (nLLf)[1]	ACCCTGYRCCGAACATGGA
C1 (EP1)[2]	GCATGGAGACCACCGTGAAC
C1 (EP2)[2]	GGAAAGAAGTCAGAAGGCAA
C2 (EP3)[2]	CATAAGAGGACTCTTGGACT
C2 (EP4)[2]	GGCAAAAAAGAGAGTAACTC
[1.] Osiowy C,et al., (2010)	[2.] Takahashi et al., (1995)

3.9.5 HBV Full genome amplification

About one quarter of the amplified samples were randomly selected for full genome amplification. Full-genome amplification was first done by a set of P1/P2 primers: as described by Gunther *et al.,*1995, amplification was done using Amplitaq Gold polymerase (Applied Biosystems). The P1 primer sequences is PhenoP1* (1821-1843) 5'-CGGAAAGCTTATGCTCTTCTTTTTCACCTCTGCCTAATCATC -3' and PhenoP2* (1825-1804) 5'-CCGGAGAGCTCATGCTCTTCAAAAAGTTGCATGGTGCTGGTG –3.' Those isolates that did not amplify using the P1/P2 primers, the Full genome of the virus was amplified using a number of different primers shown in table 2.2 and those by Osiowy *et al.,* 2008 and Kowalec *et al.,* 2013. A set of six different overlapping primers that target other viral regions were used for amplification of a single isolate. The PCR master mix and PCR profile was as shown in table 2.2.

Table 2.2 Primers used in full genome amplification and their PCR profile

Name	(nt) Position		Sequence 5′ to 3′	Region	Conditions
PS102	972	+	CCT ATT GAT TGG AAA GTA TGT CAA	ENHCII/X/Prec	94°C, 4 min-1 cycle
PA7	1770	–	TAT GCC TAC AGC CTC CTA ATA CAA	Outer primer	94°C, 40 s;
PIS102	996	+	CGT ATT GTG GAT CCT TTG GGT TT	ENHCII/X/Prec	56°C, 1 min;
PIA7	1723	–	TCA AGC TTC TCC CAG TCT TTA AAC	Nested primer	73°C, 2.5 min - 35 cycles 73°C, 8 min - 1 cycle
PS103	1642	+	GCC CAA TGT CTT ACA TAA GAG GAC	CORE	94°C, 4 min - 1 cycle
PA104	2462	–	AAA GTT TCC CAC CTT ATG AGT CCA	Outer primer	94°C, 40 s
PIS103	1667	+	CTT GGA TCC TCT GTA ATG TCA	CORE	56°C, 1 min;
PIA104	2444	–	CCA AGC TTT ACT AAC ATT GAG	Nested primer	73°C, 2.5 min - 35 cycles 73°C, 8 min - 1 cycle
PS104	2313	+	CCC TAT CTT ATC AAC ACT TCC	POL/Pre-S/S	94°C, 4 min - 1 cycle
PA100	249	–	GAA GTC CAC CAC GAG TCT AGA	Outer primer	94°C, 40 s;
PIS104	2330	+	TTC CGG ATC CTA CTG TTG TTA	POL/PRE-S/S	55°C, 1 min;
PIA100	222	–	GGT ATT GTG AGG AAG CTT GTC	Nested primer	73°C, 2.5 min - 35 cycles 73°C, 8 min - 1 cycle
PS100	3163	+	ATC CTC AGG CCA TGC AGT	Pre-S/S	94°C, 4 min - 1 cycle
PA102	1178	–	CGT CAG CGA ACA CTT GG	Outer primer	94°C, 40 s;
PIS100	15	+	CCA CCA AAC TCT TCA GGA TCC	PRES/S	56°C, 1 min;
PIA102	1134	–	AAC GGG GTA AAG CTT CAG ATA	Nested primer	73°C, 2.5 min - 35 cycles 73°C, 8 min - 1 cycle
HBVCTF	1803	+	CAC CAG CAC CAT GCA ACT TT	Quantitation primer (core region)	50°C, 2 min - 1 cycle 94°C, 10 min - 1 cycle 94°C, 15 s, 60°C, 1 min - 40 cycles
HBVCTR	1911	–	TCA ATG TCC ATG CCC CAA A		
SPF	2698	+	TGG TAC CTT ATT ATC CAG AT	Surface promoter-Rluc fusion primer	94°C, 4 min - 1 cycle 94°C, 40 s; 56°C, 1 min; 73°C, 2.5 min - 35 cycles 73°C, 8 min - 1 cycle
SPR	175	–	TCC TGA TGT GAT GCT AGC CAT		

3.9.6 Gel Electrophoresis and Visualization

Either,TAE and 2% agarose gel electrophoresis or QIAxcel-Pure excellence®electrophoresis machine (Qiagen-USA) were used for visualization of the amplified DNA depending on their availability. In Agarose gel electrophoresis; 2% agarose (Nusieve GTG, USA) dissolved in 1X TAE buffer (Sigma Eldrich, Germany) and 3µl of SYBR® Green (invitrogen-USA). The loaded gel was then transferred to electrophoresis Gel tank (Maryson-Japan) set at 100Volts, for 1hours. The gel visualization was done under 460nM ultraviolet light (Transilluminator, USA).

The QIAxcel-Pure excellence® electrophoresis machine user's manual was used in the loading the amplicon. Briefly, 1µl of the amplicon was transferred to the QIAxcel-Pure excellence gel in a PCR tube and loaded to QIAxcel machine. Depending on the number of the PCR tubes loaded, the results are displayed on the machine screen after 20-40 minutes.

3.9.7 Sequencing of the HBV positive Isolates

All PCR HBV positive Isolates were purified using the Qiagen Gel purification kit (*Appendix 4*), then quantified with Nanodrop (Thermo scientific, Wilmington USA), and finally sequenced using an automated ABI 3750 XL Genetic Analyzer (Applied Biosystems, Life Technologies). Amplification primers in Table 2.1 and 2.2 were used during sequencing. The sequences received

were assembled using DNASTAR software (Lasergene software suite v7.1.0, DNASTAR). The alignment was done using ClustalX v2.0.1 (Larkin *et al.,* 2007**).** The aligned sequences were edited using Bio-Edit software (Hall 1999) and the phylogenetic tree constructed using the Mega v.5.2 software.

3.9.8 Phylogenetic analysis

The Phylogenetic analysis of HBV Basal Core Promoter and Pre-core region (307bp; nt 1653 - 1959), and HBsAg coding region (681bp; nt 155 - 835) was done using Maximum Likelihood; the Kimura 2-parameter+γ substitution model was used as the most appropriate model. MEGA (v.5.2; Tamura *et al* 2011) was used to construct a neighbor-joining tree with 500 bootstrap replicates.

GenBank reference sequences. The sequences obtained in this study were submitted to the NCBI database (**accession numbers KP168416 to KP168435**).

3.9.9 Statistical analysis

SPSS v.16.0 was used to analyse any continuous and categorical data. Chi-test and Fisher's exact test were used to compare the proportions and P value of <0.05 was considered significant. A number of mutations at any of the four ORFs with high frequency(ies) was regarded as a mutational hot spot.

3.10.0 Objective 2: To characterize human Host P53gene among the patients attending selected hospitals in Kenya

3.10.1 Human DNA extraction from the serum

DNA of the human TP53 tumor suppressor gene that is Circulating in plasma was extracted from 200µl of the samples. The extraction was done using QIAmp DNA extraction kit (Qiagen Inc, USA) (Appendix 3), eluted in 60µl of AE buffer and stored in -30^{o}C until use.

3.10.2 TP53 PCR amplification

Five microliters (5µl) of the extracted human DNA was amplified in a conventional PCR using primers that targeted *TP53* gene exons 4 and 7. The master mix of the three primers was similar and it consisted: 5µl of 1.25mM dNTP mix, 5µl of 25 mM $MgCl_2$, 5µl of 10X PCR buffer, 5 µl of 25 mM $MgCl_2$, 0.2 µl of 5U of Taq DNA polymerase (Qiagen Inc, USA), 1.25µl of a 20µM stock of forward primer and reverse primer of sequences. The total volume was 50µl per reaction tube.

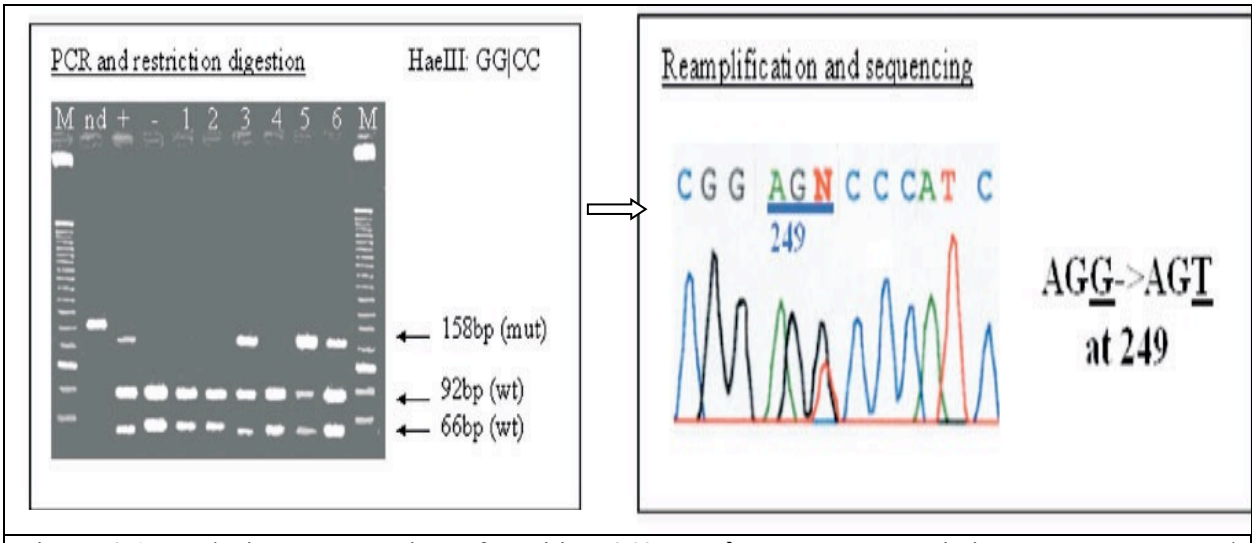


Figure 3.1 Analysis representation of Position 249 Arg→Ser HaeIII restriction enzyme on a gel electrophoresis and after sequencing. Adapted from (http://www.actabp.pl/pdf/1_2003/231s.pdf)

The PCR machine (ABI systems) profile for exon 7 was set at 95°C for 10min, 95°C for 45sec, 58°C for 30sec and 72°C 30 sec for extension. For exon 4 the machine profile was 94°C for 12 minutes, 94°C for 40sec, 56°C for 30 sec and 72°C 30 sec. Both exons had 35 cycles and a final extension of 72°C for 10mins.

Gel Electrophoresis: 4µl of the amplicons were viewed in a 2 % agarose (Fisher Scientific), 2µl of 5X Gelpilot DNA loading Dye (Qiagen Inc, USA) and 1X TBE buffer containing SYBR-safe DNA gel stain (Invitrogen, California, USA).

A 2nd nested PCR for exon 7 was done using 5µl of all negative amplicons, the PCR mix and profile of the 2nd round was similar to that of the first round. The primers used were: 7b Forward 5-AGGCGCACTGGCCTCCTT-3 and 7b reverse 5-TGTGCAGGGTGGCAAGTGGC-3. The amplified amplicons were viewed on a 2 % agarose gel using an ultraviolet trans-illuminator gel Doc-ItImager.

3.10.3 TP53 DNA sequencing and analysis of positive amplicons

Twenty (20µl) of all PCR positive amplicon for exon 4 and 7 were send to Macrogen Inc - Netherlands for sequencing after purification using the Qiagen Gel purification. The first round primers were used during the sequencing. The forward and reverse primer sequences were assembled using GENETYX v. 9.1.0 (GENETYX Co., Tokyo, Japan) software. The generated contings were aligned using Bioedit software v. 7.2.5 (Hall 1999) and a *p53* gene reference sequence from International Agency for Research on Cancer (IARC) database

(http://*p53*.iarc.fr/*p53*sequence.aspx). The mutations in each exon were analysed using MEGA v.7.0 software bioinformatics editing tool (Tamura *et al* 2011).

3.11 Ethical Considerations

Clearance to carry out the study was obtained from Scientific Ethical Review Unit (SERU) of KEMRI (KEMRI/SERU/CVR/001/3211) (*Appendix 5a*), the ERC of the MTRH hospitals (MTRH/Moi University Approval No. 001002)(*Appendix 5b),* Eldoret Cancer Registry Approval No. ECR/DRA/2017/001 and KNH and Nairobi University Ethical Committee (*Appendix 5c)*. In addition, those willing to participate in the selected hospitals were filling a consent form (A*ppendix* 1). Participation in the study were free and voluntary and if the participants choose to withdraw from the study there was no harm and it would not affect his/her relationship with the health providers at the clinic. Except of slight pain experienced when drawing blood, there was no known risk in participation. Subjects/participants in the study were kept anonymous using assigned laboratory numbers.

CHAPTER 4: RESULTS

4.0 Introduction: Social Demographic

A total of 389 blood samples were collected from patients presenting with jaundice at the three selected hospitals in Kenya. The hospitals included Kenyatta National Hospital (KNH) in Nairobi, 63.0% (245/389), Moi Teaching and Referral Hospital (MTRH) in Eldoret 7.2% (28/389) and New Nyanza Provincial Hospital (NNPH) currently called Jaramogi Oginga Odinga Teaching and Referral Hospital (Kisumu) 19.5% (76/389). The hospitals were selected based on the high number of attendees in that region. During analysis 40 samples collected at KNH were referred from Mombasa. Out of the 389 patients the gender, age, and HBsAg positivity is shown in table 4.1.

Table 4.1: Demographic and sero-marker distribution of the study population by region

	Participants		**Age**		**Gender**				**HBsAg +[1]**	
Region	**n**	**%**	**Range**	**Mean (SD)**	**M**	**%**	**F**	**%**	**n**	**%**
Nairobi	245	63.0	15-85	39.77 (14.1)	139	56.7	106	43.3	77	22.7
Kisumu	76	19.5	16-64	35.24 (11.9)	41	53.9	35	46.1	59	17.4
Mombasa	40	10.3	19-64	36.18 (12.3)	22	55.0	18	45.0	11	3.2
Eldoret	28	7.2	19-52	32.21 (10.4)	18	64.3	10	35.7	25	7.4
Total	**389**	**100.0**	**15-85**	**37.97 (13.5)**	**220**	**56.6**	**169**	**43.4**	**172**	**50.7**

[1]A total of 339 samples were tested for HBsAg.M= Male, and F = Female

Out of the 389 samples, 332 individual samples had enough volume of 2ml for all the Hepatitis B virus tests (both serological and molecular tests) (Figure 4.0). Out of the 332 samples 50.6% (168/332) were HBsAg positive. IgM anti-HBc was tested in the all patients who were HBsAg positive. Majority of the patients were negative and only 3(2.0%) were IgM anti-HBc positive, indicating possible acute infection or reactivation. Nairobi had the highest proportion among those HBsAg positive,74/168 (44.0%), followed by Kisumu, 59/168 (35.1%), Eldoret, 26/168 (15.5%)

and Mombasa, 9/168 (5.4%). The mean age and range for those HBsAg positive was 36.6±0.86 (16-64 years), with males more highly represented than females at 65.5% and 34.5%, respectively.

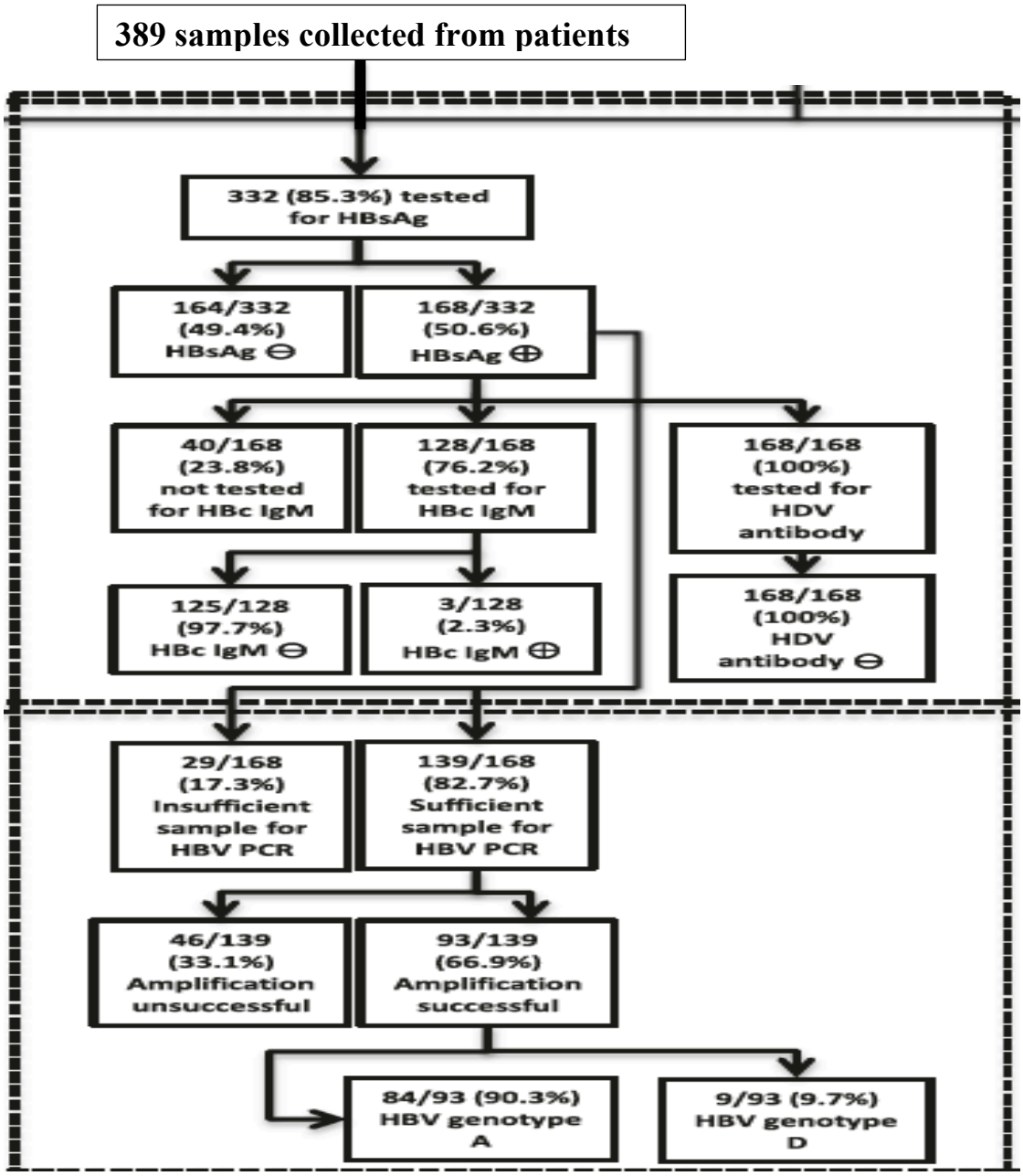


Figure 4.0: A flow chart showing a schematic number of samples tested at each stage and the outcome of the test

4.1 Objective 1 Results: To characterize hepatitis B viruses responsible for acute and chronic viral hepatitis

4.1.1HBV DNA Extraction and amplification

Viral DNA was extracted from 139 HBsAg positive samples that had sufficient serum volume of 1mL(Figure 4.0), out of which 93 (66.9%) samples successfully amplified for either the Surface region and/or BCP and PC region. For the Surface region we successfully had 86 extracts with clear PCR bands and for BCP and PC region we had a total of 79 extracts (figures 4.1a and b).

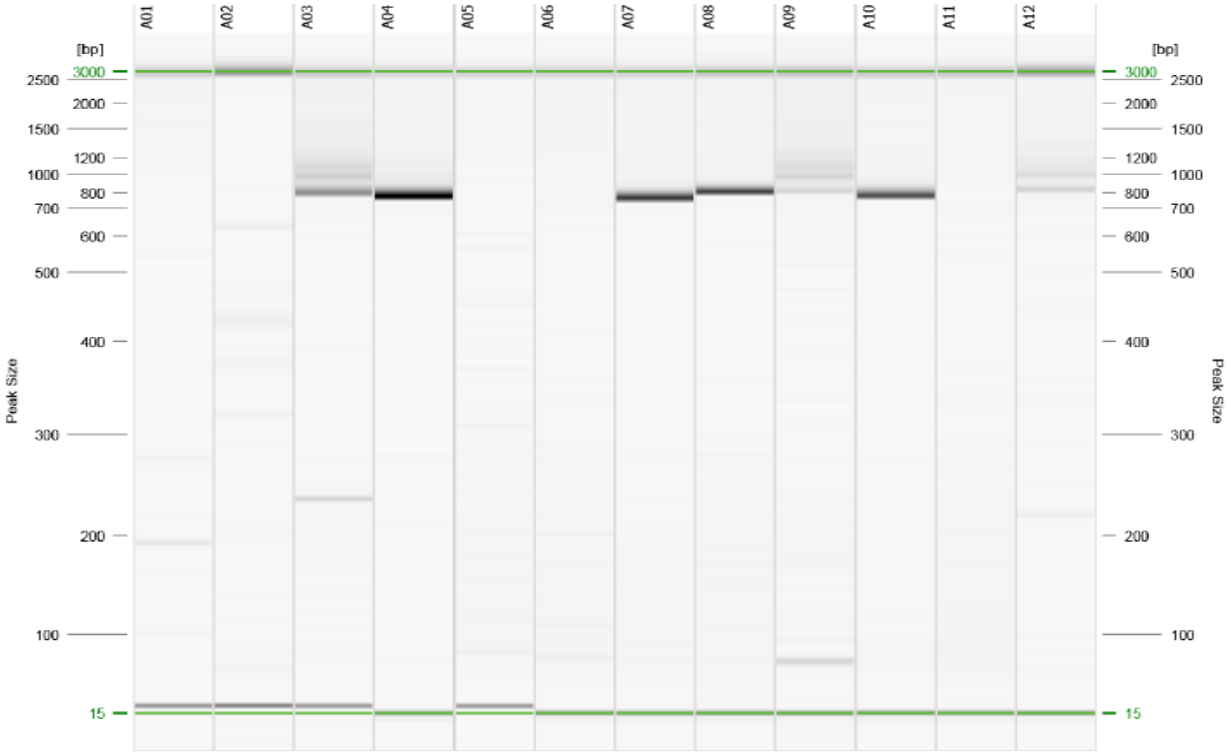


Figure 4.1a: A representative HBsAg PCR gel electrophoresis using QIAxcel ScreenGel 1.2.0. The amplified nucleotide for a positive sample was 750bp, sample A01 and A12 were negative and positive controls respectively. A band indicated that the sample was HBV surface antigen positive (e.g. A03, A04,A07, A08, A09, and A10) whereas a clear well indicated that the sample was HBV surface negative (e.g. A02, A05,A06 and A011)

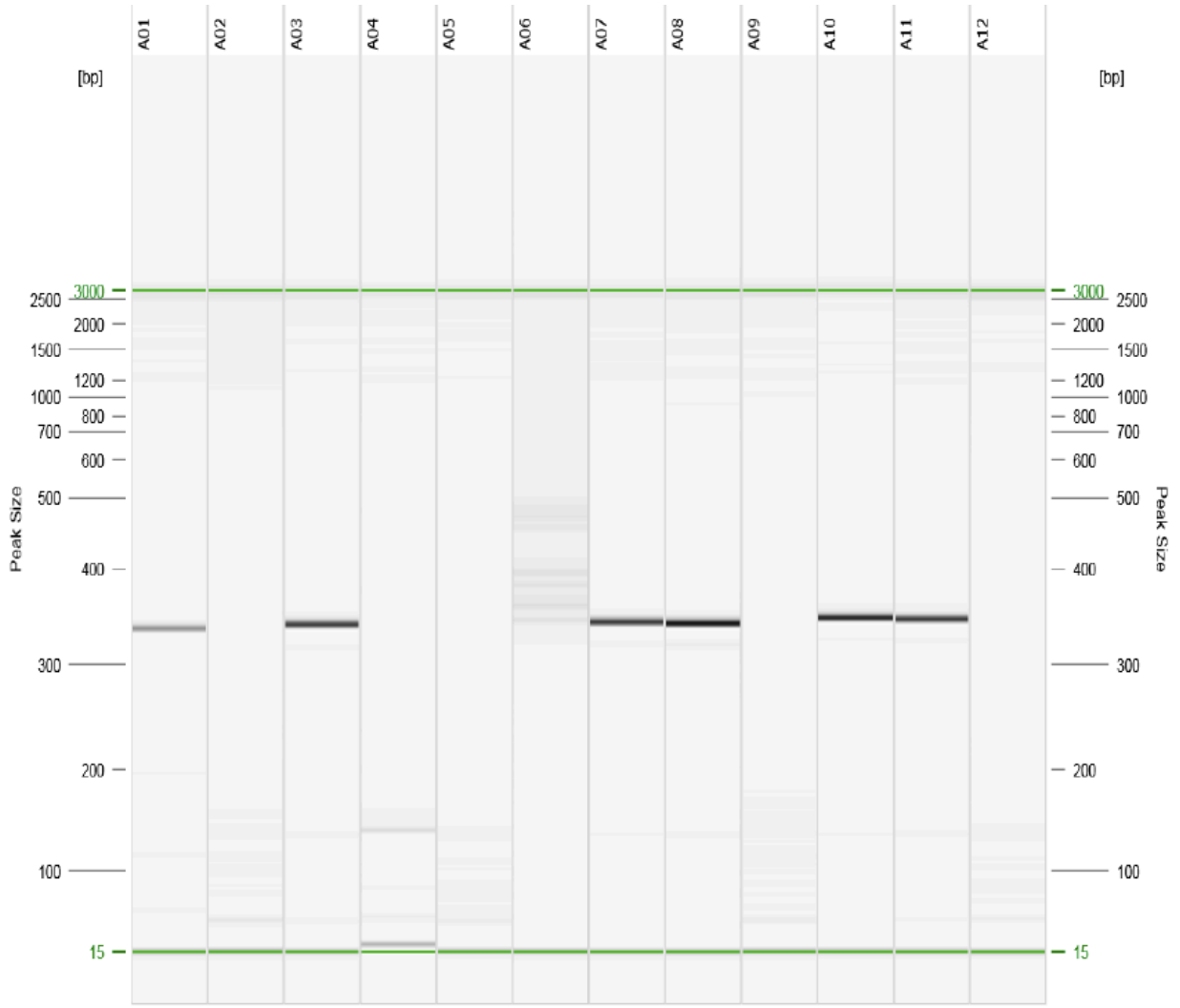


Figure 4.1b: A representative of BCP/PC region PCR gel electrophoresis using QIAxcel ScreenGel 1.2.0.
The BCP/PC region amplified 700 nucleotides, sample A01 and A12 were negative and positive controls respectively. A band indicated that the sample was HBV BCP/PC region positive (e.g. A02, A03,A05 Among others) whereas a clear well indicated that the sample was HBV BCP/PC region negative (e.g. A04)

It was observed that some samples were positive on HBsAg region and Negative on the BCP/PC region and vice-versa despite of how many times the same sample was extracted and amplified.

4.1.2Full genome amplification

For full genome sequencing, 20 samples randomly selected were amplified. Using amplification primers P1 and P2 seven (35.0%) samples had a lower band splicing

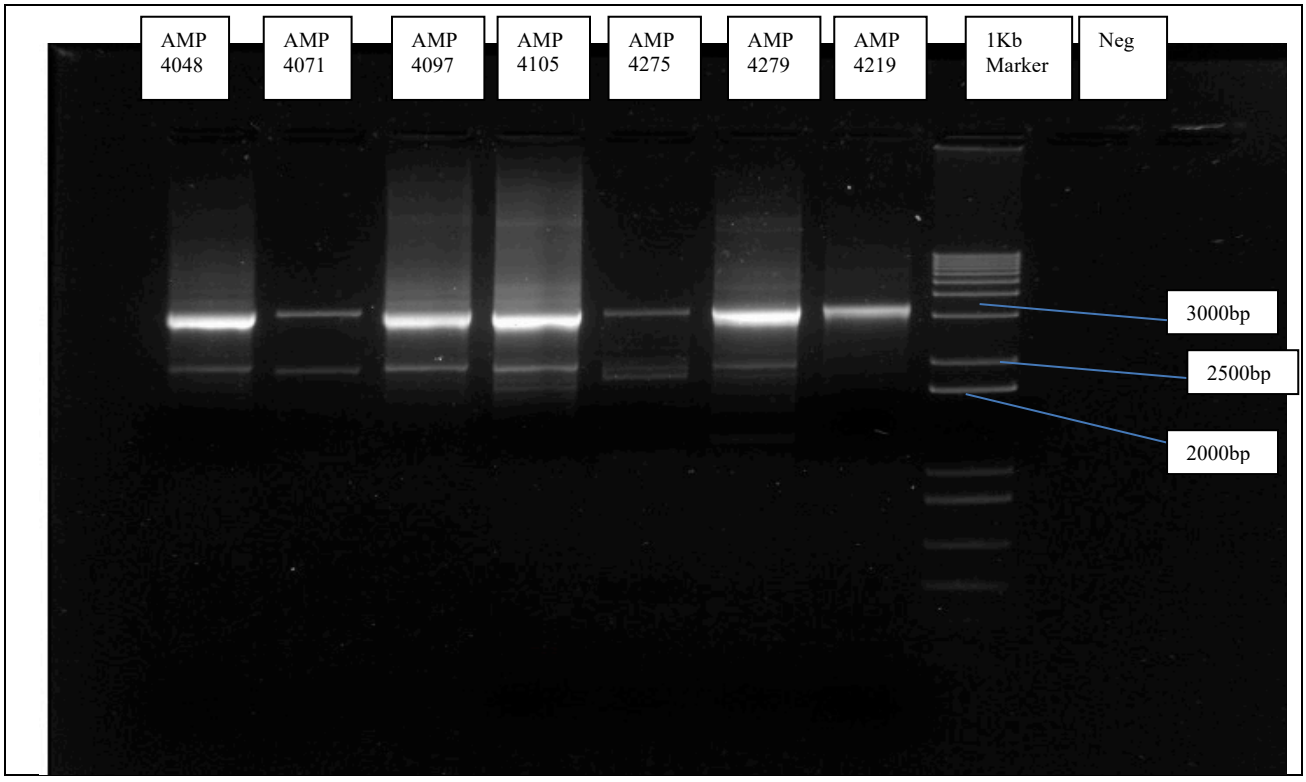


Figure 4.2: A gel electrolysis of a full HBV genome using P1 and P2 primes showing the splicing of HBV

4.2 Phylogenetic analysis of HBV isolates

HBV genotype A predominated (84/93; 90.3%) then genotype HBV/D (9/93; 9.7%) and lastly genotype E. Using the HBsAg sequences Partial genome Phylogenetic analysis revealed that genotype A sequences clustered very easily with reference sequences of sub-genotype A1. There was a clear distinction between isolates of A1 from Asia (Asian clade) and isolates of A1 from Africa (African Clade). As shown in figure 4.3 and 4.4, isolates from all the sites (Nairobi, Kisumu and Eldoret) clustered with sequences from Kenya's neighbouring countries that include Uganda Tanzania, , Congo, Rwanda, South Africa and Zimbabwe. This formed the African A1 clade. Interestingly, more than >70.0% of HBV/A1isolates from KNH -Nairobi clustered with sequences from the Bangladesh, Japan and Philippines. a statistically significant difference was observed both in geographical region and age (Fisher test, p=0.02). It was apparent that almost all patients with genotype D (8/9) were from MTRH (Fig. 4.3)

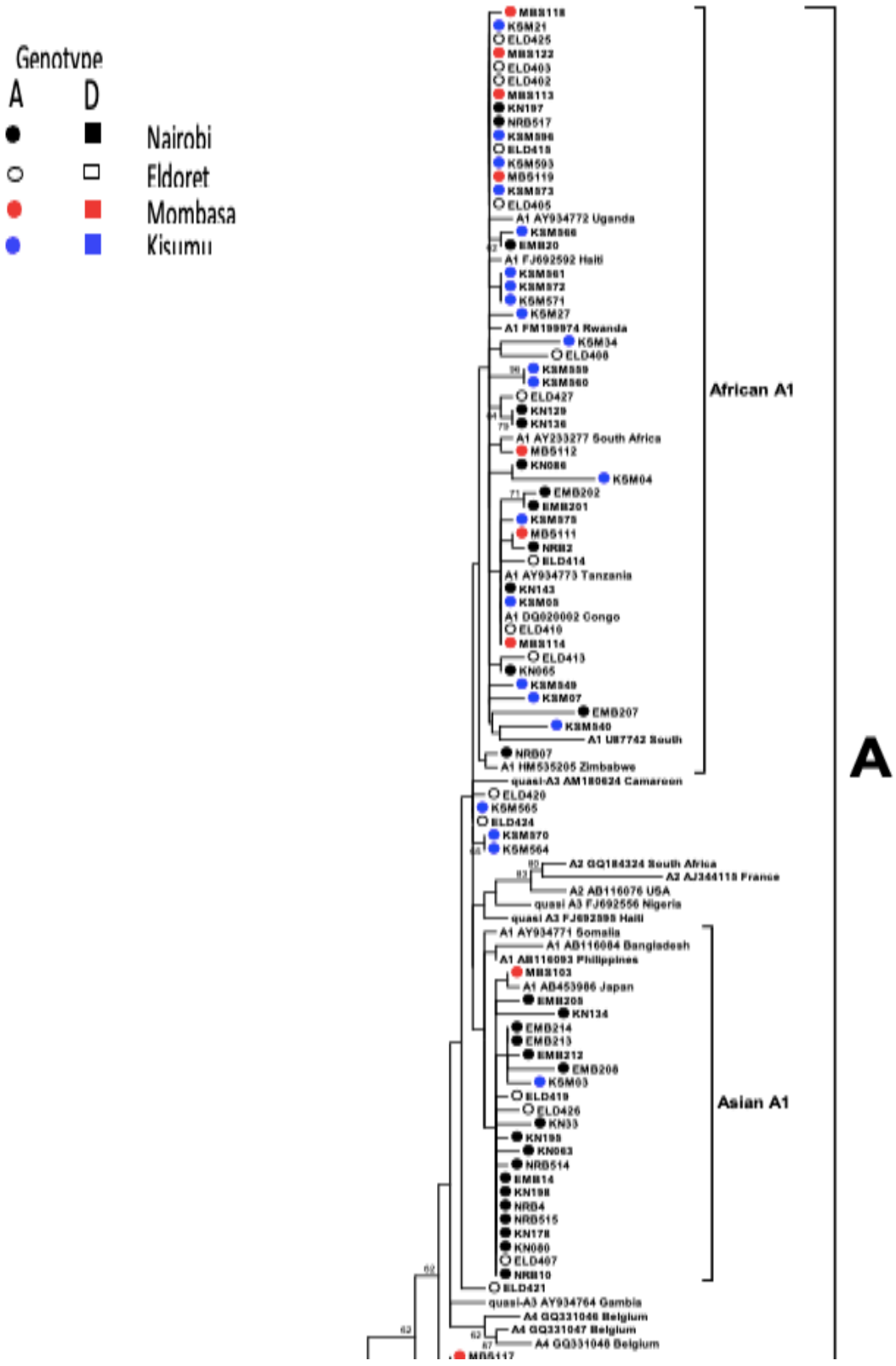

Genotype
A
D
Nairobi
Eldoret
Mombasa
Kisumu
African A1
Asian A1
A
A1 AY934772 Uganda
A1 FJ692592 Haiti
A1 AY233277 South Africa
A1 FM199974 Rwanda
A1 AY934773 Tanzania
A1 DQ020002 Congo
A1 HM535205 Zimbabwe
quasi-A3 AM180624 Cameroon
A2 GQ184324 South Africa
A2 AJ344113 France
A2 AB116076 USA
quasi A3 FJ692556 Nigeria
quasi A3 FJ692555 Haiti
A1 AY934771 Somalia
A1 AB116084 Bangladesh
A1 AB116093 Philippines
A1 AB453986 Japan
quasi-A3 AY934764 Gambia
A4 GQ331046 Belgium
A4 GQ331047 Belgium
A4 GQ331048 Belgium

Figure 4.3: HBsAg Phylogenetic analysis of HBV subgenomic sequences using Maximum likelihood analysis and neighbor-joining tree construction.

Among the 20 samples selected for full genome, majority (fourteen) were genotype A and only six were genotype D. (Fig. 4.4). When genotype A nucleotide divergence of the 14 full genome sequences was calculated, it was found to be 3.47%±0.25%. This divergence was close to 4% that describes a new subtype. The high nucleotide divergence was mainly contributed to the unusual sequence of isolate MBS117 (see Figure 4.7). When the sequence (MBS117) was excluded from the calculation the mean distance was 2.71% ± 0.23%. The sequence had 3 nucleotide deletion at the beginning of the PreS leading to loss of PreS1 amino acid 11.

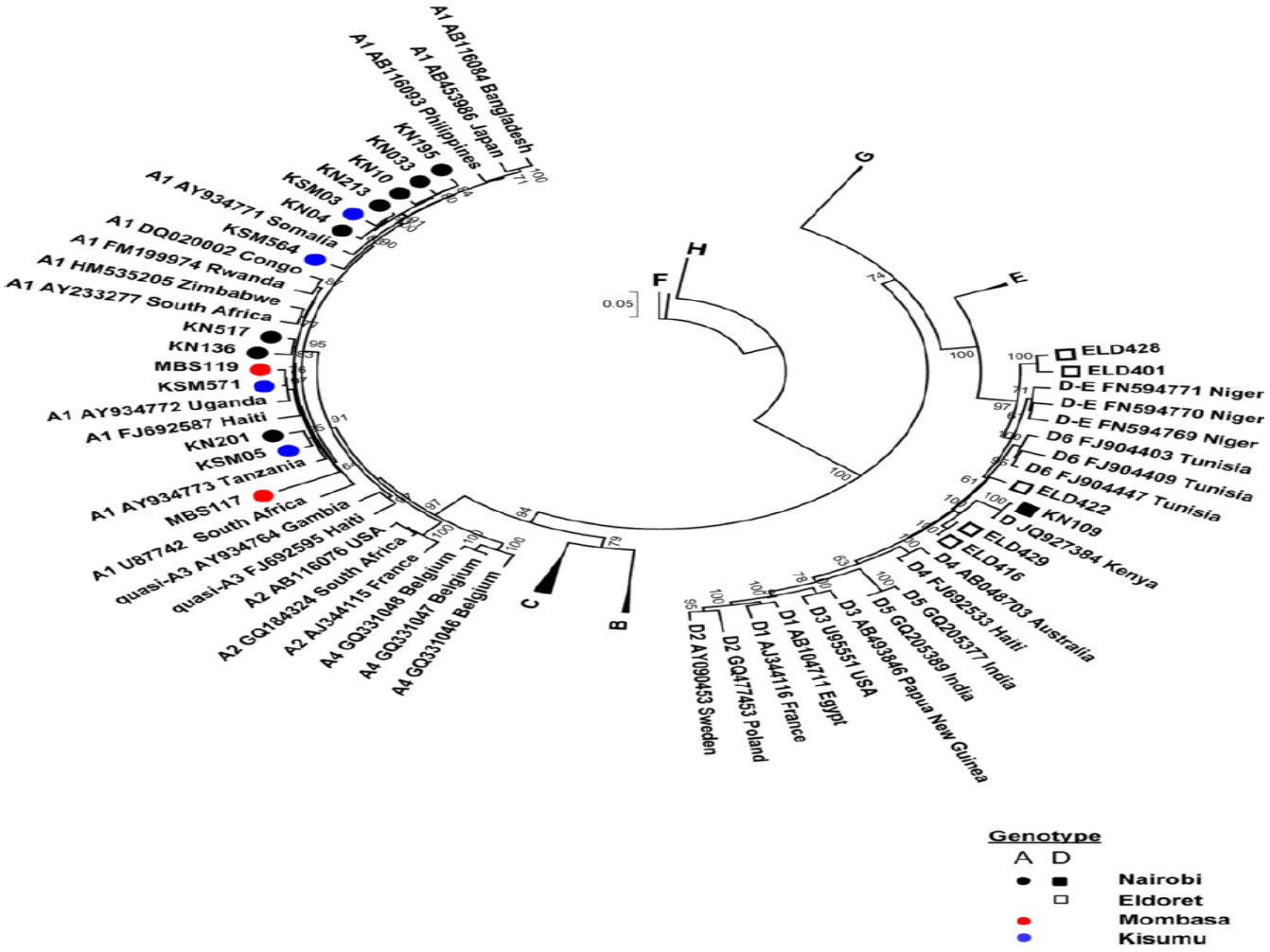


Figure 4.4: Maximum likelihood Phylogenetic analysis of 20 Kenyan full genome isolates

Four genotype D isolates namely KN109, ELD422, ELD429, and ELD416, did not cluster with the reference sequence for genotype D. instead they all clustered with a sequence GenBank

accession No. JQ927384 that was submitted from Kenya as genotype D6 strain (Fig. 4.4). Two isolates; ELD401 and ELD428 clustered separately from all other genotype D subgenotypes, indicating they could be different from the others HBV/D sequences. When the Nucleotide distance measurements was calculated for HBV genotype D(Table 4.2) was clear that isolates ELD422, ELD416, and ELD429 had mean pairwise nucleotide distance of less than 0.04 for sub-genotypes D4 and D6, and HBV D/E recombinant reference sequences. This suggested that these isolates resembled the reference sequences of D4, D6 or they could be recombinants of either, and that the isolates were very different from isolates D1, D2, D3 and D5. Isolate KN109 was a subtype D6 because it had a nucleotide distance identity of less than 0.04 that is falling within reference subgenotype D6 sequences. For isolate to be considered HBV genotype D6 or a D/E recombinant sequences it must contain amino acid rt237T according to Yousif and Kramvis, 2013 and the isolates considered genotype D6 had that signature amino acid rt237T (Yousif and Kramvis, 2013).

Table 4.2: A Mean pairwise nucleotide distances between genotype D sub genotypes and Kenyan complete genome genotype D sequences

		Genotype D subgenotype*						
		D1	**D2**	**D3**	**D4**	**D5**	**D6**	**D-E**
Genotype D full genome sequences	**ELD401**	**0.061**	**0.065**	**0.061**	**0.053**	**0.068**	**0.049**	**0.048**
	ELD416	**0.041**	**0.044**	0.038	0.032	**0.047**	0.030	0.033
	ELD422	**0.043**	**0.048**	**0.043**	0.033	**0.051**	0.033	0.033
	ELD428	**0.054**	**0.058**	**0.054**	**0.045**	**0.061**	**0.041**	**0.042**
	ELD429	**0.041**	**0.045**	0.038	0.032	**0.047**	0.031	0.034
	KN109	**0.050**	**0.053**	**0.051**	**0.044**	**0.058**	0.039	**0.047**

*GenBank reference sequences used were as follows: D1 (AF280817, AJ344116, AF151735, GU456684, AB104711), D2 (AY090453, X72702, GQ477453, JF754597, AB078032), D3 (U95551, AY233296, AJ627217, EU921419, AB493846), D4 (HQ700500, AB033559, AB048703, FJ692533, HE974378), D5 (GQ205377, GQ205385, GQ205389, AB033558, GQ205382), D6 (FJ904409, FJ904447, FJ904403, FJ904433, FJ904438, FJ904410, FJ904395, FJ904442, KF170740, JQ927384), D-E recombinant (FN594771, FN594769, FN594770); values in bold represent nucleotide diversity >4%.

Complete HBV genome of genotype D must have a 33nt deletion which is characteristic for HBV/D Figure 4.4b. sequence analysis of ELD401 and ELD428 revealed that despite the sequences having a resemblance to HBV/D they did not have the 33nt deletion (Figure 4.4b). Similar observation was also made on sequences from west Africa (Chekaraou *et al.,* 2010). The

deletion is as a result of a deletion of the preS1 start codon of genotype D. When Mean pairwise nucleotide distances was calculated it is interesting to note that both ELD428 and ELD401 full genome sequences had a nucleotide distance >4% from all HBV/D subgenotypes and D/E recombinant reference sequences. In HBV subgenotype classification, a sequence is categorized unique and new if the nucleotide distance is >4% (Norder *et al.,* 2004 and Kramvis *et al.,* 2008) (Table 4.2).

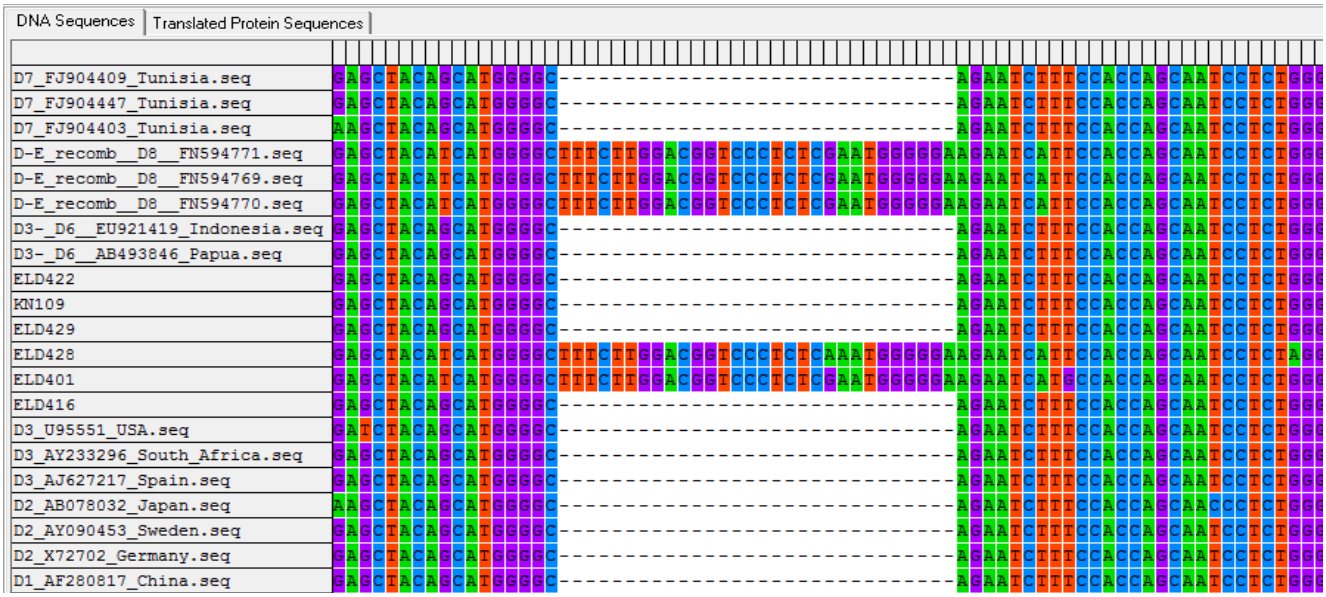
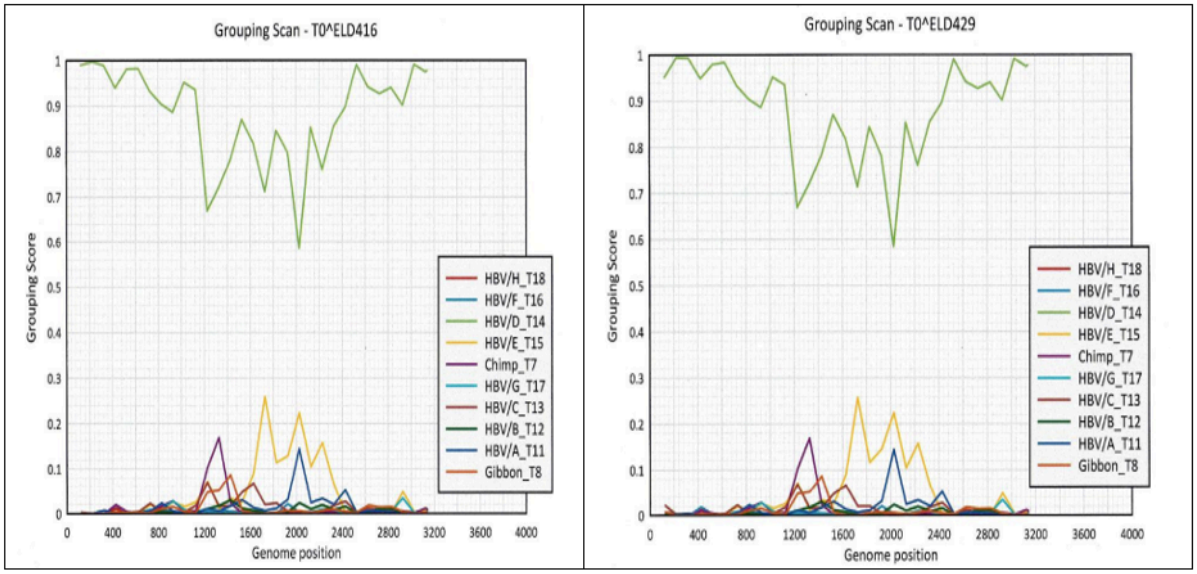


Figure 4.4b: HBV sequences of Genotype D showing two (ELD428 and ELD401) of Kenyan HBV isolates missing the 33nt deletion which is a characteristic for HBV/D

4.3Recombination Analysis

Since isolates of genotype D complete genome sequences never had a specific cluster and some had more than 4% nucleotide distance (Table 4.2) Recombination Simmonic softwarepackage(SSE v1.1) was used to conduct a recombination analysis with KN109, ELD429, ELD416 and ELD422, full genome sequences. It was observed that all the sequences (KN109, ELD429, ELD416 and ELD422) were genotype D and they did not have any recombination with other genotypes in their sequences (Figure 4.5(a) and (b))



The complete genome was scanned against GenBank reference sequences of human HBV genotypes A-H and chimpanzee- and gibbon-derived strains (n=295 sequences). Association values ≥0.5 indicate phylogenetic clustering with the specified genotype reference group, while those <0.5 indicate an out group position having no phylogenetic association with the genotype reference groups.

Figure 4.5 *Full genome Grouping Scan analysis of (a) ELD416 and (b) ELD429 showing no evidence of recombination*

Group scan recombination analysis of ELD428 and ELD401 showed that the isolates were putative recombination of genotype D and genotype E (D/E recombinants) (Figure 4.6(a) and 4.6(b)). Isolate ELD401 demonstrated having a recombination of grouping scan value approaching 50% with genotype A at nucleotide positions 1840 to 2240 (Figure 4.6a) and with Genotype E at nt 2800-3000 which happened to be within the preS1 region of the virus. These two sequences had similarity to previously described D/E recombinant isolates from west Africa (Chekaraou *et al*., 2010). It was equally observed that the two putative D/E recombinants had a nucleotide distance of >4% among recombinant sequences in GenBank (FN594769, FN594770, FN594771).

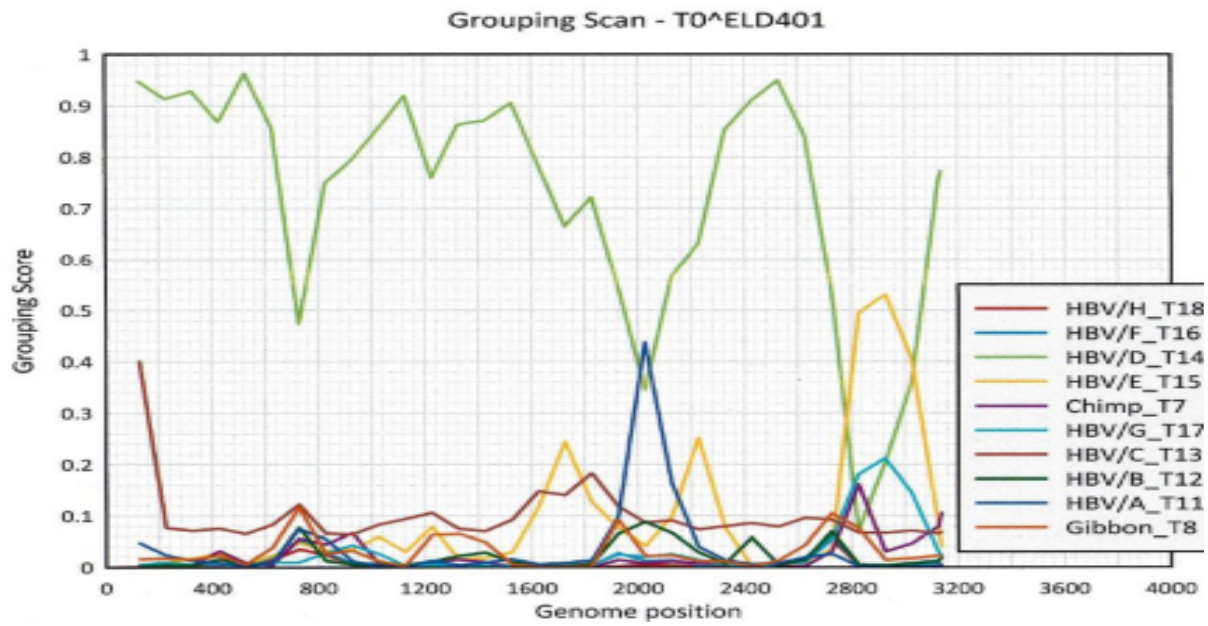


Figure 4.6(a) Grouping Scan analysis of the complete genome of ELD401(Green line) showing recombination at position 2000 with HBV genotype A and at position 2800 – 3200 with HBV genotype E.

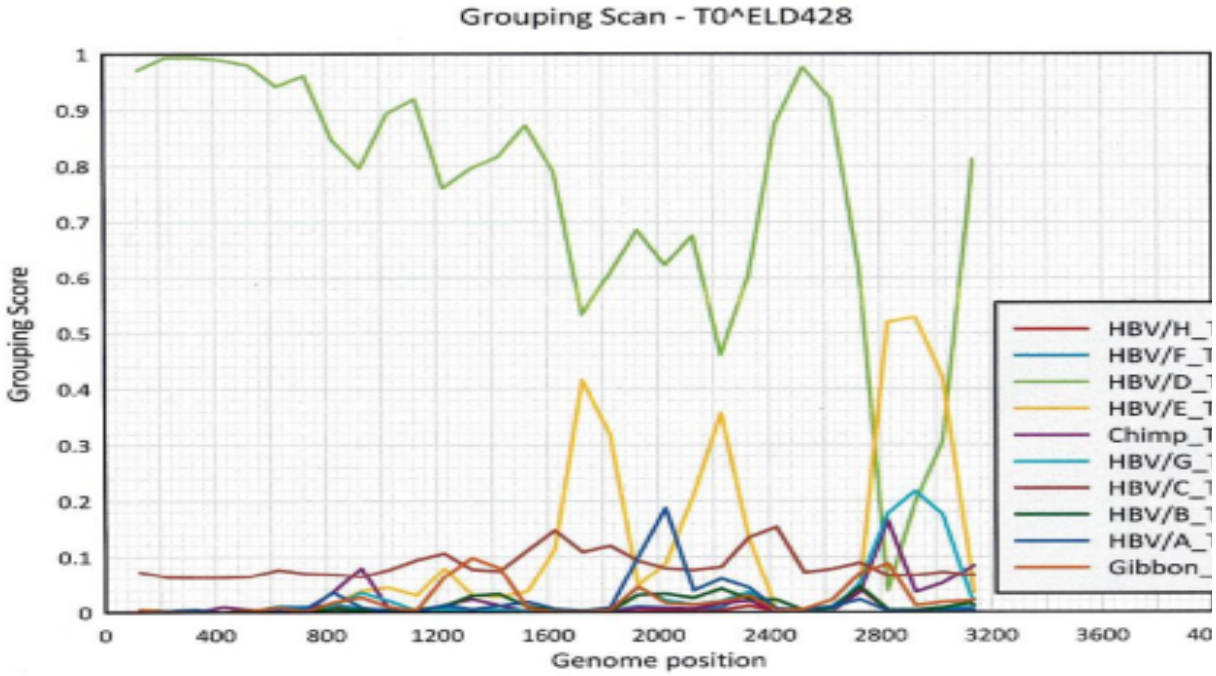


Figure 4.6: *Grouping Scan analysis of the complete genome of ELD401 and ELD428*

Isolate MBS117 also showed unique characteristics and did not cluster readily with Genotype A isolates. Grouping scan analysis of MBS117 showed a number of recombination at various regions of all the open reading frames. A significant recombination occurred at the PreS1 region (nt 2800-3000) with genotype D and Genotype E. It was not easy to categorize the recombination of the virus as at position 1600 there was recombination with genotype F, at position 600-700 and position 1000-1500 there was recombination with genotype B as shown in Fig. 4.7.

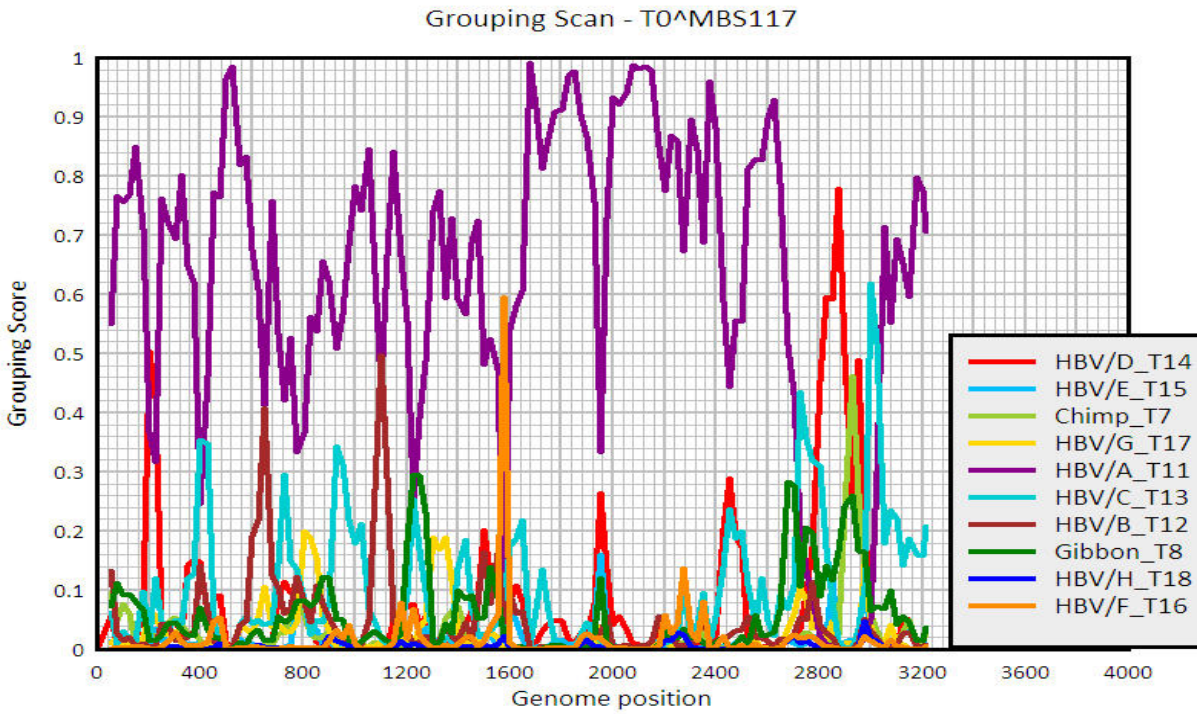


Figure 4.7: *Grouping Scan analysis of the complete genome of MBS 117 showing unique recombination of genotype A at multiple locations. The combination was complex at position 2600 to 3200*

4.4 Distribution of HBV genotypes in Kenya

The HBV genotypes A is most prevalent genotype in Kenya and it was observed in all facilities among the patients. Kisumu has only genotype A whereas Mombasa has genotype A but also there was a unique recombination with genotype E (MBS117 isolate). Genotype was followed by genotype D which according to our finding it found in Nairobi and Eldoret (North Western region). The putative recombinants and genotype E were only observed in Eldoret (Figure 4.8)

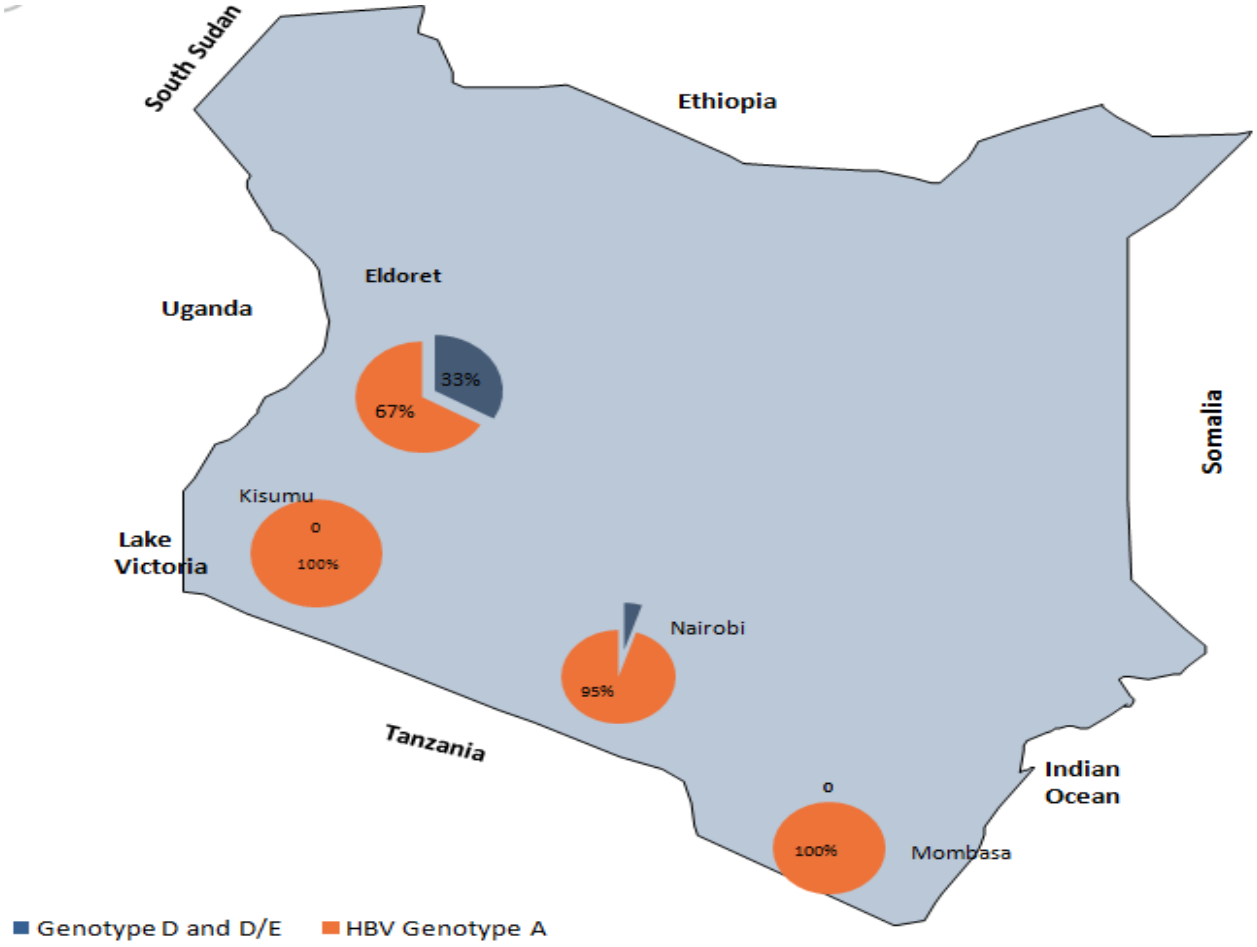


Figure 4.8*:showing Distribution of HBV genotypes in Kenya*

4.5Profiling Mutations in the HBV genome

4.5.1 BCP/PC region substitutions

Basal core promoter (BCP) and Precore (PC) sequences that could be analysed were 85 sequences. Genotype A had (76 samples) and 9 were genotype D and only one genotype E. Genotype E isolate results are indicated in table 4.4. Double mutation A1762T /G1764A was 33.0% in genotype A isolates and 44% in genotype D isoaltes. A higher proportion of mutations at BCP/PC insertion or deletion was observed in in 5/76 (7%) of genotype A and none in genotype D. Other mutations are as shown below (table 4.3)

Table 4.3: Prevalence of HBV substitutions/mutations observed among genotype A and D sequences

	Substitution/mutation(%)*	
Nucleotide mutation	**Genotype A**	**Genotype D**
HBsAg amino acid substitutions†	13/81 (16)	0
BCP/PC insertion/deletions‡	5/76 (7)	0
C1653T	2/76 (3)	3/9 (33)
A1752C	0	1/9 (11)
T1753V	3/76 (4)	2/9 (22)
A1762T/G1764A§	25/76 (33)	4/9 (44)
G1809T	70/76 (92)	0
A1811T/C	8/76 (11)	0
C1812T	66/76 (87)	0
C1858T	4/76 (5)	9/9 (100)
G1862T/C	27/76 (36)	0
G1888A	52/76 (68)	0
G1896A	3/76 (4)	4/9 (44)

*Genotype A: 81 and 76 samples could be amplified for HBsAg and BCP/PC sequences, respectively; Genotype D: Sequence was available in both regions for all 9 samples. †The following substitutions within the antigenic determinant region (amino acids 100-160) of the HBsAg gene were observed: M103I, L109P, I110L, G112R, T114S, T115A, T118K, Q129R, G130N, M133I, F134I/V, P135H, T143M, S155R; ‡see text; §includes A1762/A1764 (6 genotype A and 2 genotype D) and T1762/G1764 (3 genotype A).

Table 4.4 shows mutations that were observed in HBV/D and HBV/E isolates showing the co-existence of the mutation in an individual. Mutations on BCP and pre-core region (1750-1900) shown in the table include the double mutation A1762T and G1764A, 'Kozak' region mutation 1809-1812 TCAT, G1896A, G1862T, C1858T and G1888A. There was a unique nucleotide T insertion at position 1841of HBV/E isolate and another insertion of - - AACAATT - - that translated to ...PTI. amino acids at position 2698

4.5.2 Mutations and Co-existence with each other

There was a significant co-existence between mutations A1762T /G1764A and 1809-1812 (p=0.002), between A1762T /G1764A and 1858C (P= 0.007), between 1809-1812 and 1888 (χ^2, p= 0.004), and between 1862 and 1888. Four, (8.7%) of 10 patients with C1858T co-existed with G1896A. One patient with HCC had an additional mutation at position T1753C along with A1762T /G1764A, C1858T, G1896A and T1768A. Mutation G1899A always co-existed with G1896A. Mutant A1762T co-existed with mutant G1764A (double mutation), however, in three isolates, mutation G1764A existed alone without 1762 (single Mutation) (Table 4.4).

Table 4.4: BCP and Pre-Core Mutations of HBV/D

s. no	**Sample AMP**	**1753**	**1762, 1764**	**1802-1803**	**1809-1812**	**1814, 1816**		**1858**	**1862**	**1884**	**1888**	**1896**	**1899**
1	AMP4047_D1	T	AG	CG	GTAC	TG		T	G	T	G	A	G
2	AMP4070_D6	T	AG	CG	GCAC	AT		T	G	T	G	A	A
3	AMP4226_D1	T	AG	CG	GCAC	AG		T	G	T	G	G	G
4	AMP4071_D6	T	AG	CG	GCAC	AT		T	G	T	G	A	A
5	AMP4167_D6	G	TA	CG	GCAC	AA		T	G	T	G	A	A
6	AMP4275_D/E	T	TA	CG	GCAC	AG		T	G	T	G	A	G
7	AMP4020_E	T	AG	CG	GCAC	AG	T INSERTION AT 1841	C	G	Y	G	G	G

4.5.3 HBsAg-coding region substitutions

Mutation observed in the Pre-S2 and in the S region of genotype D did not follow any specific pattern, each patient had distinct mutations unlike those observed in genotype A. Genotype D and E had very minimal mutation as compared to genotype A. All HBV genotype D were *Ayw2* serotype. One patient did not have any mutation on the Pre-S/S Region. One patient (AMP4047) had R18X, A39V and P41H, the second (AMP4167) had F22L, P41H and D51V, another one (AMP4226) had A39V and P52L and the last had four mutations T37S, T38S, R48K and L54X (Table 4.5)

In the S-gene of the HBV/D patients, two patients did not have any mutation, but in the four remaining patients, one patient (AMP4047) had only one mutation; G10X, another one (AMP4070) had two mutations R79H and S204T whereas patient AMP4275 had four mutations V15A, C69X, S114T and L213I and finally AMP4226 had seven mutations I4V, T23E, L26H, Q30X, D33N, S204I S207R (Table 4.5)

No known mutations associated with occult or vaccine escape mutants were observed in genotype D.

Table 4.5: Mutations in Pre-S, S and Polymerase regions of HBV/D

No.	Isolates	Genotype (serotype)	Pre-S2	S- gene	Polymerase region
1	AMP4047	D1(Ayw2)	R18X, A39V, P41H	G10X,	R18X
3	AMP4226	D1(Ayw2)	A39V, P52L	I4V, T23E, L26H, Q30X, D33N, S204I S207R	H12R, D31G, E39X, R41K, V43L, K212N
2	AMP4070	D6(Ayw2)	None	R79H, S204T,	Q149K, R153W, P237T, Q267H
4	AMP4071	D6(Ayw2)	None	None	V27I, Q149K, R153W, P237T, Q267H, I254D, G255X,
5	AMP4167	D6(Ayw2)	F22L, P41H, D51V,		Q149K, R153W, P237T, Q267H
6	AMP4275	D/E(Ayw2)	T37S, T38S, R48K and L54X	V15A, C69X, S114T and L213I	A7X, S78T, F122H, H124N, M129L and V191I
7	AMP4020	E(Ayw4)	None	None	None

4.5.4 Mutation Frequency within the Pol region of Genotype D isolates

HBV subtype D1 did not show any distinctive amino-acids as compared to other D1 subtypes. Isolates of subtype D6 there was distinctive amino acids in POL open reading frame. The isolates had K149, W153, T237 and H267 instead of Q149, R153, P237 and Q267 usually found in other subtypes (Table 4.5).

4.6 Characterization of Occult Hepatitis B (OBI) among Jaundiced Population in Kenya

Sixty-seven specimens previously determined to be HBsAg negative were selected for investigation of OBI. The findings are as shown in figure 4.9.

All specimens determined to be HBV DNA positive by real-time PCR underwent nested PCR for sequence analysis. The single OBI specimen from the jaundiced (12/19; 63.2%) OBI specimens provided sufficient amplicon following nested PCR for sequence analysis, likely due to the very low HBV viral load levels consistently observed with occult infection (Locarnini and Raimondo, 2018). The resulting sequences aligned with reference sequences representing subgenotypes of HBV from GenBank and were subjected to phylogenetic analysis. All study sequences were determined to be genotype A. (GenBank accession no. **MK487133-MK487155**).

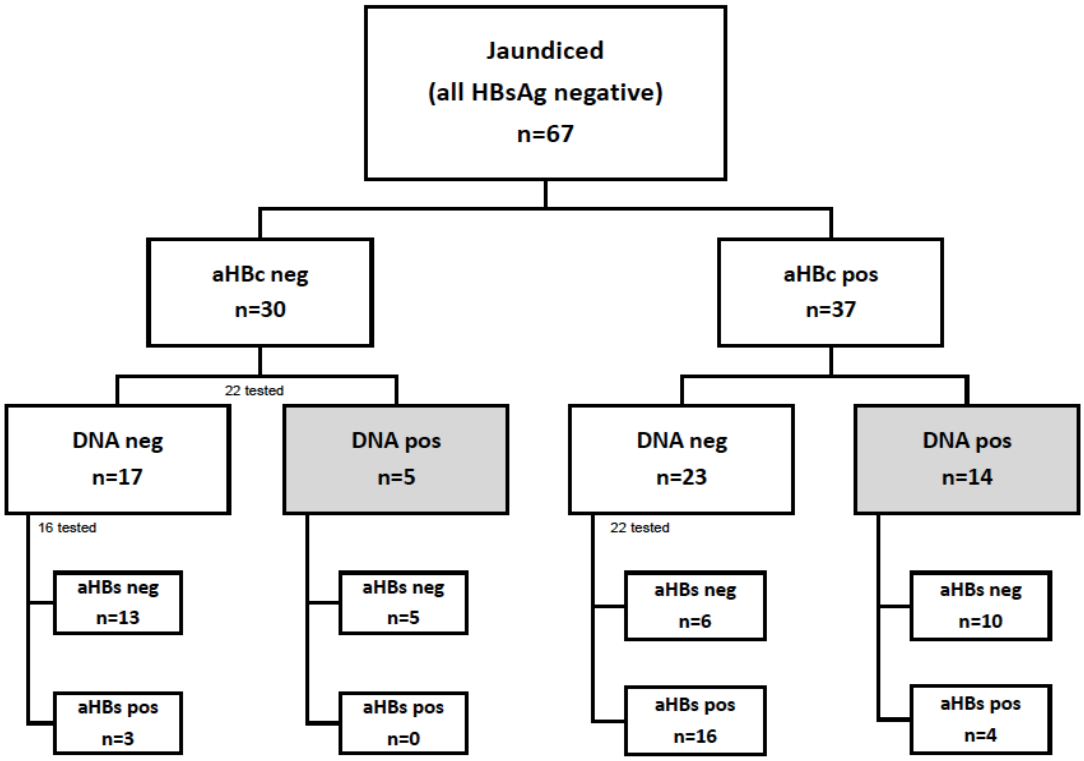


Figure 4.9 *Schematic flow of the occult samples tested*

Definitive classification of the sub-genotype of each sequence could not be determined due to sub-genomic region analysis (Pourkarim et al. 2014). The sequences from the patients who were jaundiced were similar to sub genotype A1 although this was not necessarily established by branch support. The patients had mutations that were associated with impaired HBsAg or virion secretion (Zhu et al. 2016) were equally observed among the OBI sequences.

4.7 Objective 2 and 3: To characterize human Host P53gene among the patients attending selected hospitals in Kenya and determine its association with chronic HBV

4.8 Demographic of Participant demographics

The total number of patients that were positive for HBsAg, chronically infected and had clear sequence results for primers exon 4, 6 and 7 were 33. Their demographic characteristics are as shown in table 4.6. there was no difference in gender as the number of male to female was almost equal; 17:16 (51.5%:48.5%). Out of the 33 patients 25(75.8%) were having hepatocellular carcinoma (HCC), chronically infected and positive for HBsAg and the remaining 8(24.2%) were

without HCC but were positive for HBsAg. Among the HCC patients, 48.0% were female and 52.0% were male and among the non-HCC patients the male to female ratio was one to one (1:1).

Table 4.6. Clinical and molecular characteristics of the subjects

Characteristics		**Participants**	
		n	**Frequency, %**
Gender	Male	17	51.5
	Female	16	48.5
HCC status	HCC	25	75.8%
	Without HCC	8	24.2%
Codon 72 Polymorphisms	Arg/Arg	4	12.1
	Pro/Pro	17	51.5
	Pro/Arg	12	36.4
Ser 249 mutation	AGG (Arg)	25	75.8%
	AGT (Ser)	8	24.2%

4.9 TP53 polymorphism at Exon 4 codon 72

Out of the 33 samples that were amplified with clear sequences for forward and reverse primers for exon 4 codon 72, the most common allele frequency 54.5% was homozygous proline (P) (CCC), as compared to 12.1% who were homozygous Arg/Arg (R) (CGC) and the least common. Other patients had the heterozygous Arg/Pro (33.3%) indicating that the patients had both the Proline and arginine in either the forward or reverse strand (Figure 4.10). It was observed that all patients who were homozygous Arg/Arg were also having HCC

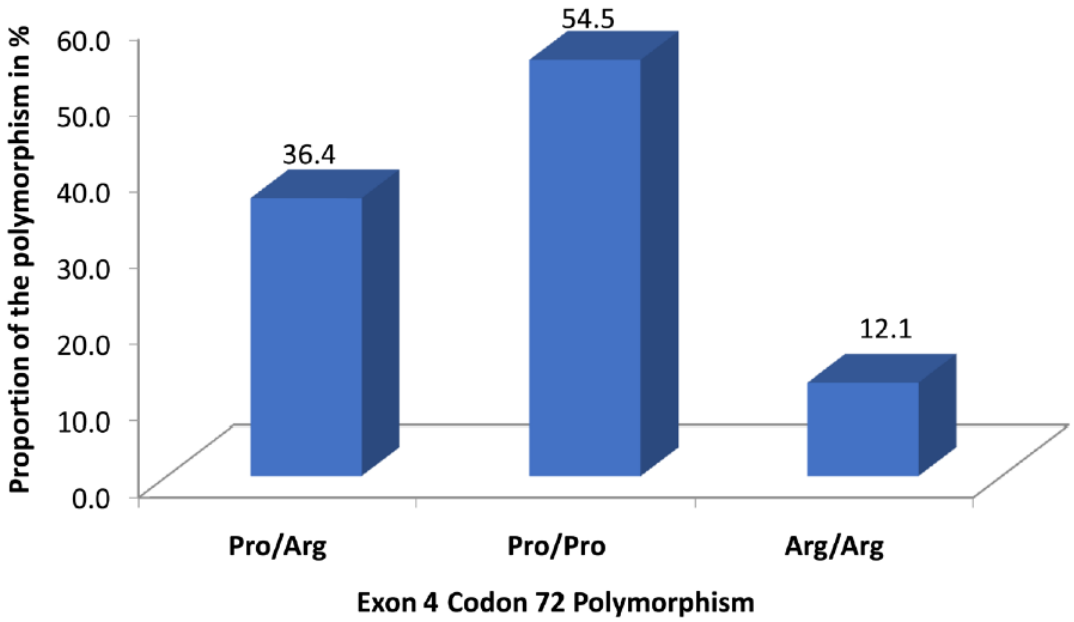


Figure 4.10: *codon 72 Allele polymorphism frequencies of the selected patients*

Various polymorphisms combinations were observed When the sex of the subject was compared to the codon polymorphisms. All Male patients had homozygous Arg/Arg whereas 64.7% female patients had homozygous Pro/Pro (Table 4.8). Among heterozygous Pro/Arg More male were heterozygous Pro/Arg than female (58.3% vs 41.7%). It was observed that there was a statistically significant association (Fisher test=5.4 and p=0.04),

Most patients (88.2%) who had HCC also had homozygous Pro/Pro polymorphism at TP53 codon 72. Among the HCC patients Pro/Arg and homozygous Arg/Arg polymorphism were 66.6% and 50.0% respectively (Table 4.7). However, there was no association between patients who had the HCC and the TP53 polymorphisms observed (Fisher test=3.58 and p=0.12) Table 4.7)

Table 4.7 Association between p53 Codon 7 polymorphisms, gender and HCC

Polymorphisms	**Female**	**Male**	**Fisher value**	**P-value**
Arg/Arg	0	4 (100%)	5.4	0.04
Pro/Pro	11 (64.7%)	6 (35.3%)		
Pro/Arg	5 (41.7%)	7 (58.3%)		
Polymorphisms	**Without HCC**	**HCC**	**Fisher value**	**P-value**
Pro/Pro	2 (11.7%)	15 (88.2%)	3.58	0.12
Pro/Arg	4 (33.3%)	8 (66.6%)		
Arg/Arg	2 (50.0%)	2 (50.0%)		

When codon polymorphisms of patients who had HCC were compared with those without the HCC it was observed that homozygous Pro/Pro allele was more frequent in the HCC group and patients wth Arg/Arg alleles were without HCC (Table 4.8), although there was no significant association between P53 arg72 Pro/Arg and the patients with or without HCC Fisher Test=0.68 P=0.57 (table 4.8).

Table 4.8: Association between gender, and allele polymorphism at *TP53* Arg72.

Polymorphisms	**Sex**	**HCC status, n (%)**		**Total**	**Fisher value**	**P-value**
		Without HCC	**HCC**			
Arg/Arg	Male	2 (50.0%)	2 (50.0%)	4	-	-
	Female	-	-	-		
	Total	2 (50.0%)	2 (50.0%)	4 (100%)		
Pro/Arg	Female	2 (40.0%)	3 (60.0%)	5 (100.0%)	0.68	0.57
	Male	2 (28.6%)	5 (71.4%)	7 (100.0%)		
	Total	4 (33.3%)	8 (66.7%)	12 (100.0%)		
Pro/Pro	Female	2 (18.2%)	9 (81.9%)	11 (100.0%)	0.27	0.40
	Male	-	6 (100.0%)	6 (100.0%)		
	Total	2 (11.8%)	15 (88.2%)	17 (100.0%)		

4.10 Mutations at exon 7, codon 249

Majority 24/33 (75.8%) of the patients who were positive for HBsAg and amplified at exon 7, did not have serine 249 mutation, only eight (24.2%) had this mutation where arginine amino acid was substituted with serine (Arg→Ser) at codon 249. Out of the eight who had the mutation, there were more males (87.5%) than females (12.5%). It was observed that gender and serine mutation at codon 249 of TP53 exon 7 were not associated. (Fisher's exact test =5.47, P-value=0.039) and

males were at higher risk of having the mutation compared to females (OR=10.5, 95% CI =1.1-98.9%) (Table 4.9 and figure 4.11 (a) and (b)

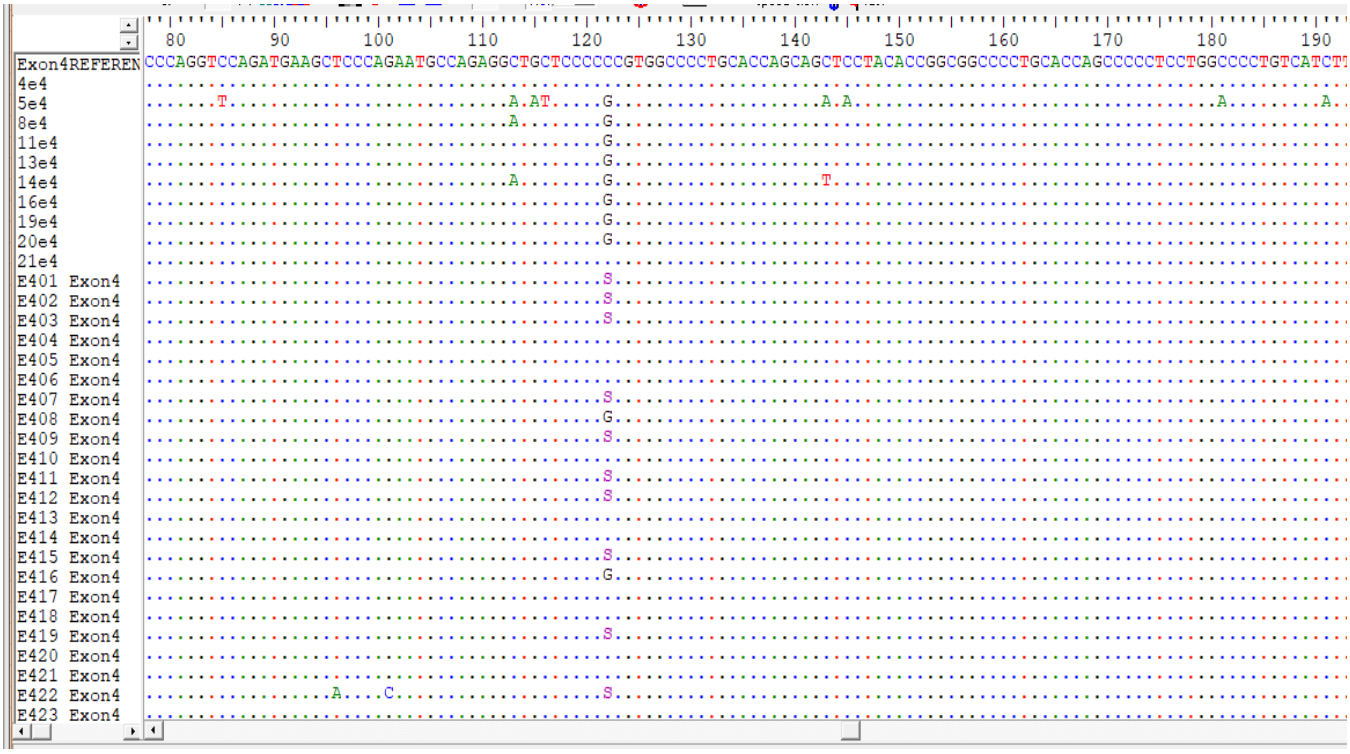


Figure** 4.11(a): **A figure showing sequenced Nucleotides at codon 72:
*Sequence CCCC**C**CGTGGCCCCT to represent - **proline** (CCC), Sequence CCCC**G**CGTGGCCCCT to represent - **Arginine** (CGC) and CCCC**S**CGTGGCCCCT to represent a mixture of C and S at codon 72.*

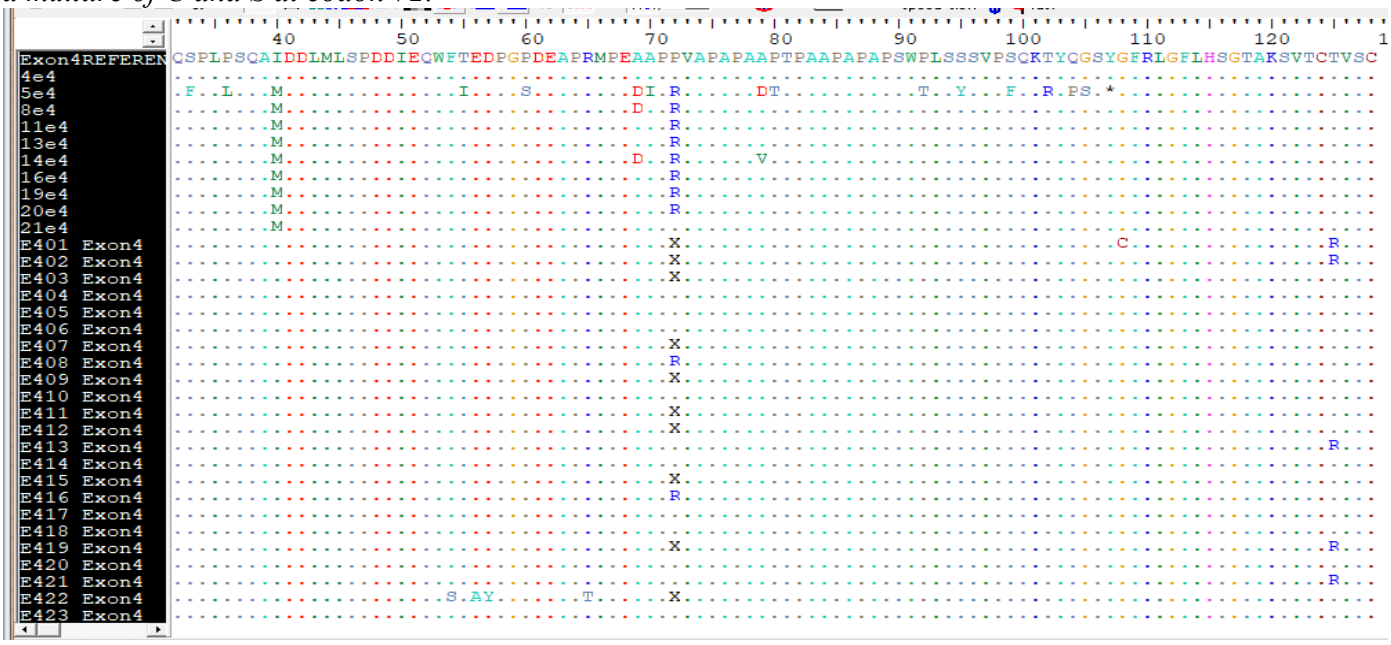


Figure 4.11(b): A figure showing sequenced amino acids at codon 72

Sequence EAAPPVAPAPA to represent - ***Proline*** *(P), Sequence EAAPRVAPAPA to represent -* ***Arginine*** *(R) and EAAPXVAPAPA to represent a mixture of* ***Proline*** *and* ***Arginine*** *at codon 72.*

Table 4.9HCC status and Codon 249 mutation

Variable		Codon 249 mutation		Total
		AGG	AGT	
Sex	Female	15 (93.8%)	1(6.20%)	
	Male	10(58.8%)	7(41.2%)	
HCC status	Acute Hepatitis	6(24.0%)	2 (25.0%)	8 (24.0%)
	Chronic Hepatitis	19(76.0%)	6(75.0%)	25(76.0%
	Total	25(100.0%)	8(100.0%)	33(100.0%)

Mutation: substitution of nucleotide Guanine (G)-to-Thymine (T) in the third base of codon 249 of *TP53* gene resulting to change of Arginine (Arg) amino acid to Serine (Ser) amino acid.

4.11 Codon 249 mutation and HCC

Majority 6(75.0%) of the patients with Ser-249 mutation were having HCC and a very small proportion 2(25.0%) of those with the mutation did not have HCC. On the other hand, among the patients without ser-249 mutation, 76.0% of them had HCC and only 6(24.0%) did not have HCC. When the presence of Ser-249 mutation at codon 249 was compared with HCC status of the patient (with or without HCC) the results showed that there was no significant association (Fisher's exact test =4.23, P-value=0.15).

CHAPTER 4: DISCUSSION

Kenya as a country is geographical located at a junction of HBV genotypes with genotype A to the south and west, genotype D to the northern and E to the Northern and Western. All these genotype contribute significantly to the genotypes in Kenya (Ochwoto *et al.,* 2013 and Kwange *et al.,* 2013 and Mwangi *et al.,*2008).This study, successfully characterized 132 HBV positive samples, from patients attending 3 main referral hospitals selected in Kenya.

The main HBV genotype circulating in Kenya is Genotype then, genotype D and finally genotype E. Genotype A was distributed throughout the country whereas genotypes D and E were mainly observed in western regions of Kenya, among samples collected in Eldoret (figure 4.8). This finding are comparable to previous findings that showed genotype A to be predominant in Kenya followed by genotypes D and E. Subgenotype D6 has been reported among liver patients (Ochwoto *et al.,* 2013) and subgenotypes D4 has been reported among blood donor (Mwangi *et al.,* 2008, Kwange *et al.,* 2013 Ochwoto *et al.,* 2013).

Although Genotype A1 remain the major HBV genotype in the country among patients and blood donors (Nyairo *et al.,* 2016, Kwange *et al.,* 2013 and Mwangi *et al.,* 2008). Studies conducted in the southern part of the country show that the only genotype in circulation is genotype A (Nina Kim*et al*., 2011., Kibaya *et al.,* 2015, Nyairo *et al.,* 2016 and Mabeya *et al.,* 2017) which agree with the prodeminant genotypes in the neighbouring countries: Tanzania and Uganda (Forbi *et al.,* 2013 and Forbi *et al.,* 2017). In this study it was observed that the prevalence of HBV/D and HBV/E increases as you move towards the North near Ethiopia and South Sudan where the predominant HBV Genotypes are D and E. (Mahgoub *et al.,* 2011, Hundie *et al.,* 2017). We observed that the HBV virus could have crossed the borders from or to the neighbouring countries (Tatematsu*et al.,* 2009, Norder *et al.,* 2004 and Kramvis *et al.,* 2008).

Recombination of HBV genotype is one of the ways of forming a unique subgenotype. Recombinant strains may develop following HBV intergenotype dual infections or coinfection of subgenotypes (Ding *et al.,* 2001 and Hannoun *et al.,* 2002). The formed recombinant may be not be able to withstand the host immunity and therefore extinct or it can survive and persist as a predominant circulating strain in a population (Hannoun *et al.,* 2002). In this study, only three samples were recombinants of D/E (ELD 401, ELD 428 and AMP4275 . On the other hand genotype A variant having putative recombination with genotype E and/or D (MBS117) collected

from coastal Kenya, was also observed within the study population. The A/E recombinant had multiple insertions of other genotypes. All the observed Kenyan recombinants have fragments of genotype E in their genome. Interestingly, the proportion of genotype E in the jaundice population was minimal. Genotype A and D are often aggressive as compared to genotype E, a reason that may explain its low proportion among chronic population, but a high proportion of its recombinants. Among blood donors Mwangi et al 2008 observed genotype E who were asymptomatic (Mwangi *et al.,* 2008). Genotype E is the most reserved genotype without any subgenotype (Yousif and Kramvis, 2012), however, in this study we observed it to be easily combined with other genotypes to form recombinants.

In Kenya HBV genotype A strains are all of subgenotype A1. Analysis of HBV genotype D strains indicated that they are subgenotypes D6. This was confirmed since D6 characteristic amino acid signature; E161 of the polymerase terminal protein was observed in the study sequences (Yousif and Kramvis, 2012). Although the same sequences showed close similarity with D4, the subgenotype D4 signature V161 amino acid was missing in the sequences. (Yousif and Kramvis, 2012).

Viruses co-evolve within human beings and continue to diverge; this is one of the key successes of the viral infections and mutation (Geoghegan *et al.,* 2017). In HBV, mutation rates are due to the HBV replicative strategy that includes viral fitness and genetic barrier of the drugs (Caligiuri *et al.,* 2016). HBV has a mutation rate estimated to be 2×10^4 base substitutions/site/year. (Yano *et al.,* 2015) and can be even higher (Osiowy *et al.,* 2006). The mutations occur throughout the genome because of high viral replication rate, overlapping reading framesand it contains a polymerase that lacks a proofreading activity, (Caligiuri *et al.,* 2016). The data presented in this study showed a higher number of mutations in Genotype A isolates as compared with the genotype D and E isolates in the PreS/S and overlapping S gene. Despite the fact that all sequences were obtained from Chronic HBV patients, these finding may not be conclusive due to low number of sequences obtained for HBV/D genotype. Genotype E has the least divergence in all the HBV genotypes and it has no other known genotypes. This is first HBV/E full genome to be isolated in Kenya and there were very minimal mutations on genotype E isolate

Some mutations among HBV genotype C, have been associated with elevated ALT levels, such as A1762T/G1764A and T1753V (Liu *et al.,* 2008) but this study did not find such association. The

ALT levels in most patients were normal except for mutations G1888T and G1862T that registered high frequencies. More research is needed to clarify the effect of these mutations to ALT levels.

On BCP/pre-core the nucleotide exchange mutation of prominence were double A1762T (A=T; changing lysine to methionine) and G1764A (G=A; changing valine to isoleucine), which have also been detected from other parts of the world in severe liver disease patients (Kramvis and Kew 1999). The,T1762/A1764 mutation was observed in isolates of genotype A and D, the .prevalence of this double mutation was comparable to that in other studies (Yuan *et al.,* 2009, Chen *et al*., 2012). Several reports suggests that, double mutation A1762T/ G1764A is associated with HCC and liver diseases, In South Africa it accounts for 66% (Baptista *et al.,* 2003) in Chinese it accounts for 72% (Chen *et al.,* 2000) and in patients with chronic hepatitis it has been often reported (Kramvis and Kew, 1999; Liu *et al.,* 2008) The mutation is also associated with reduced expression of HBeAg but enhanced viral genome replication (Buckwold *et al.,* 1996, Tong *et al.,* 2007, Pang *et al.,* 2004, Chauhan*et al.,* 2006). In our study population, there were both HBeAg positive and negative patients, this could indicate that the mutation emerges before or during HBeAg sero–conversion. Other studies found out that the mutation emerges sometimes earlier before the onset of HCC (Guo *et al.,* 2008).

A number of mutations on the polymerase region were observed in this study. All patients in this study did not have any known primary or secondary drug resistance mutations (rt184 and rt204) or (rt80 and rt180) in their Polymerase gene. Previous studies by Nina Kim et al working among HIV co-infected patients reported similar finding before the initiation of treatment (Nina Kim et al 2011). The resistance to treatment occurred during and after treatment as observed by Nina Kim's and Mabeya's study (Mabeya *et al.,* 2017). None of the patients that was on nucleos (t) ide analogues treatment due to resource limitations, the patients' result on biochemical marker (ALT or AST) test could not be accessed in some health facilities. Elsewhere in the world patients naive to nucleos (t) ide analogues treatments have reported Hepatitis B virus resistance (Zoulim, and Locarnini 2009) and Kim *et al.,* 2017). The mutations reported in this study have never been associated with dug resistance and their effect on hepatitis B polymerase protein either directly or indirectly remain unknown.

Occult hepatitis B infection (OBI) is when Hepatitis B virus is present in the liver or serum but the HBsAg or other serological markers of HBV infection or exposure cannot be detected (Wu *et al*. 2017). This is the first description of occult hepatitis B among jaundiced populations of Kenya.

The findings of this study confirm the high prevalence of HBsAg positivity in jaundiced with nearly a third of the cohort demonstrating past exposure (aHBc positive, HBsAg negative). The presence of occult is usually associated with very low serum DNA viral loads of less than 2000 U/mL (Locarnini and Raimondo, 2018). In some patients the presence of HBsAg may be below the diagnostic detection limit (Satoh et al. 2008)**.** The risk of OBI is dependent on various factors including the sensitivity or specificity of the detection kit, the HBV prevalence in that population and whether the person is HIV co-infected among other factors (Zhu et al. 2016). Consistent with the endemic prevalence of HBV in Africa, high rates (>6%) of OBI have been reported, in Nigerian blood donors (Oluyinka *et al.* 2015, Akintule *et al.* 2018) and HIV positive patients in Botswana (Ryan et al. 2017) or Cameroon (Gachara *et al.* 2017).Therefore the OBI rate of 19.6% (27/138) among all specimens analyzed in the present study is not entirely surprising considering the population tested comprised individuals at high risk of HBV infection.

This is the first study in Kenya on human TP53 polymorphisms at exon 4 and 7 of Hepatitis positive patients in a midst of the controversy regarding the association between TP53 and HCC Studies focusing at codon 72 demonstrate two different wild-type structures of allele that differ in their biological and biochemical characteristics (Thomas et al., 1999). One structure contain proline (Pro) and the other one Arginine (Arg). The prevalence of these different structures differ from country to country and from study to the other. A number of studies have associated one form of the structure to HCC whereas other studies show no association at all. In Kenya this study found that among patients with hepatitis B the most common structure was homozygous Pro/Pro genotype with 54.5% then heterozygous pro/arg and the least is homozygous Arg/Arg. Similar results have been reported among the Taiwanese, Egyptian and Chinese populations (Mah *et al.,* 2011, Neamatallah *et al.,* 2014 and Wang *et al.,* 1999). In the Kenyan population, we observed that most patients with Pro/Pro (88.2%) also had HCC a similar observation made among Moroccan population (Ezzikouri *et al.,* 2010) and Egyptian HCV patients (Koushik *et al.,* 2004; Neamatallah*et al.,* 2014).

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At TP53 codon 249 in DNA isolated the study showed that substitution of guanine (G) - to - thymine (T) in the third base of codon 249 corresponding to arginine-to-serine substitution was not associated with HCC in the Kenyan population. Similar findings have been reported among Guangxi, Taiwan and Gambian populations where similar mutations were reported (Mah*et al*.,

2011; Özdemir*et al*., 2010). Contrary results have been reported among the European studies indicating absence of this mutation among their population. (Kirk *et al*., 2000). Some developed countries like Japan, United States, Australia and China, the presence of the mutation is remarkably low (Bruix*et al*.,2011; Montesano *et al*., 2010; Özdemir*et al*., 2010). There are a number of factors reported by Genetic of cancer - National Cancer Institute (2017), that may contribute to absence or presence of the mutation; ethnicity, differences in geographical location, hereditary disorders, and excessive exposure to mutation-inducing agents as well as study size. These factors may explain the differences observed in our Kenyan population and the European studies (National Cancer Institute (2017). Some studies have shown association of 249mutation with HCC however our study did not find such association (Fisher's exact test =4.23, P-value=0.15) however, the presence of codon 249 mutation might be considered a predisposing factor for HCC (OR=0.5278: 95% CI 0.0584-4.7736). Similar results have been reported in Taiwan, United states, Japan, Australia, Gambian and Guangxi populations (Mah*et al*., 2011; Montesano *et al*., 2010; Özdemir*et al*., 2010; Stern *et al*., 2001; Kirk *et al*., 2000). In Gambia, the presence of the mutation was highly associated with HCC and it was considered a promising candidate for HCC early diagnosis. This study observed that more male than female had the mutation categories both in HCC subjects and non-HCC. This may explain the possible reason for male having severe HCC and progressing faster to last phase of HCC as compared to females counterparts (Li *et al.,* 2016). There was an association between gender and ser-249 mutation in the Kenyan population.

Studies by Kirk *et al.* (2000) and Ozturk *et al*. (1994) have observed the presence of codon 249 mutation in both HCC and non-HCC. In our Kenyan population similar results were observed. A possible explanation of such finding would be that generally, it is established that codon 249 is an important hotspot for aflatoxin B1 (AFB1) modification and AFB1-induced mutation for patients residing in high-risk regions, where chronic hepatitis B virus (HBV) as well as hepatitis C (HCV) infections and exposure to dietary aflatoxin B1 are endemic (Özdemir*et al*., 2010, Adjiri, 2017).. In the report compiled by the International Agency for Research on cancer (IARC) *p53* mutation database (http://www.iarc.fr/p53), shows that up to 66% of codon 249 mutations occur in patients with HCC originating from regions with a high incidence of HCC and high exposure to dietary AFB1. This study did not test the presence or absence of aflatoxin or their exposure among the patients with jaundice. This array of probable analysis may perhaps implicate that the existence of the mutation in one of the controls paralleled excessive exposure to aflatoxin B1 and chronic HBV

infection. Alternatively, it may be suggestive of an early genetic event in hepatocellular carcinogenesis.

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

5.1: Conclusion

A number of mutations have been observed in this study in almost all the four open Reading Frame (ORF), their Diversity in the country should be considered when designing prevention, diagnosis and treatment program interventions for HBV.

OBI was determined to be highly prevalent among jaundiced patients in Kenya presenting for medical care (32.2%). OBI was significantly associated with a HBc positive status and a lower mean a HBs titre in jaundiced patients.

5.2 Recommendation and way Forward

The clinical manifestations and disease progression of the recombinant strains identified in this study remain poorly documented. Genotype A/E recombinants are rare; and as such, the impact of such a recombinant; A/E and D/E variant needs to be assessed.

A number of mutations and molecular diversity have been observed in this study in almost all the four open Reading Frame (ORF), we recommend that HBV Diversity in the country should be considered when designing interventions for HBV.

The study investigated exons 4 and 7 it will be of interest to document the polymorphism other exons of TP53, understand their diversity and to properly understand the TP53 gene polymorphism and its association with the Hepatocelullar carcinoma. There is equally a need to explore other tumor suppressor molecular markers with a high risk of recurrence or disease progression.

The mutation reported for TP53 and HBV in this study were from patients' blood serum. Frozen fixed liver tissues from the cancer patients did not amplify at all and we did not obtain fresh tumor tissues from the patients for verification. We therefore recommend a similar study with fresh liver tissues for comparison.

5.3 Limitation of the study

This study was a cross sectional study where the sample was only taken once from the participant. It was therefore not possible to determine when the mutation occurred, whether it was before or after the onset of Hepatitis Chronic infection.

We never expected a low incidence HBV that was at acute infection stage, This observation was because the participants had jaundice due to symptomatic flares of chronic infection. The participants may have missed to test during acute infection may be because of poor healthcare seeking behaviour (Burton, *et al.,* 2011).

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5.0 Appendices

Appendix 1 (a): Consent Form

Title of the study: MOLECULAR CHARACTERIZATION OF ACUTE AND CHRONIC HEPATITIS B VIRUS AMONG PATIENTS ATTENDING SELECTED HOSPITALS IN KENYA

CONSENT FORM

Good morning/ evening?

My name is **Missiani Ochwoto** from the Kenya Medical Research Institute (KEMRI) and university of Nairobi. I am currently carrying out a Viral Hepatitis study among patients with jaundice attending selected hospitals in Kenya, in order to find out their risk factors and how many person are infected (prevalence) and their impact to cause cancer of the liver (Hepatocellular Carcinoma). The viruses that cause hepatitis B can stay in the body shortly (acute) or for many months/year (chronic) leading to cancer of the liver.

How many people will take part in the study? This study will involve 350 patients attending hospital with liver disease infection or liver cancer or liver related complications (jaundice).

Procedure: Participation in the study is voluntary. If you choose to participate, I will provide a questionnaire for you to fill to the best of your knowledge. The answer you give to these questions will be kept completely confidential and will only be accessed by the principal investigators. To ensure confidentiality, we will not use your name anywhere. The questionnaires are coded and only your Hospital admission number (OP) will be used to relay the results to you. You can answer all questions in the questionnaire, but In case there is a question that you are not comfortable to answer you have the rights to do so.

In addition to the questionnaire, we will also take approximately 7mL of blood sample from your blood vein to be used for testing whether you are infected with which hepatitis later we will find the strain or the type of the Hepatitis and their quantities in your body.

What are the risks of the study? There is no known risk in participating in this study except the slight pain that you will experience when drawing blood.

What are the costs? There will be no costs for the participants in the study. It is FREE.

What are the benefits in taking part in the study? The overall results will be used to advice the public and the government health sector on the impact of Hepatitis and Liver Cancer.

How long will the study take? The study is projected to take two to three years. For viral hepatitis disease progression the patient will be followed for 24 months (2 years) where blood sample will be drawn at interval of three or four months up to a minimum of four times. The results will be relayed to the patient each time for treatment purposes. Once you begin the exercise and you wish to stop at any time, you are free to do so; you will not be penalized in any way.

Where will the sample be analyzed? The blood sample will be transported to KEMRI for some laboratory analysis. Any positive sample will be transported to **University of Manitoba** in **Canada** for genotyping using pyro-sequencing, viral load determination and other molecular assays.

It is important to emphasize that your information will be very useful in establishing these viral Hepatitis in Kenya. Your acceptance is most appreciated. To answer all questions in this form, it might take 10-15 minutes. For any clarification feel free to contact **Missiani Ochwoto (the principal investigator),** on mobile **+254 ……..,** or email: **omissiani@gmail.com**or the following contact will be available:

The Secretary

KEMRI Ethics and Research committee

P.O. BOX 54840-00200 Nairobi

Tel. (020) 2722541 or 0722 205901 or 0733 400003

Subject Permission:

I acknowledge that have read this consent form and understood it, I therefore make my decision

Consent to fill the questionnaire **YESNO** ☐ ☐

Consent for blood collection **YESNO** ☐ ☐

Consent to transport the samples to Canada **YES** ☐ **NO** ☐

Signature/fingerprints of participant _____

Date: _____

Signature of the principle investigator _____ Date _____

Appendix 1 (b): Karatasi ya kufahamisha Idhini

Kichwa cha utafiti: ACUTE AND CHRONIC VIRAL HEPATITIS IN SELECTED HOSPITALS IN KENYA

Hujambo?

Mimi ni Missiani Ochwoto na nafanya kazi na taasishi ya utafiti ya Kenya (KEMRI)

Kwa wakati huu tunafanya utafiti wa kuchunguza maradhi ya ini yaletwayo na virusi. Hivi virusi vya Hepatitis vyaweza kuwa vya muda mfupi *(acute)* au vya muda mrefu *(chronic)*na vyaweza luleta saratani

Idadi ya washiriki

Utafiti huu tahusisha washiriki miatatuna hamsini (350) wanaohudhuria hospitali za kutibu magonjwa ya ini

Taratibu za Utafiti

Lengo la karatasi hii ni kukuomba idhini ya kushiriki. Iwapo utakubali kushiriki basi utajaza fomu ya maswali ya maswali na kisha utatolewa damu (kiasi cha millilita saba; 7mL) na wauguzi ambayo itatumika kwa uchunguzi. Damu hiyo itasafirishwa hadi taasisi ya uchunguzi wa matibabu (KEMRI) ambapo ni watu wangapi wana hivi virusi na idadi yao. Matokeo hayo yatawasilishwa kwa zahanati hii. Na utajulishwa hali yako. Wale ambao wakuwa na virusi tutawachunguza kwa watafuata kwa muda wa miaka miwili ambapo watakuwa wanajaza fomu na kutolewa damu kila baada ya miezi mitatu au nne. Kila wakati majibu yao watafahamishwa.

KUSHIRIKI : Wagojwa wote wanaoudhulia Zahanati za kutibu magonjwa ya ini katika miji mikuu (Kisumu na Nairobi) na Eldoret wana uhuru wa kushiriki.

FAIDA: Faida ya binafsi mshiriki atakayo pata ni kuwa, majibu ya utafiti huu yatatumwa kwa madaktari wa Zahanati yake ili wachukue hatua zifaazo. Faida nyingine ni kuwa, matokeo hayo yatasaidia secta ya Afya katika kufahamu idadi ya maambukizo hayo na kuweka mikakati inayofaa.

MADHARA: Kando na uchungu mdogo mshiriki atahisi wakati wa kutolewa damu, hakuna madhara mengine. Hata hivyo utaratibu wa kutoa damu utafanyika katika mahali safi na hatua zote za kapunguza uchungu zitatumika.

UTAFITI HUU UTAFANYWA KWA MDA GANI?

Utafiti huu utafanywa kwa mda wa miaka minne. Na mshiriki anaruhusiwa kutolewa damu mara nne tu; kila baada ya miezi tatu au nne.

HABARI ZAIDI AU MASWALI

Iwapo utakuwa na swali lolote kuhusu mradi huu linastahili kuelekezwa kwa wafuatao: **Missiani Ochwoto** (**mchunguzi mkuu),** rununu (mobile) **+254 ……..,** ua barua pepe: **omissiani@gmail.com**au waweza kuwasilina kwa kuandika baura au kupiga simu kwa:

KwaKarani,

KEMRI Ethics and Research committee

S.L.P. 54840-00200. Nairobi Simu. 2722541 au 0722 205901 au 0733 400003

Code:

Appendix 2: PATIENT QUESTIONNAIRE CONFIDENTIAL

Project Title:
Molecular Characterization of Acute And Chronic Hepatitis B Virus and TP53 Tumour Suppressor Gene in Human Cancers; Among Patients Attending Selected Hospitals In Kenya

Date _____

Patient's Hospital number (OP) …….. Registry number ……..

Enumerator administering the questionnaire_____ **Sign**_____

You are asked to fill this questionnaire to the best of your knowledge. It will take about ***5-10 minutes.*** *The answer you give to these questions will be kept completely confidential. The questionnaire has two sections part A and B. As a participant you will only fill part A.*

A. Demographics

Household Questions:

1. Gender Male; ☐ Female... ☐

2. Age of the patient .....

2a. Year and Month when you were born? Year…… Month……

3. Your area of residence? (county)..... ....

(Location) . ... (village).....

4. How long have you lived there? .....Years

5. Place of birth.....

Level of Education

6. Did you ever attend school? Yes …….No…….

7. What was the highest education level attained?

None ☐

Primary school ☐

Secondary School ☐

College ☐

Marital Status

8. Marital status (tick one)

Married (monogamous) ☐

Never married (Single) ☐

Divorced/separated ☐

Widowed ☐

9.Religion 1 – Christian 2 – Muslim 3 – Hindu 4 – Other ……..

11 (a)Is there anyone in your family or extended family who has HBV?

Yes .....No .....

11 (b) If the answer is yes, how are you related to him/her?

My: brother.....☐parent.....☐ sister.....☐ uncle.....☐ aunt .....☐ cousin.....☐

Other (Specify).....

11c Is there anyone in your family or extended family with Liver cancer

.....

B. Stage of Tumour (To be filled at Histology lab)

12 (a) Date of when first diagnosed (dd/mm/yyyy) ……..

(b) Basis of the Diagnosis.....

.....

(c) Was Histology done ……Date ……..…….. Lab No. …

13. The type of tumour .....

.....

14. Primary Site of the tumour_____

Behaviour of the tumour _____

_____

15. The size of the tumour (in cm)_____

16. Disease stage.....

.....

17. Concurrent illnesses. .....

. .....

18. Other descriptions of the disease/tumour .....
....

19. Treatment done.....

20. Outcome of the disease/tumour 1. Survived ☐ 2. Died..... ☐ 3. unknown..... ☐

Thank you for your Participation

Ahsante kwa kushiriki

########################## End (Mwisho) ##########################

Appendix 3: QIAamp® DNA blood mini kit manufacturer's manual

Blood or Body Fluid Spin Protocol

Protocol: DNA Purification from Blood or Body Fluids (Spin Protocol)

This protocol is for purification of total (genomic, mitochondrial, and viral) DNA from whole blood, plasma, serum, buffy coat, lymphocytes, and body fluids using a microcentrifuge. For total DNA purification using a vacuum manifold, see "Protocol: DNA Purification from Blood or Body Fluids (Vacuum Protocol)" on page 29.

Important points before starting

- All centrifugation steps are carried out at room temperature (15–25°C).
- Use carrier DNA if the sample contains <10,000 genome equivalents (see page 17).
- 200 µl of whole blood yields 3–12 µg of DNA. Preparation of buffy coat (see page 18) is recommended if a higher yield is required.

Things to do before starting

- Equilibrate samples to room temperature (15–25°C).
- Heat a water bath or heating block to 56°C for use in step 4.
- Equilibrate Buffer AE or distilled water to room temperature for elution in step 11.
- Ensure that Buffer AW1, Buffer AW2, and QIAGEN Protease have been prepared according to the instructions on page 16.
- If a precipitate has formed in Buffer AL, dissolve by incubating at 56°C.

Procedure

1. **Pipet 20 µl QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube.**
2. **Add 200 µl sample to the microcentrifuge tube. Use up to 200 µl whole blood, plasma, serum, buffy coat, or body fluids, or up to 5 x 10^6 lymphocytes in 200 µl PBS.**

   If the sample volume is less than 200 µl, add the appropriate volume of PBS.

   QIAamp Mini spin columns copurify RNA and DNA when both are present in the sample. RNA may inhibit some downstream enzymatic reactions, but not PCR. If RNA-free genomic DNA is required, 4 µl of an RNase A stock solution (100 mg/ml) should be added to the sample before addition of Buffer AL.

   Note: It is possible to add QIAGEN Protease (or proteinase K) to samples that have already been dispensed into microcentrifuge tubes. In this case, it is important to ensure proper mixing after adding the enzyme.

3. **Add 200 µl Buffer AL to the sample. Mix by pulse-vortexing for 15 s.**

   To ensure efficient lysis, it is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution.

   If the sample volume is larger than 200 µl, increase the amount of QIAGEN Protease (or proteinase K) and Buffer AL proportionally; for example, a 400 µl sample will require 40 µl QIAGEN Protease (or proteinase K) and 400 µl Buffer AL. If sample volumes larger than 400 µl are required, use of QIAamp DNA Blood Midi or Maxi Kits is recommended; these can process up to 2 ml or up to 10 ml of sample, respectively.

   Note: Do not add QIAGEN Protease or proteinase K directly to Buffer AL.

4. **Incubate at 56°C for 10 min.**

   DNA yield reaches a maximum after lysis for 10 min at 56°C. Longer incubation times have no effect on yield or quality of the purified DNA.

5. **Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.**

6. **Add 200 µl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.**

   If the sample volume is greater than 200 µl, increase the amount of ethanol proportionally; for example, a 400 µl sample will require 400 µl of ethanol.

7. **Carefully apply the mixture from step 6 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.***

   Close each spin column to avoid aerosol formation during centrifugation.

   Centrifugation is performed at 6000 x *g* (8000 rpm) to reduce noise. Centrifugation at full speed will not affect the yield or purity of the DNA. If the lysate has not completely passed through the column after centrifugation, centrifuge again at higher speed until the QIAamp Mini spin column is empty.

   Note: When preparing DNA from buffy coat or lymphocytes, centrifugation at full speed is recommended to avoid clogging.

8. **Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.***

   It is not necessary to increase the volume of Buffer AW1 if the original sample volume is larger than 200 µl.

* Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach. See page 6 for safety information.

Blood or Body Fluid Spin Protocol

9. **Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min.**
10. **Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.**

    This step helps to eliminate the chance of possible Buffer AW2 carryover.
11. **Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.**

    Incubating the QIAamp Mini spin column loaded with Buffer AE or water for 5 min at room temperature before centrifugation generally increases DNA yield.

    A second elution step with a further 200 µl Buffer AE will increase yields by up to 15%.

    Volumes of more than 200 µl should not be eluted into a 1.5 ml microcentrifuge tube because the spin column will come into contact with the eluate, leading to possible aerosol formation during centrifugation.

    Elution with volumes of less than 200 µl increases the final DNA concentration in the eluate significantly, but slightly reduces the overall DNA yield (see Table 5, page 25). For samples containing less than 1 µg of DNA, elution in 50 µl Buffer AE or water is recommended. Eluting with 2 x 100 µl instead of 1 x 200 µl does not increase elution efficiency.

    For long-term storage of DNA, eluting in Buffer AE and storing at –30 to –15°C is recommended, since DNA stored in water is subject to acid hydrolysis.

    A 200 µl sample of whole human blood (approximately 5 x 10^6 leukocytes/ml) typically yields 6 µg of DNA in 200 µl water (30 ng/µl) with an A_{260}/A_{280} ratio of 1.7–1.9.

Appendix 4: Qiagen Gel purification kit the manufacturers' Manual

Procedure

1. **Add 5 volumes of Buffer PB to 1 volume of the PCR sample, and then mix. It is not necessary to remove mineral oil or kerosene.**

   For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).
2. **If pH Indicator I has been added to Buffer PB, check that the mixture's color is yellow.**

   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.

3. **Place a QIAquick spin column in a provided 2 ml collection tube.**
4. **To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.**
5. **Discard flow-through. Place the QIAquick column back into the same tube.**

   Collection tubes are reused to reduce plastic waste.
6. **To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.**
7. **Discard flow-through and place the QIAquick column back into the same tube. Centrifuge the column for an additional 1 min.**

   IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
8. **Place QIAquick column in a clean 1.5 ml microcentrifuge tube.**
9. **To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.**

   IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volumes are 48 µl from 50 µl elution buffer volume and 28 µl from 30 µl elution buffer.

   Elution efficiency is dependent on pH. Maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C because DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
10. **If the purified DNA is to be analyzed on a gel, add 1 volume Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting it up and down before loading the gel.**

    Loading Dye contains 3 marker dyes – bromophenol blue, xylene cyanol and orange G – that facilitate estimation of DNA-migration distance and optimization of the agarose gel run time. Refer to Table 2 (page 17) to identify the dyes according to migration distance and agarose gel percentage and type.

Appendix 5: Kemri SERU approval (SSC No. 2436)

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200 NAIROBI - Kenya
Tel: (254) (020) 2722541, 254 (020) 2713349, 0722-205901, 0733-400003 Fax (254) (020) 2720030
Email: director@kemri.org info@kemri.org Website: www.kemri.org

KEMRI/RES/7/3/1 **December 20, 2015**

TO: **OCHWOTO MISSIANI, (PRINCIPAL INVESTIGATOR)**

FOR DIRECTOR
CENTRE FOR VIRUS RESEARCH
P.O. BOX 54628
NAIROBI.

THROUGH : **DR. GEORGE NAKITARE,**
DIRECTOR, CVR,
NAIROBI

forwarded
Raphael Ithae
21st Dec. 2015

Dear Sir,

RE: **SSC PROTOCOL NO. 2436 *(RESUBMITTED REQUEST FOR ANNUAL RENEWAL WITH PROTOCOL DEVIATION):* ACUTE AND CHRONIC VIRAL HEPATITIS IN SELECTED HOSPITALS IN KENYA**

Reference is made to your letter dated 9 November 2015 (predated). The KEMRI Scientific and Ethics Review Unit (SERU) acknowledge receipt of the revised document on 10 December 2015.

This is to inform you that the Committee determines that the issues raised at the 245th Committee B meeting of the SERU held on 18th November 2015 have been adequately addressed.

This study is granted approval for continuation effective this **December 15, 2015.** Please note that authorization to conduct this study will automatically expire on **December 14,2016** If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the SERU Secretariat by **November 2, 2016.**

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the SERU for review prior to initiation.

You may continue with the study.

Yours faithfully,

RAB

PROF. ELIZABETH BUKUSI,
ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

CENTRE FOR VIRUS RESEARCH
18 DEC 2015
P.O. Box 54628 - NAIROBI

Appendix 7: sequences submitted to NCBI

Hepatitis B virus isolate EMB220 S protein (S) gene, partial cds

GenBank: MK487135.1

GenBank Graphics

>MK487135.1 Hepatitis B virus isolate EMB220 S protein (S) gene, partial cds
GGTATGTTGCCCGTTTGTCCTCTAATTCCAGGATCCACAACAACCAGCACGGGACCCTGCAAAACCTGCA
CGACTCCTGCTCAAGGCAACTCTATGTTTCCCTCATGTTGCTGTACAAAACCTACGGATGGAAATTGCAC
TTGTATTCCCATCCCATCATCTTGGGCTTTCGCAAAATACCTATGGGAGTGGGCCTCAGTCCGTTTCTCT
TGGCTCAGTTTACTAGTGCCATTTGTTCAGTGGTTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGCTA
TATGGATGATGTGGTACTGGGGGCCAAGTCTGTACAACATCTTGAGT

Hepatitis B virus isolate KN88 S protein (S) gene, partial cds

GenBank: MK487136.1

GenBank Graphics

>MK487136.1 Hepatitis B virus isolate KN88 S protein (S) gene, partial cds
GGTATGTTGCCCGTTTGTCCTCTAATTCCAGGATCCACAACAACCAGCACGGGACCCTGCAAAACCTGCA
CGACTCCTGCTCAAGGCAACTCTATGTTTCCCTCATGTTGCTGTACAAAACCTACGGATGGAAATTGCAC
CTGTATTCCCATCCCATCATCTTGGGCTTTCGCAAAATACCTATGGGAGTGGGCCTCAGTCCGTTTCTCT
TGGCTCAGTTTACTAGTGCCATTTGTTCAGTGGTTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGCTA
TATGGATGATGTGGTACTGGGGGCCAAGTCTGTACAACATCTTGAGT

Hepatitis B virus isolate EMB7 S protein (S) gene, partial cds

GenBank: MK487137.1

GenBank Graphics

>MK487137.1 Hepatitis B virus isolate EMB7 S protein (S) gene, partial cds
GGTATGTTGCCCGTTTGTCCTCTAATTCCAGGATCCACAACAACCAGCACGGGACCCTGCAAAACCTGCA
CGACTCCTGCTCAAGGCAACTCTATGTTTCCCTCATGTTGCTGTACAAAACCTACGGATGGAAATTGCAC
CTGTATTCCCATCCCATCATCTTGGGCTTTCGCAAAATACCTATGGGAGTGGGCCTCAGTCCGTTTCTCT
TGGCTCAGTTTACTAGTGCCATTTGTTCAGTGGTTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGCTA
TATGGATGATGTGGTACTGGGGGCCAAGTCTGTACAACATCTTGAGT

Hepatitis B virus isolate KN168 S protein (S) gene, partial cds

GenBank: MK487138.1

GenBank Graphics

>MK487138.1 Hepatitis B virus isolate KN168 S protein (S) gene, partial cds
GGTATGTTGCCCGTTTGTCCTCTAATTCCAGGATCCACAACAACCAGCACGGGACCCTGCAAAACCTGCA
CGACTCCTGCTCAAGGCAACTCTATGTTTCCCTCATGTTGCTGTACAAAACCTACGGATGGAAATTGCAC
CTGTATTCCCATCCCATCATCTTGGGCTTTCGCAAAATACCTATGGGAGTGGGCCTCAGTCCGTTTCTCT
TGGCTCAGTTTACTAGTGCCATTTGTTCAGTGGTTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGCTA
TATGGATGATGTGGTACTGGGGGCCAAGTCTGTACAACATCTTGAGT

Hepatitis B virus isolate KN120 S protein (S) gene, partial cds

GenBank: MK487139.1

GenBank Graphics

>MK487139.1 Hepatitis B virus isolate KN120 S protein (S) gene, partial cds

GGTATGTTGCCCGTTTGTCCTCTAATTCCAGGATCCACAACAACCAGCACGGGACCCTGCAAAACCTGCA
CGACTCCTGCTCAAGGCAACTCTATGTTTCCCTCATGTTGCTGTACAAAACCTACGGATGGAAATTGCAC
CTGTATTCCCATCCCATCATCTTGGGCTTTCGCAAAATACCTATGGGAGTGGGCCTCAGTCCGTTTCTCT
TGGCTCAGTTTACTAGTGCCATTTGTTCAGTGGTTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGCTA
TATGGATGATGTGGTACTGGGGGCCAAATCTGTACAACATCTTGAGT

Hepatitis B virus isolate KN194 S protein (S) gene, partial cds

GenBank: MK487140.1

GenBank Graphics

>MK487140.1 Hepatitis B virus isolate KN194 S protein (S) gene, partial cds

GGTATGTTGCCCGTTTGTCCTCTAATTCCAGGATCCACAACAACCAGCACGGGACCCTGCAAAACCTGCA
CGACTCCTGCTCAAGGCAACTCTATGTTTCCCTCATGCTGCTGTACAAAACCTACGGATGGAAATTGCAC
CTGTATTCCCATCCCATCATCTTGGGCTTTCGCAAAATACCTATGGGAGTGGGCCTCAGTCCGTTTCTCT
TGGCTCAGTTTACTAGTGCCATTTGTTCAGTGGTTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGCTA
TATGGATGATGTGGTACTGGGGGCCAAATCTGTACAACATCTTGAGT

Hepatitis B virus isolate KSM590 S protein (S) gene, partial cds

GenBank: MK487141.1

GenBank Graphics

>MK487141.1 Hepatitis B virus isolate KSM590 S protein (S) gene, partial cds

GGTATGTTGCCCGTTTGTCCTCTAATTCCAGGATCCTCAACAACCAGTACGGGACCCTGCAAAACCTGCA
CGACTCCTGCTCAAGGCAACTCTATGTTTCCCTCATGTTGCTGTACAAAACCTACGGATGGAAATTGCAC
CTGTATTCCCATCCCATCATCTTGGGCTTTCGCAAAATACCTATGGGAGTGGGCCTCAGTCCGTTTCTCT
TGGCTCAGTTCACTAGTGCCATTTGTTCAGTGGTTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGTTA
TATGGATGATGTGGTATTGGGGGCCAAGTCTGTACAACATCTTGAGT

Hepatitis B virus isolate KN160 S protein (S) gene, partial cds

GenBank: MK487142.1

GenBank Graphics

>MK487142.1 Hepatitis B virus isolate KN160 S protein (S) gene, partial cds

GGTATGTTGCCCGTTTGTCCTCTAATTCCAGGATCCACAACAAACAGTGCGGGACCCTGCAAAACCTGCA
CGACTCCTGCTCAAGGCAACTCTATGTTTCCCTCATGTTGCTGTACAAAACCTACGGATGGAAATTGCAC
CTGTATTCCCATCCCATCATCTTGGGCTTTCGCAAAATACCTATGGGAGTGGGCCTCAGTCCGTTTCTCT
TGGCTCAGTTTACTAGTGCCATTTGTTCAGTGGTTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGTTA
TATGGATGATGTGGTATTGGGGGCCAAGTCTGTACAACATCTTGAGT

Hepatitis B virus isolate KN126 S protein (S) gene, partial cds

GenBank: MK487143.1

GenBank Graphics

>MK487143.1 Hepatitis B virus isolate KN126 S protein (S) gene, partial cds

GGTATGTTGCCCGTTTGTCCTCTAATTCCAGGATCCACAACAAACAGTGCGGGACCCTGCAAAACCTGCA
CGACTCCTGCTCAAGGCAACTCTATGTTTCCCTCATGTTGCTGTACAAAACCTACGGATGGAAATTGCAC

CTGTATTCCCATCCCATCATCTTGGGCTTTCGCAAAATACCTATGGGAGTGGGCCTCAGTCCGTTTCTCT
TGGCTCAGTTTACTAGTGCCATTTGTTCAGTGGTTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGTTA
TATGGATGATGTGGTATTGGGGGCCAAGTCTGTACAACATCTTGAGT

Hepatitis B virus isolate KSM585 S protein (S) gene, partial cds

GenBank: MK487144.1

GenBank Graphics

>MK487144.1 Hepatitis B virus isolate KSM585 S protein (S) gene, partial cds
GGTATGTTGCCCGTTTGTCCTCTAATTCCAGGATCCACAACAACCAGTACGGGACCATGCAAAACCTGCA
CGACTCCTGCTCAAGGCAACTCTATGTTTCCCTCATGTTGCTGTACAAAACCTACGGATGGAAATTGCAC
CTGTATTCCCATCCCATCATCTTGGGCTTTCGCAAAATACCTATGGGAGTGGGCCTCAGTCCGTTTCTCT
TGGCTCAGTTTACTAGCGCCATTTGTTCAGTGGTTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGTTA
TATGGATGATGTGGTATTGGGGGCGAAGTCTGTACAACATCTTGAGT

Hepatitis B virus isolate KSM583 S protein (S) gene, partial cds

GenBank: MK487152.1

GenBank Graphics

>MK487152.1 Hepatitis B virus isolate KSM583 S protein (S) gene, partial cds
GGTATGTTGCCCGTTTGTCCTCTAATTCCAGGATCCTCAACAACCAGYACGGGACCCTGCAAAACCTGCA
CGACTCCTGCTCAAGGCAACTCTATGTTTCCCTCATGTTGCTGTACAAAACCTACGGATGGAAATTGCAC
CTGTATTCCCATCCCATCATCTTGGGCTTTCGCAAAATACCTATGGGAGTGGGCCTCAGTCCGTTTCTCT
TGGCTCAGTTTACTAGTGCCATTTGTTCAGTGGTTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGYTA
TATGGATGATGTGGTATTGGGGGCCAAGTCTGTACAACATCTTGAGT

Hepatitis B virus isolate KN113 S protein (S) gene, partial cds

GenBank: MK487153.1

GenBank Graphics

>MK487153.1 Hepatitis B virus isolate KN113 S protein (S) gene, partial cds
GGTATGTTGCCCGTTTGTCCTATAATTCCAGGATCCACAACAACCAGCACGGGACCCTGCAGAACCTGCA
CGACTCCTGCTCAAGGCAACTCTATGTTTCCCTCATGTTGCTGTACAAAACCTACGGAAGGAAATTGCAC
ATGTATTCCCATCCCATCATMTTGGGCTTTCGCAAAATTCCTATGGGAGTGGGCCTCAGTCCGTTTCTCT
TGGCTCAGTTTACTAGTGCCATTTGTTCAGTGGTTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGCTA
TATGGATGATGTGGTATTGGGGGCCAAGTCTGTGCAACATCTTGAGT

Papers published

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RESEARCH ARTICLE Open Access

Hepatitis B infection is highly prevalent among patients presenting with jaundice in Kenya

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Abstract

Background: Viral hepatitis is a major concern worldwide, with hepatitis A (HAV) and E (HEV) viruses showing sporadic outbreaks while hepatitis B (HBV) and C (HCV) viruses are associated with chronic hepatitis, cirrhosis and hepatocellular carcinoma. The present study determined the proportion, geographic distribution and molecular characterization of hepatitis viruses among patients seeking medical services at hospitals throughout Kenya.

Methods: Patients presenting with jaundice at four selected hospitals were recruited ($n = 389$). Sera were tested for the presence of antibody to hepatitis viruses A through E, and HBV surface antigen (HBsAg). Nucleic acid from anti-HAV IgM antibody and HBsAg positive samples was extracted, amplified and sequenced.

Results: Chronic HBV infection was the leading cause of morbidity among patients with symptoms of liver disease seeking medical help. Incident HCV, HEV and HDV infection were not detected among the patients in this study, while the proportion of acute HAV was low; HAV IgM positivity was observed in 6.3 % of patients and sequencing revealed that all cases belonged to genotype 1B. HCV seropositivity upon initial screening was 3.9 % but none were confirmed positive by a supplementary immunoblot assay. There was no serological evidence of HDV and acute HEV infection (anti-HEV IgM). HBsAg was found in 50.6 % of the patients and 2.3 % were positive for IgM antibody to the core protein, indicating probable acute infection. HBV genotype A was predominant (90.3 %) followed by D (9.7 %) among

2.

2015 International HBV Meeting

The Molecular Biology of Hepatitis B Viruses

Bad Nauheim
Hessen, Germany

Meeting Dates:
October 4 – 8, 2015

July 13, 2015

Dear Missiani Ochwoto ,

Thank you for submitting your abstract to the HBV International Meeting 2015
Hepatitis B Virus Genotypes and Unique Recombinants Circulating Among outpatients in Selected

Hospitals in Kenya - Oral Presentation

ID:
101130

Text:
HBV causes 780,000 deaths yearly worldwide. In Africa HBV infection is endemic and of the ten known HBV genotypes (A-J), A, D and E are prominent. Kenya is geographically located at the junction of these three genotypes. Genotypes are important in both clinical and manifestation of HBV infection, however, there is paucity of genotyping data in Kenya. In order to determine the prevalence and molecular characterization of HBV, 339 serum samples were obtained from patients with jaundice seeking medical services in four selected hospitals in Kenya. Hepatitis B surface Antigen and Antibody to the core antigen (HBsAg and HBcAg) were serologically detected. Sub-genomic regions of the virus genome (basal core promoter/precore (BCP/PC and HBsAg) from the positive specimen were amplified and sequenced with 20 specimen selected at random for full genome. Isolates of the unique sequences were cloned to rule out strand switching during PCR amplification. The prevalence of HBsAg positivity in this population was 51.6% (175/339) of which 101/175 were positive for HBV DNA, and 2.0% were positive for IgM antibody to the core protein indicating acute infection. HBV sub genotype A1 was predominant (91%) followed by genotype D (9%). There was a statistically significant difference observed in age and geographical region between patients infected with genotype D and A (p=0.02) with genotype D infected persons (8/9) being mainly male and over 40 years of age, from western Kenya. Recombination analysis of the full genome sequences showed that there were two unique recombinants circulating in Kenya. In the coastal region, possible recombination between genotypes A and E (A/E) were observed in one isolate whereas in the western region, putative recombination between genotype D and E was in 4/9 (44.4%). Interestingly, D/E recombinant sequences had a nucleotide divergence >4% from previous subgenotypes (D1-D8) including D/E recombinants in GenBank/DDBJ. The recombination occurred primarily within the preS region. All genotype D isolates had the signature amino acid rt237T, which is characteristic of genotype D6. The PC stop codon mutation A1896 and BCP mutation T1762/A1764, was identified in 4% and 33% in genotype A isolates respectively, while 44% of HBV/D strains had either the A1896 mutation or BCP mutation, or a variation of it (T1762/G1764 or A1762/A1764). In conclusion: Recombination of different HBV genotypes may enhance their virulence, the finding of novel HBV recombinant strains in Kenya indicates that further investigation is required as the morbidity and progression of infection with such strains is not known.

Keywords:
Viral Variants and Variability, A/E recombinants, D/E Recombinants, HBV genotypes in Kenya.

3. https://f1000researchdata.s3.amazonaws.com/manuscripts/21284/d01ea98c-d2c7-41ad-9868-2faf0e3e71cc_19416_-_missiana_ochwoto.pdf?numberOfBrowsableCollections=17&numberOfBrowsableInstitutionalCollections=4&numberOfBrowsableGateways=22