

**MOLECULAR CHARACTERIZATION OF HEPATITIS B VIRUS
IN RURAL AND SEMI-URBAN AREAS IN THE CENTRAL
REGION OF GHANA**

BY

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DECLARATION

I, CAROLINE AGYARE BOATEMAA do hereby declare that the work presented in this thesis is solely the results of my own research undertaken under the supervision of Dr. Osbourne Quaye with advice from Dr. Samuel Duodu (Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon-Accra) and that, except previous research works which have been duly acknowledged, this work has never been submitted to this University or elsewhere in part or whole for the award of any degree.

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ABSTRACT

Hepatitis B virus (HBV) infection is a major cause of liver inflammation accounting for 887,000 deaths annually. The introduction of HBV vaccines has significantly reduced new cases of the infection, but there are still 65 million people living with the virus in Sub-Saharan Africa. Currently, 10 genotypes (A-J) of HBV have been classified. Several studies have shown that the different HBV genotypes differently influence clinical presentation, progression of liver diseases and response to treatment. In Ghana, studies on HBV circulating genotypes and their implications on epidemiology is limited, however few available data have been obtained mostly in the urban areas of the Ashanti and Greater Accra regions. This study is aimed at characterizing HBV genotypes in patients attending three district hospitals in the Central Region of Ghana (Agona-West, Effutu Municipal and Gomoa-West district hospitals). Questionnaires were administered to the patients before sample collection which included their gender, age, educational levels and risk factors. There was no association between socio-demographic factors and HBV infectivity and transmissibility, except educational levels. HBV DNA was extracted from the blood of 173 HBsAg seropositive patients using QIAamp DNA Blood mini kit; 70 (40%) samples from the Gomoa-West district, 50 (29%) samples from the Effutu Municipal and 53 (31%) from the Agona-West district. The extracted DNA was confirmed by amplifying the S region of the HBV genome using conventional PCR. A total of 115 (66.5%) patients were found to be PCR positive, with 58 (33.5%) negative for HBV DNA. The HBV was genotyped by nested-multiplex PCR using type-specific primers that amplifies the preS/S region of the HBV genome. To confirm the specificity of the nested-multiplex PCR assay, 31 of the samples obtained from the S gene amplification were sent for Sanger sequencing in one direction. The nucleotide sequences obtained

were submitted into the HBV HepSeq genotyping software to confirm the genotypes. The genotype results obtained from both the software and the nested -multiplex PCR assay included; 67.1% genotype E which was the most dominant genotype, followed by genotypes A, D and G with 1.7%, 0.6% and 0.6%, respectively. The phylogenetic analysis of the sequenced isolates from the three districts clustered together with the reference strains. The characterization of the HBV genotypes will help understand the transmission dynamics of the virus in the rural and semi-urban setting.



DEDICATION

I dedicate this work to the Agyare family for helping me bring this work to a success.



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TABLE OF CONTENTS

CONTENTS

DECLARATION	i
ABSTRACT.....	ii
DEDICATION.....	iv
ACKNOWLEDGEMENT	v
TABLE OF CONTENTS.....	vi
LIST OF FIGURES/MAPS	x
LIST OF TABLES.....	xii
LIST OF ABBREVIATIONS.....	xiii
CHAPTER ONE	1
1.0 INTRODUCTION.....	1
1.1 Background	1
1.2 Problem statement.....	5
1.3 Hypothesis.....	6
1.4 Aim.....	6
1.5 Specific objectives.....	6
CHAPTER TWO	7
2.0 LITERATURE REVIEW.....	7
2.1 The liver	7

2.2 Hepatitis infection	7
2.3 Biology of the hepatitis B virus (HBV)	8
2.4 The hepatitis B virus life cycle.....	8
2.5 Morphology and genome organization of HBV.....	11
2.5.1 Description of the morphology	11
2.5.2 Genome organization and associated proteins.....	13
2.5. 3 Features of the viral proteins	14
2.6 The role of HBV genes and proteins in the clinical manifestation of HBV.....	16
2.7 Clinical manifestation of mutations in the HBV genome	17
2.8 Antigenicity	19
2.9 HBV genotypes	21
2.9.1 Characteristics of HBV genotypes	21
2.9 2 Geographic distribution of Hepatitis B virus genotypes	22
2.10 Importance of epidemiology and molecular characterization of HBV	24
2.11 Clinical outcomes of HBV genotypes.....	26
2.12 Pathogenesis.....	29
2.13 HBV Co-infections.....	30
2.14 Mode of transmission	31
2.15 Diagnostics of HBV	33
2.15. 1 Molecular diagnostics.....	34
2.15.2 Next Generation Sequencing (NGS)	35

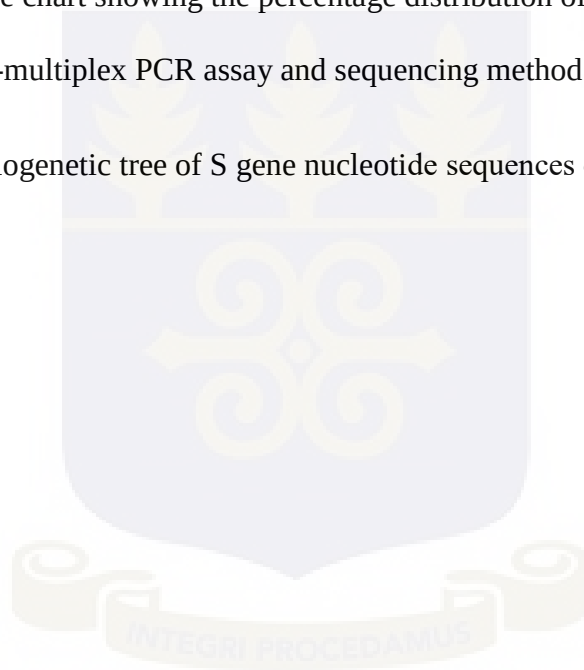
CHAPTER THREE	36
3.0 METHODOLOGY.....	36
3.1 Study design and patients selection.....	36
3.2 Study sites description.....	36
3.3 Population size	37
3.4 Sampling method.....	38
3.5 Inclusion criteria.....	38
3.6 Exclusion criteria.....	38
3.7 Sample collection	38
3.8 Serological testing and storage.....	39
3.9 Deoxyribonucleic Acid (DNA) extraction	39
3.10 Polymerase Chain Reaction (PCR) analysis	40
3.11 Genotyping of HBV by nested-multiplex PCR.....	41
3.12 Agarose gel electrophoresis and visualization of HBV PCR products	43
3.13 Sequencing of PCR products.....	43
3.14 Statistical analysis	44
CHAPTER FOUR.....	45
4.0 RESULTS.....	45
4.1 Baseline characteristics and seroprevalence of HBsAg in patients.....	45
4.2 Amplification of S gene among HBsAg infected patients	46
4.3 S gene amplification (HBV DNA positivity) and patient gender	47

4.4 S gene amplification (HBV DNA positivity) and age groups of patients	47
4.5 Association between marital status and HBV infectivity.....	48
4.6 S gene amplification (HBV DNA positivity) and educational levels of the patients	49
4.7 Risk factors associated with Hepatitis B infection.....	49
4.8 Gel electropherogram of the presence of HBV DNA in the three districts.....	50
4.9 Genotyping of HBV by nested-multiplex PCR.....	52
4.10 The percentage distribution of the HBV genotypes	53
CHAPTER FIVE	57
5.0 DISCUSSION	57
CHAPTER SIX.....	64
6.0 CONCLUSION	64
6.1 RECOMMENDATIONS	64
REFERENCES	65
APPENDIX.....	85

LIST OF FIGURES/MAPS

Figure 2.1: A schematic diagram of the replication cycle of hepatitis B virus	11
Figure 2.2: Electron micrographs of the morphological forms of the hepatitis B virion	12
Figure 2.3: The genomic organization of HBV	14
Figure 2.4: A schematic representation of HBV, showing the virion surface antigens (L, M, S HBsAg) embedded in the outer lipid envelope	16
Figure 2.5: A phylogenetic tree of the whole genome sequences of nine HBV genotypes (A-I) and their respective serological subtypes	20
Figure 2.6: Worldwide geographical distribution of HBV genotypes (A-J)	23
Figure 2.7: World map of HBV infection endemicity	25
Figure 3.1: The three study sites; Effutu Municipal Assembly, Agona- West district and Gomoa-West district	37
Figure 4.1: Distribution of HBsAg seropositivity in patients attending hospitals in the Gomoa-West and Agona-West districts, and the Effutu Municipal Assembly of the Central Region of Ghana	46
Figure 4.2: A graph of HBV DNA positivity across different age groups.....	48
Figure 4.3: Representative gel electrophoresis pattern of HBV DNA by PCR amplification of the S gene in HBsAg seropositive samples from Gomoa-West district.	51

Figure 4.4: Representative gel electrophoresis pattern of HBV DNA by PCR amplification of the S gene in HBsAg seropositive samples from Effutu Municipal.....	51
Figure 4.5: Representative gel electrophoresis pattern of HBV DNA by PCR amplification of the S gene in HBsAg seropositive samples from Agona-West district.....	52
Figure 4.6: Genotyping of HBV by nested-multiplex PCR assay.....	52
Figure 4.7: A pie chart showing the percentage distribution of HBV strains typed using the nested-multiplex PCR assay and sequencing method.....	53
Figure 4.8: Phylogenetic tree of S gene nucleotide sequences of HBV strains.....	56



LIST OF TABLES

Table 3.1: Nested-multiplex PCR primers and their sequences	42
Table 4.1: Distribution of HBsAg seropositive patients in the districts with respect to gender.....	46
Table 4.2: HBV DNA positivity in HBsAg seropositive patients in the three districts.....	47
Table 4.3: Amplification of S gene (HBV DNA positivity) among males and females with HBsAg seropositivity.....	47
Table 4.4: Association between S gene amplification (HBV DNA positivity) and marital status	48
Table 4.5: Association between S gene amplification and educational levels of HBsAg seropositive patients	49
Table 4.6: Association between risk factors and HBV DNA positivity.....	50
Table 4.7: Distribution of HBV genotypes of HBsAg seropositive patients in the three districts of the Central Region of Ghana.....	53
Table 4.8: Age distribution of HBV genotypes.....	54
Table 4.9: Distribution of HBV genotypes among males and females.....	54
Table 4.10: Relation between HBV genotypes and HBV DNA positivity (S gene amplification)	54

LIST OF ABBREVIATIONS

HBsAg – Hepatitis B surface antigen

HCC- Hepatocellular carcinoma

BCP- Basal core promoter

YMDD- tyrosine-methionine-aspartate-aspartate

HIV/AIDS- Human Immunodeficiency Virus Infection and Acquired Immune Deficiency Syndrome

NTCP-Sodium taurocholate co-transporting polypeptide

rc DNA – relaxed circular DNA

cccDNA- covalently closed circular DNA

HNF- Hepatocyte nuclear factors

C/EPF- CCAAT/enhancer-binding

SVP- Subviral envelope particles

HBx – Hepatitis B protein X

MHC- Major-histocompatibility complex

DR- Direct repeats

CTL- Cytotoxic T-lymphocytes

ALT- Alanine aminotransferase

NRT I- Nucleotide reverse transcriptase inhibitor

Ig – Immunoglobulin g

CHB- Chronic hepatitis B

EDTA- Ethylene diamine tetra acetic acid

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Infectious microorganisms in the blood that get to the liver are normally cleared rapidly by stimulation of immune response mechanism. However, some pathogenic microorganisms (such as hepatitis viruses) are able to establish hepatitis infections (Protzer *et al.*, 2012). These pathogens are provided with favourable conditions in the liver and persist in hepatocytes as a result of T-cell exhaustion due to high viral antigen loads. The immune evasion strategies by the viral antigens due to mutations also contribute to the infection. These can be seen in the case of chronic hepatitis virus infections such as Hepatitis B Virus and Hepatitis C Virus infections (Protzer *et al.*, 2012). Viral hepatitis pathogens have caused significant morbidity and mortality worldwide by the establishment of chronic and acute viral hepatitis infections.

Viral Hepatitis is a liver inflammation caused by an infection with a virus. Hepatitis viruses have been categorized into five main types; hepatitis A virus (HAV) and hepatitis E virus (HEV) are transmitted by oral-fecal route while hepatitis B virus (HBV), hepatitis C virus (HCV) and delta hepatitis virus (HDV) are transmitted through contact with infected body fluids (WHO, 2016). The hepatitis viruses are of global health importance because of their potential to cause outbreaks and epidemics. Acute hepatitis infection can result in no or limited symptoms. Symptoms of acute infection include loss of appetite, weakness, fever, abdominal pains, anorexia, nausea, malaise,

vomiting, headaches, extreme fatigue, this is followed by jaundice (yellowing of skin and sclera) and dark urine in one or two weeks later. Other clinical symptoms include skin rashes, itches, arthralgia, arthritis, hepatomegaly and splenomegaly. All hepatitis viruses cause acute infection, but the hepatitis B and C are the leading cause of end-stage liver diseases, such as cirrhosis and hepatocellular carcinoma (HCC), in hundreds of millions of people worldwide (WHO, 2015). There are about 2 billion people worldwide (one-third of the world's population) with Hepatitis B virus (HBV) infection, with 887,000 resulting in deaths annually from liver diseases due to HBV infection (WHO, 2017). In Sub-Saharan Africa, HBV is the leading cause of chronic liver diseases and has an estimated prevalence rate of 12.3% in Ghana (Ofori-Asenso & Agyeman, 2016).

Hepatitis B virus belongs to the family Hepadnaviridae and genus Orthohepadnavirus. It has a genome of approximately 3,200 base pairs. The viral particle is made up of a lipid envelope, an icosahedral nucleocapsid core protein enclosing a double-stranded DNA, and a viral polymerase (Locarnini, 2004). The lipid envelope has embedded in it, the HBV small (S), middle (M) and large (L) surface antigens (HBsAg) and the HBV protein X (HBx) (Glebe & König, 2014). HBV infection has two modes of transmission; the vertical and horizontal transmissions. Majority of HBV infections in African countries occur by vertical transmission whiles the main route of transmission in the Western countries and Asia is horizontal transmission (Candotti *et al.*, 2007). HBV infection in highly endemic areas occurred mostly in infancy and childhood until the introduction of vaccines by the World Health Organization (WHO) for infants (WHO, 2015). The HBV vaccine has significantly reduced new cases of HBV infection, but there are still at least 257 million people worldwide who are chronically infected (WHO, 2017), with 65 million from Sub-Saharan Africa (Kramvis & Kew, 2007a). In

Ghana, the highest HBV prevalence rates have now been recorded in persons within the age group of 16-39 years (Ofori-Asenso & Agyeman, 2016).

The clinical presentation and severity of HBV infection have been shown to be associated with some viral factors such as viral load, viral mutation and HBV genotypes. The HBV genotypes, however, do not only affect the clinical severity of HBV but also affect response to antiviral treatment. Phylogenetic analysis of the whole genome has resulted in the classification of HBV into 10 genotypes (A-J) based on more than 8% divergence nucleotide sequence, and 40 sub-genotypes based on 4-8% divergence nucleotide sequence (Pourkarim *et al.*, 2014). The genetic diversity in the different genotypes results from error-prone reverse transcription step which is mediated by the viral polymerase (Lauring & Andino, 2010). The HBV genotypes are geographically distributed with genotypes A, D and E (HBV/A, HBV/D, HBV/E) being prevalent in African countries. There are also pathogenic differences among the HBV genotypes which can influence the severity of chronic liver diseases, chronicity rates, seroclearance of HBsAg, seroconversion rate of HBeAg, interferon therapy, viral load and mutation rates (Lin & Kao, 2011). The persistence of HBV infection and the risk of progression to liver diseases are influenced by an acute infection of certain genotypes (Suzuki *et al.*, 2005). The persistence can also result from host-viral interaction and the transmission route (Suzuki *et al.*, 2005). Patients infected with HBV/A, for example, are said to have a higher rate of chronicity, followed by genotype B (HBV/B) and genotype C (HBV/C) (Suzuki *et al.*, 2005).

HBeAg seroconversion and HBsAg seroclearance are critical factors in the clinical outcome of chronic hepatitis (Liaw, 2009; Park & Lee, 2016). Patients with HBeAg seroconversion present favourable advantage over those with late or absent seroconversion, as persistence of HBeAg may contribute to the advancement of chronic

hepatitis to end-stage liver diseases (Liaw, 2009). HBV/C and HBV/D infected patients have a slower rate of HBeAg seroconversion, HBsAg seroclearance and higher duration of HBV replication compared to HBV patients infected with HBV/B and HBV/A (Sánchez-Tapias *et al.*, 2002) thus making HBV/A and HBV/B to be associated with minor liver injuries than HBV/C and HBV/D (Kramvis & Kew, 2005; McMahon, 2009). A combination of higher viral load and naturally occurring basal core promoter (BCP) mutation A1762T/G1764A in HBV genotypes are also contributing factors to the severity of HBV-associated chronic liver diseases (Kao, 2007; Lin & Kao, 2008; Yang *et al.*, 2008). HBV/C and HBV/D have been established to confer a higher prevalence of A1762T/G1764A mutation in the BCP region, which results in high viral loads and increases the risk to severe liver disease progression (e.g. hepatocellular carcinoma (HCC)) than the other genotypes (Kao *et al.*, 2000; Yu *et al.*, 2005). In terms of therapy, the HBV/C and HBV/D have been found to be less susceptible to interferon antiviral treatment than HBV/A and HBV/B (Erhardt *et al.*, 2005; Wiegand *et al.*, 2008). Resistance to lamivudine therapy is associated with the evolution of tyrosine-methionine-aspartate-aspartate (YMDD) mutations in the genotypes, and for instance HBV/A has been associated with the emergence of higher frequency of YMDD mutants than HBV/D (Schaefer, 2007).

Unlike genotypes A-D infections, genotypes E-J infected patients are rare and clinical studies on infections are limited (Lin & Kao, 2011).

Identification of the various genotypes has relied greatly on the diversity that exists within the pre-S and S regions. The availability of vaccines, treatment of affluent areas and migration are responsible for altering the geographical distribution of HBV genotypes (Tong & Revill, 2016). Thus, studies on the virus will serve as a tool for understanding the transmission pattern of the virus, clinical severity of the virus, the

source of infection, identifying factors influencing treatment failure and vaccine response.

1.2 Problem statement

Sub-Saharan Africa has been categorized as a highly endemic region for hepatitis B infection with high morbidity and mortality rates due to liver diseases (WHO, 2017). The high endemicity might be due to the high infectivity rate of the virus which is 50 to 100 times more than HIV/AIDS, making the virus a major risk factor of liver diseases in the Ghana (Adoga *et al.*, 2010). Severity of the liver diseases have been shown to be influenced by HBV genotypes, and co-infections with two or more genotypes can present even more severe clinical outcomes (Blankson *et al.*, 2005). Information about HBV genotypes will therefore be very useful in the management of hepatitis B infection in Ghanaian patients. Despite the high number of cases of hepatitis B infection in Ghana, the implications of HBV genotypes in the epidemiology and natural history of the infection have not been well defined. Most HBV prevalence studies in Ghana are based on HBsAg seropositivity (Ofori-Asenso & Agyeman, 2016), albeit molecular studies in general suggest that disease progression, treatment response and virulence of the virus are genotype specific. The very few molecular studies on HBV have been concentrated in urban areas in the Ashanti and Greater Accra Regions of the country (Kramvis & Kew, 2005; Ofori-Asenso & Agyeman, 2016). Also, these studies, normally focus on specific groups such as blood donors and disease-specific (e.g. Sickle cell) patients but not on the general population (Ampah *et al.*, 2016). Thus, there are no molecular studies of HBV in the general population of the rural settings in Ghana. This research was consequently designed to determine the genotypes of HBV in rural and semi-urban areas in the Central Region of Ghana; the importance is to establish the circulating strains, and detect and characterize unidentified genotypes in these areas.

Understanding the molecular epidemiology will be critical in improving control and treatment of the infection in the study locations.

1.3 Hypothesis

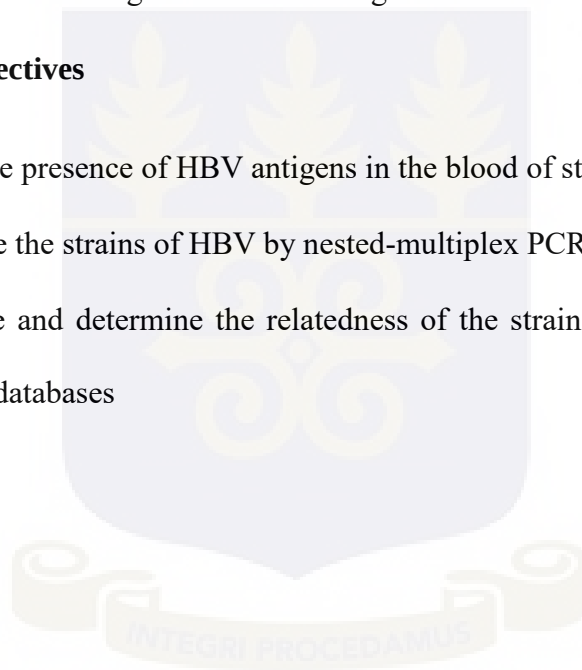
New strains of HBV are responsible for hepatitis infection in patients from rural and semi-urban areas in three districts in the Central Region of Ghana?

1.4 Aim

To characterize HBV from HBV positive subjects attending three Municipal or district hospitals in the Central Region of Ghana using molecular tools.

1.5 Specific objectives

- Detect the presence of HBV antigens in the blood of study participants
- Genotype the strains of HBV by nested-multiplex PCR assay
- Sequence and determine the relatedness of the strains to reference strains in existing databases



CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The liver

The liver is an essential organ that is involved in metabolic and clearance functions. By metabolism, macronutrients (lipids, peptides, carbohydrates) and micronutrients (e.g. iron) are transported by portal blood to the hepatocytes to be metabolized (Protzer *et al.*, 2012). In terms of immune clearance functions, the liver induces immunity when hepatocytes take up and express antigens on their cell surfaces, and directly present them to MHC molecules for recognition by T-cells (Thomson & Knolle, 2010). These roles played by the liver predisposes it to infectious pathogens; pathogens are able to escape T-cells mediated immunity by targeting the liver other than the lymphoid tissues. Example of such pathogens are viruses that cause hepatitis infection (Thomson & Knolle, 2010).

2.2 Hepatitis infection

Hepatitis simply means an inflammation of the liver. This condition can resolve on its own (self- limiting) or progress into a chronic state leading to chronic liver diseases (fibrosis, cirrhosis and hepatocellular carcinoma (HCC)) (Bernal & Wendon, 2013). It can be caused by viral and non-viral factors. Non-viral factors include toxics (alcohol, carbon tetrachloride, vinyl chloride and poisonous mushrooms), certain drugs (large dosage of pain reliever; acetaminophen) and autoimmune diseases (WHO, 2016). Viral hepatitis on the other hand is the most common form of hepatitis (WHO, 2016).

Currently, the introduction of modern diagnostic tools have led to the classification of viral hepatitis into five main types; Hepatitis A virus (HAV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Hepatitis D virus (HDV) and Hepatitis E virus (HEV) (Mahoney, 1999; Robinson, 1995).

2.3 Biology of the hepatitis B virus (HBV)

Although both HBV and HCV are found in Sub-Saharan Africa, HBV is the most predominant causative agent of liver diseases in Africa (Ganem & Schneider, 2001). Hepatitis B virus can affect both human and non-human primates (Sa-Nguanmoo *et al.*, 2009). HBV belongs to the family hepadnaviridae because of their hepatotropism (Persistent infection of the hepatocytes with HBV). It is one of the smallest viruses with a diameter of 42-47 nm and a DNA genome, which is a partially double stranded relaxed circular structure (rc-ds) (Locarnini, 2004). Acute infection of the virus is self-limiting, antibodies are developed against HBV to enable clearance of the virus. However HBV infection can progress to chronic infection.

2.4 The hepatitis B virus life cycle

Attachment: When HBV enters the body, by reversible and non-cell type specific binding, the virion binds to receptors, including heparin sulfate proteoglycans, transferrin receptor, the asialoglycoprotein receptor, human liver endonexin, the primary receptor hepatic bile acid transporter and sodium taurocholate co-transporting polypeptide (NTCP) (Figure 2.1; 1) (Yan *et al.*, 2012).

Irreversible binding: There is irreversible binding of HBV ligand to an unknown hepatocyte-specific preS1 receptor. This activates the virus which exposes the myriostolyted N-terminus of the L-protein (Figure 2.1; 2).

The virus then undergoes two entry pathways; the nucleocapsid is endocytosed into the hepatocyte from endocytic vesicles and the viral envelope fuses at the plasma membrane (Figure 2.1; 3a and 3b) (Urban, 2008).

Release of the viral nucleocapsid: The viral nucleocapsid is then released into the cytoplasm of the host's hepatocyte. In the nucleocapsid, the relaxed circular partially double stranded DNA (rcDNA) is connected with the viral polymerase (Figure 2.1; 4).

Transport: The nucleocapsid is transported along microtubules where the capsid accumulates at the nuclear envelope. Accumulation of the capsid enables its interactions with adaptor proteins of the nuclear pore complex (Figure 2.1; 5).

Release of rcDNA: The nucleocapsid is trapped in the nucleus and breakdown to release the viral rcDNA genome into the nucleoplasm (Figure 2.1; 6) (Kann *et al.*, 2007).

Repair: Cellular enzymes mediate the repair of the incoming rcDNA and the removal of the polymerase and short RNA primers from the 5' end of the (-) strand DNA. These products are used for the synthesis of the plus strand. (Figure 2.1; 7) (Urban, 2008).

Covalently closed circular (ccc) DNA formation: After synthesis, the plus strand is ligated to the minus strand to form the covalently closed circular DNA (cccDNA). The cccDNA is organized into a chromatin-like structure (Levrero *et al.*, 2009). A study done on the hepatocytes of HBV-infected transgenic mice showed the absence of cccDNA, suggesting that hosts factors might be responsible for cccDNA formation in humans (Figure 2.1; 8).

Transcription: The cccDNA produces all viral RNAs using the host's cellular transcription machinery/factors such as hepatocyte nuclear factors (HNF), CCAAT/

enhancer-binding (C/EPF) and viral proteins (core and the regulatory X-protein). These host's transcription factors modulate viral gene expression (i.e. protein production and viral replication) by the interaction of viral promoters of the four major overlapping open reading frames (ORF); the pre-core/core gene, the polymerase gene, RNase H and terminal protein domains and X-gene (Pollicino *et al.*, 2006). All mRNAs use a common polyadenylation signal. The host factors such as La RNA binding protein is involved in processing, stabilization and nuclear export of viral RNAs (Figure 2.1; 9).

Translation: The viral RNAs are then exported from the nucleus into the cytoplasm. There are two types of RNAs that are translated, the pregenomic RNA (pgRNA) and subgenomic RNAs (Figure 2.1; 11).

Complex formation: Here, the pgRNA forms a complex structure with the core protein and the viral polymerase at its epsilon stem loop structure (Figure 2.1; 10).

Reverse transcription and Maturation: Within the nucleocapsid, the pgRNA is reverse transcribed into a plus strand DNA. This pgRNA is called the RNA-containing nucleocapsid while the plus strand DNA is called DNA-containing nucleocapsids (Figure 2.1; 13).

Re-importation and secretion: The DNA-containing nucleocapsids can form the cccDNA and re-imported into the nucleus or can be enveloped and secreted. There is also co-translation of the envelope proteins in the endoplasmic reticulum (ER), where they bud out from the ER and secreted as either the 22 nm subviral envelope particles (SVPs) or as 42 nm infectious virions (DNA particles). The 42 nm infectious virions (Dane particles) develop when virions have the DNA-containing nucleocapsids enveloped before budding (Urban *et al.*, 2010). The sera of HBV- infected patients may contain as many as 10^{10} infectious virions per ml. The spherical and filamentous SVPs

do not have envelope and nucleocapsid hence they are non-infectious (Figure 2.1; 14a, 14b, 15, 16, 18).

In a study, hepatocytes infected with duck hepatitis B virus contained cccDNA molecules coming from newly synthesized nucleocapsids. Each hepatocyte contained 1-50 cccDNA molecules, however, the dynamics and efficiency of the cccDNA molecules were different from that of Human hepatitis B virus. These cccDNA amplification is suppressed by negative feedback mechanism involving the L-protein. Also decreased levels of cccDNA results from loss of incoming viruses from blood or inhibition of viral DNA synthesis in the cytoplasm. However, HBV polymerase inhibitors do not directly contribute to the declined levels of cccDNA (Zoulim, 2005).

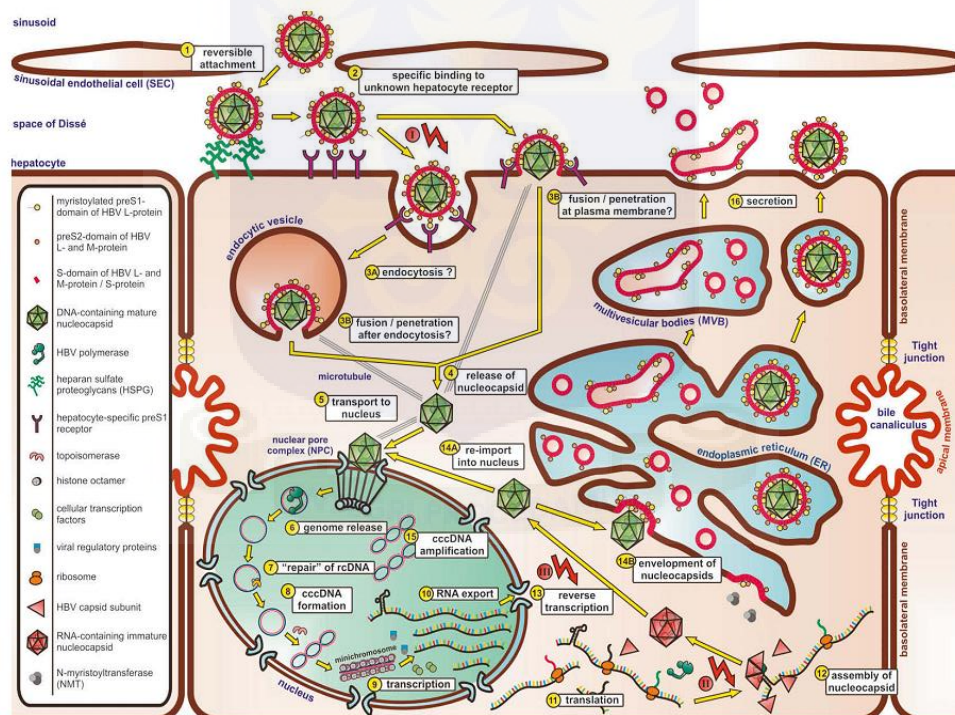


Figure 2.1 A schematic diagram of the replication cycle of hepatitis B virus (Urban *et al.*, 2010)

2.5 Morphology and genome organization of HBV

2.5.1 Description of the morphology

The outer surface envelope (viral coat) is made of protein, lipid and carbohydrate which encloses an icosahedral nucleocapsid, which consists of the genomic DNA of the virus

(HBV DNA), endogenous DNA polymerase, HBV core antigen (HBcAg) and the HBV protein X. The proteins embedded in the outer envelope are used by the virus for attachment and entry into susceptible hepatocytes. The outer envelope (outer lipoprotein coat) contains lipids derived from susceptible hosts and polypeptides of the S genes (i.e. large (L), middle (M) and small (S) surface proteins also known as the pre-S1, pre-S2 and the HBsAg) (Figure 2.7) (Locarnini, 2004).

Researches have also categorized HBV into three distinct morphologies; the spherical non-infectious particles which are highly detected in the sera at a concentration of 10^{13} particles per ml and contains the outer envelope layer (Figure 2.7) (Luckenbaugh *et al.*, 2015), the filamentous forms which are tubular with a diameter similar to that of the small particles, HBsAg. It also contains the HBsAg polypeptides (Figure 2.7) (Luckenbaugh *et al.*, 2015) and the 42 nm hepatitis B virion or Dane particles which are spherical in shape with a complex morphology. They are made of an outer envelope and an inner core nucleocapsid with a diameter of 27 nm (Asabe *et al.*, 2009).

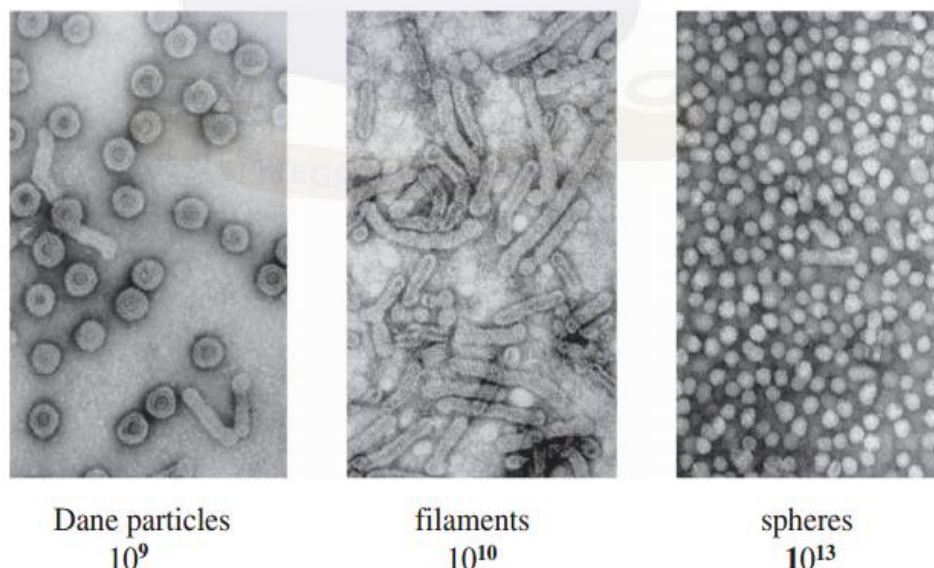


Figure 2.2 Electron micrographs of the morphological forms of the hepatitis B virion (Gerlich, 2013).

2.5.2 Genome organization and associated proteins

The double stranded HBV DNA genome has a fixed 5' end and a variable 3' end. The double stranded region consists of a minus and a plus strand. The plus overlaps the 5' and 3' extremities of the minus strand causing the circularity of the HBV DNA. The circularity of the HBV DNA is maintained by its 5' ends (Minor & Slagle, 2014). Both strands are made up of gaps although the minus strand is almost complete and a unit length while the other is incomplete and less than a unit length. The 5' end of the minus strand is covalently linked to a protein and maps within the region termed as DR1 (direct repeat). The plus strand, however, begins with DR2 and has an oligonucleotide capped to its 5' end. These repeats serve as primers for the synthesis of their respective DNA strands (Minor & Slagle, 2014). The single-stranded region also known as gap has a fixed polarity but variable length. The viral polymerase is able to repair these gaps and generate a full double genome (Minor & Slagle, 2014). The negative or minus strand acts as a template for the four partially overlapping open reading frames (ORFs) or mRNA transcripts synthesis. HBV has a very organized coding structure in which the ORFs are translated into seven proteins. Each of the four ORFs encodes different proteins; the ORF P codes for the polymerase of the virus and a terminal protein linked to the minus strand DNA, the ORF C codes for the nucleocapsid (structural protein), HBeAg (non-structural secreted protein) and HBcAg, the ORF S/ pre-S codes for the viral surface glycoproteins and the ORF X which codes for the HBV small protein X (HBx). Non-coding regions are not present (Minor & Slagle, 2014). These mRNA transcripts can further be divided into the genomic and the sub-genomic. The sub-genomic RNAs make up ORF S and ORF X, whereas the genomic RNAs are bi-functional and act as a template for viral DNA synthesis. It consists of ORF C and ORF

P. There are four promoters of the virion; pre-S1, S, genomic and X (Minor & Slagle, 2014).

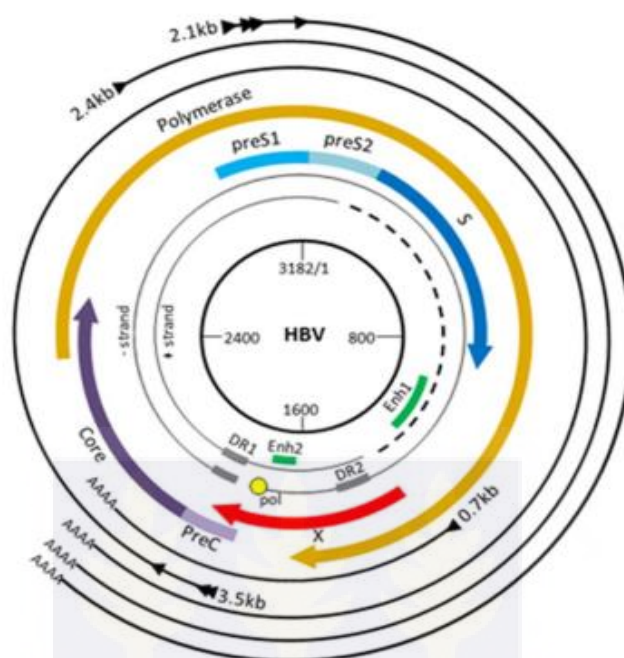


Figure 2.3: The genomic organization of HBV (Minor & Slagle, 2014).

2.5. 3 Features of the viral proteins

HBsAg

It contains three proteins; Small, Large and Medium proteins. S domain also called the S protein is 24 kD. Glycosylation of the S domain releases two protein isoforms. L protein which contains the pre-S1, pre-S2 and S regions is 39 kD. M protein which is 31 kD contains the pre-S2 and S regions. HBsAg is the most abundant antigen. The expression levels of the L and M are about 5-15% whereas the S protein is expressed in levels of about 1-2% (Kramvis, 2016). These three enveloped proteins are not uniformly distributed amongst the different types of HBV particles. For instance, the sub-viral 22 nm particle is predominantly made up of S proteins with some amount of M and L proteins. The viral or Dane particle on the other hand is mainly made of L

protein, this is because the L protein contains a receptor recognition domain that allows effective binding to cell surface receptors on hepatocytes (Kramvis, 2016).

HBcAg and HBeAg

The ORF C has two AUG codons; a more internal start codon and an upstream AUG. The HBcAg or core particle is 21 kD in size and a product of the internal start codon. It is a highly conserved polypeptide found in hepadnaviruses that infect mammals. It is expressed on the surface of hepatocyte and initiates cellular immunity. HBeAg on the other hand is an immunologically distinct antigen of 16-18 kD, and the product of the upstream AUG. It is secreted and accumulated in the serum of an HBV-positive patient and not incorporated into the virions (Robinson, 1995).

The polymerase protein (reverse transcriptase activity and a protein kinase activity)

It is a multifunctional enzyme, made of a DNA-dependent DNA polymerase, reverse transcriptase and RNase enzymes. It acts as a primer by binding to the 5' end of the HBV genome where an intermediate RNA is reverse transcribed into the negative strand DNA. It also plays a role in enclosing the viral pre-genomic RNA in a capsid. The polymerase can induce an immune response during chronic or acute infection (Ganem & Prince, 2004).

Protein X

The X protein (HBx) is encoded by the smallest ORF X of the genome with a size of 17 kD. It acts as a transactivator for both the viral S promoters and core protein. It also serves as a regulatory protein that enhances the expression of cellular genes (Robinson,

1995). It is a non-structural protein made up of 154 amino acids (Bouchard & Navas-Martin, 2011). The sequences of the X protein are the least conserved.

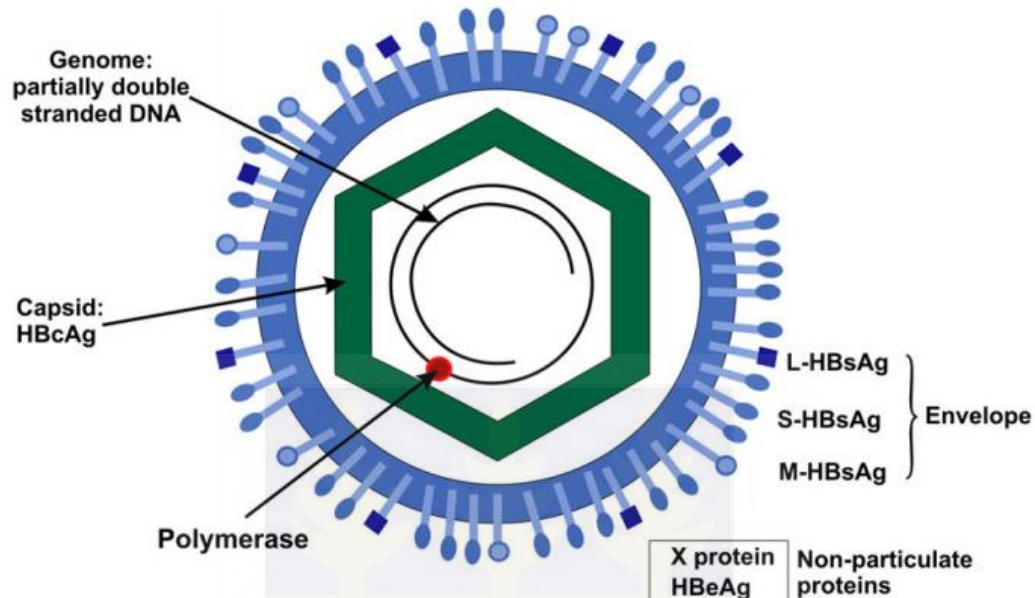


Figure 2.4: A schematic representation of HBV, showing the virion surface antigens (L, M, S HBsAg) embedded in the outer lipid envelope. The lipid envelope encloses the capsid, comprising the HBcAg. The capsid surrounds the partially duplex stranded viral DNA. The DNA polymerase is indicated as the red dot. The virion also expresses the X protein and the HBeAg (Kramvis, 2016)

2.6 The role of HBV genes and proteins in the clinical manifestation of HBV

HBsAg

An increase in expression levels of HBsAg is a contributing factor to the progression of hepatitis B infection to chronic liver diseases. A longitudinal study done on 2,946 HBsAg seropositive patients suggested that the risk of developing HCC reduces with declined levels of serum HBsAg (Liu *et al.*, 2013). Recent novel mutations in the trans-membrane C terminal domain of the S gene is critical for the reduction of HBsAg secretion and retention of intracellular HBsAg accumulation, leading to an increase in cellular proliferation and increased risk of HCC development (Salpini *et al.*, 2017).

HBeAg

It is involved in transmissibility and active viral replication, thus, it is associated with active progression of liver diseases. A study has suggested that HBeAg positivity is involved in 11.2 fold increased risk of cirrhosis and 6.7 fold increased risk of HCC (Mendy *et al.*, 2010). Seroconversion of HBe to hepatitis B envelope antibodies (anti-HBe) leads to decline in viral replication (Candotti *et al.*, 2007). However some studies have shown an increase in viral replication even in the presence of anti-HBe, and this is as a result of pre-core and basal core mutations (Chowdhury, 2004).

Protein X

Studies of HBV replication in model systems suggest that HBx strongly influences viral replication (Lim *et al.*, 2010). Analyses performed in HBx-transgenic mice suggested that HBx has the potential to cause oncogenesis (Koike *et al.*, 1994). HBx can also affect cytosolic calcium levels, regulate signal transduction and transcription pathway, DNA repair, degradation of proteins and apoptosis (Clippinger *et al.*, 2009; Rawat & Bouchard, 2015).

2.7 Clinical manifestation of mutations in the HBV genome

There have been the discovery of genetic variations in the envelope, pre-core, core and polymerase genes of the wild-type HBV (Shen & Yan, 2014), with the mutants having a survival advantage over the wild types. For example, antigenic variation in the envelope leads to the evolution of a vaccine-induced mutant which has an increased chance of survivability during immune selective pressure (e.g. during hepatitis B Ig treatment and HBV vaccination). The vaccine-immune mutants are able to replicate in the presence of the vaccine and prolong antiviral treatment (Torresi, 2002; Yang *et al.*,

2002). There is also a pre-core mutant (HBV83) produced from a point mutation from nucleotide G to A at position 83 in the basic core promoter (BCP) or pre-core gene (Okamoto *et al.*, 1994). The mutant, HBV83, with an amino acid change at position 144, is the most common mutation (Okamoto *et al.*, 1994). This nucleotide mutation of mutant HBV83, also introduces a stop codon at position 28 of the HBeAg, thus preventing HBeAg synthesis as observed in the case of HBeAg carriers, thus resulting in active viral replication in these carriers, although the patients are HBeAg negative or anti-HBe positive. Since the HBeAg cannot be detected in the subjects, liver inflammation and fibrosis can easily result (Chowdhury, 2004; Hadziyannis & Vassilopoulos, 2001). These forms of pre-core mutation are normally seen in fulminant hepatitis, active chronic hepatitis and asymptomatic chronic carriers (Gitlin, 1997). These mutations are found in regions of B and T cells epitopes though there is no study of their involvement in immune evasion mechanism.

There is also a duplex mutation in the BCP region at positions A1762T and G1764A that results in the production of HBeAg, and a down regulation of the transcription of pre-core mRNAs. Studies have shown that the duplex mutation is associated with the development of HCC (Kao *et al.*, 2003). A longitudinal study done by Iloeje and colleagues on 3,582 Taiwanese patients with chronic hepatitis B infection showed that 11 years without treatment caused an increase in HBV DNA levels and increased incidence of cirrhosis in the duplex mutant subjects (Iloeje *et al.*, 2006).

The polymerase of the HBV is error-prone and therefore causes mutation. However, other mutations can compensate for the impaired function. These mutations occur in the region of the reverse transcriptase (rt) in the HBV polymerase gene at positions rt 180 and rt 204, and are responsible for resistance in lamivudine antiviral therapy

(Niesters *et al.*, 2002). Pre-S deletions have also been thought to hasten the storage of large envelope proteins in the cytoplasm of the hepatocytes, which are associated with the rapid progression of liver disease and HCC (Liu & Kao, 2007; Sinn *et al.*, 2013). In Ghana, Ampah *et al.* in their research discovered a series of gene mutations in Ghanaian patients with HBV genotype E infection (Ampah *et al.*, 2016). They discovered a Ser143Leu substitution in the “a” determinant of the HBsAg. This particular codon mutation is responsible for false negativity during HBsAg immune-assay diagnostic. They also discovered the presence of the mutations Leu216 (sLeu216*) and Trp182 (sTrp182*) of the S gene which have been reported to have oncogenic properties and therefore a contributing factor to the development of HCC (Ampah *et al.*, 2016). A study done to show the interactions between basal core promoter mutation, pre-core mutation and pre-S deletions at the various stages of HBV-acquired liver diseases in chronic carriers suggested that all three mutations caused a greater risk of rapid progression of liver diseases than a single mutation (Xu *et al.*, 1997).

The HBV also encodes for an oncogenic protein, the HBx, which has been shown to promote carcinogenesis in chronic hepatitis B carrier patients (Liu & Kao, 2007). The exact mechanism by which this protein contributes to carcinogenesis is understudied, but few studies done in transgenic mice demonstrated that over expression of HBx leads to carcinogenesis (Liu & Kao, 2007; Wu *et al.*, 2006). HBV genotypes that encode HBx have been established to have a higher hepatocarcinogenic potential than those without HBx (Datta, 2008).

2.8 Antigenicity

The HBcAg and core particles are internal components, thus do not present in the blood as free forms. The HBcAg is found on the surface of the core particles. The core antigen

share sequences with the “e” antigen (HBeAg). Both the HBeAg and the HBcAg are called soluble antigens but they do not cross react with each other (Robinson, 1995).

In terms of immunity, HBsAg induces the anti-HBs by humoral immunity while the viral structural proteins induce cytotoxic T-cells by cellular immunity that help eliminate HBV infected hepatocytes. HBsAg is heterogeneously antigenic and this is due to the fact that it has antigenic determinants that result from antigenic epitopes present on the envelope proteins. The antigenic determinants include a common antigen designated by the letter “a” and any two of these antigens; d and y, w and r. These sub-determinants give rise to four subtypes; adw, ayw, adr and rare ayr (Kramvis, 2016). These subtypes are geographically distributed. Protection against one subtype confers the same protection for all other subtypes, thus there is no significant differences in their clinical features, and this is due to the common “a” sub-determinant (Robinson, 1995).

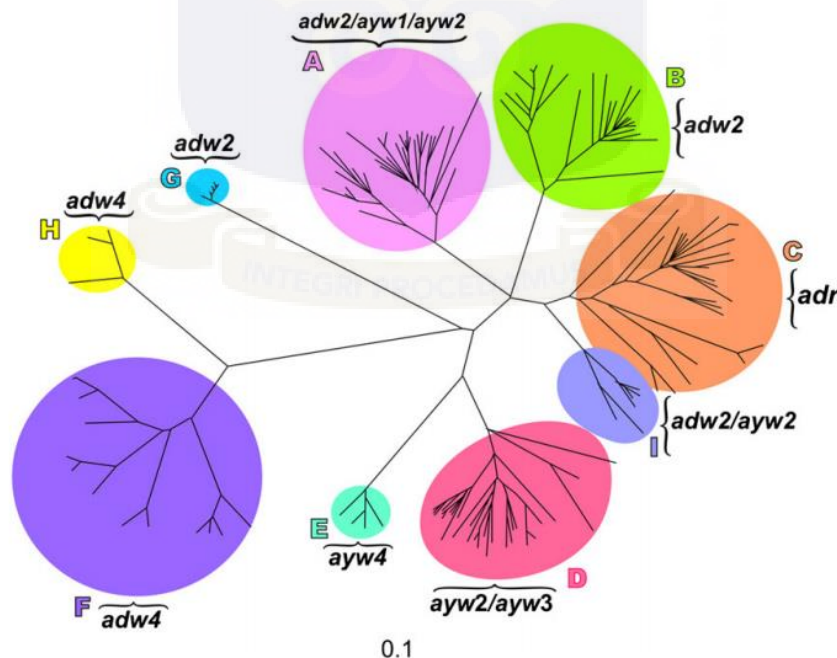


Figure 2.5: A phylogenetic tree of the whole genome sequences of nine HBV genotypes (A-I) and their respective serological subtypes (Kramvis, 2016).

2.9 HBV genotypes

The wide diversity in the HBV genome has led to evolution of the 10 recognized genotypes (A-J). The HBV strains are further classified into sub-genotypes except for E, G and H genotypes (Pourkarim *et al.*, 2014).

2.9.1 Characteristics of HBV genotypes

Genotype A (HBV/A): It was discovered in 1988 and characterized by 6 nucleotides insertion at the carboxyl end of the core gene (Okamoto *et al.*, 1988; Shi *et al.*, 2013).

Genotype B (HBV/B): It was also discovered in 1988. It is made of sub-genotypes with recombination with genotype C in the pre-core/ core region and those that show no recombination (Shi *et al.*, 2013).

Genotype C (HBV/C): It is the oldest HBV genotype with the highest number of sub-genotypes. The presence of the many sub-genotypes reflects its long existence of being endemic in humans (Pancawardani *et al.*, 2012).

Genotype D (HBV/D): It is widely distributed globally and characterized by 33-nucleotides deletion at the amino terminus of the pre-S1 region (Murhekar *et al.*, 2008).

Genotype E (HBV/E): It is characterized by three nucleotides deletion at the pre-S1 region. It has a low degree of genetic diversity and hence does not divide into sub-genotypes (Mulders *et al.*, 2004).

Genotype F (HBV/F): It was discovered by Norder *et al.* and has a wide intra-genotypic diversity (Norder *et al.*, 1994).

Genotype G (HBV/G): In 2000, this genotype was defined as the seventh HBV genotype when it was isolated from a French patient with hepatitis B infection (Stuyver

et al., 2000). It has 36 nucleotides insertion in the core region and has a genome length of 3,248 base pairs.

Genotype H (HBV/H): Phylogenetic analysis has revealed a close relation between HBV/H and HBV/F and has been proposed that it might be a sub-genotype of HBV/F (Arauz-Ruiz *et al.*, 2002).

Genotype I (HBV/I): In 2008, this genotype was isolated from a Vietnamese male with HBV infection. After a complete genome analysis, it was established that it is closely related to HBV/A, HBV/C and HBV/G (Huy *et al.*, 2008).

Genotype J (HBV/J): It was isolated from a Japanese male with hepatocellular carcinoma. Phylogenetic analysis revealed that its preS-S region is closely related to that of orangutan (with mean divergence of 10.9% and 10.7%) and the core region to that of humans (Tatematsu *et al.*, 2009).

2.9 2 Geographic distribution of Hepatitis B virus genotypes

The HBV genotypes are geographically distributed within and between regions and serve as an important tool for determining the severity, vaccine efficacy and treatment response (Norder *et al.*, 2004).

For instance, genotype A (HBV/A) is predominant in Sub-Saharan Africa. The genetic diversity of A is wider in Africa than in other parts of the world. There are seven subtypes of HBV/A, A1-A7 (Kramvis & Kew, 2007b). A1 dominates in Asia and Africa (Kramvis & Kew, 2007a) and A2 is found in Northern Europe and United States (Norder *et al.*, 2004). Genotype B (HBV/B) is mostly found in Indonesia, China and Vietnam and the genotype C (HBV/C) is predominant in East Asia; Korea, China, Japan, Polynesia and Vietnam. HBV/B has 9 sub-genotypes. In one part, the HBV/C is recombined into the core region of the HBV/B to form sub-genotypes B2 to B5 whiles

the B1 and B6 are not recombinants (Hadziyannis & Vassilopoulos, 2001; Sakamoto *et al.*, 2007). Genotype D (HBV/D) has 9 sub-genotypes which is mostly predominant worldwide (Norder *et al.*, 2004). The D1 sub-genotype mostly occurs in the Middle East and the D2 has been reported in Northeast Europe (Lazarevic *et al.*, 2007; Olinger *et al.*, 2008). The D3 is found in Italy and D4 in Oceania (Hübschen *et al.*, 2009; Olinger *et al.*, 2008). The D5 to D9 are distributed in Guinea, Tunisia, Nigeria and India (Kitab *et al.*, 2011). The genotype E (HBV/E) is mainly limited to Africa especially Western Africa, but also found in people with African decent in other continents (Vray *et al.*, 2006). The genotype F (HBV/F) is found in Indigenous America (Livingston *et al.*, 2007). Genotypes G (HBV/G) and genotype H (HBV/H) are prevalent in Atlanta (Georgia, USA) and Lyon (France), respectively (Lok *et al.*, 1987). The two new strains, genotype I (HBV/I) and genotype J (HBV/J) evolved from Laos-Vietnam and Japan, respectively. HBV/I is a novel recombinant of HBV/A, HBV/C and HBV/G whiles the HBV/J has a mixed recombination (Tatematsu *et al.*, 2009).

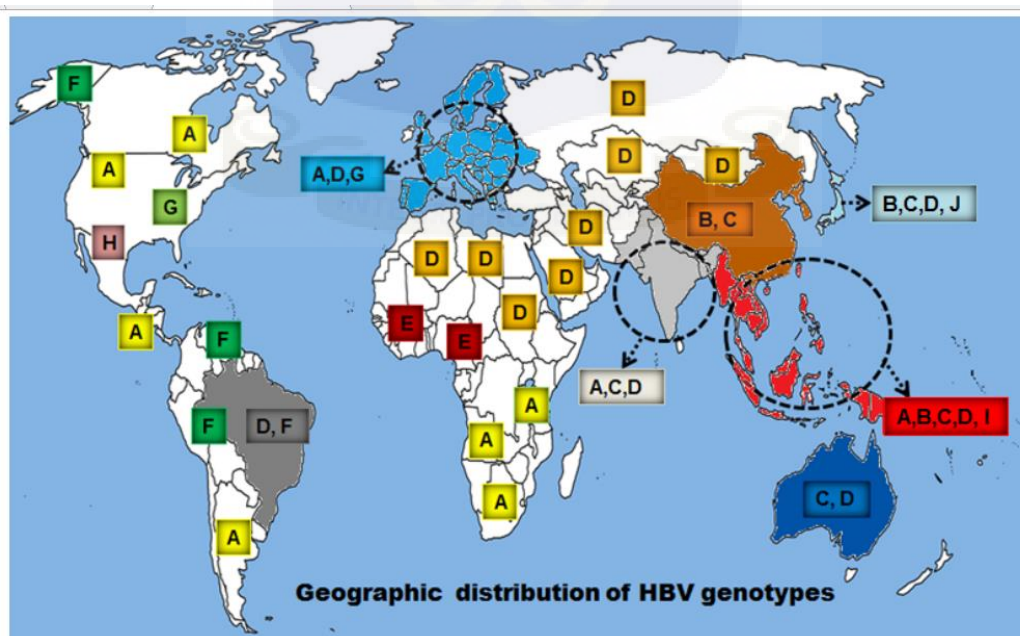


Figure 2.6 Worldwide geographical distribution of HBV genotypes (A-J) (Tatematsu *et al.*, 2009).

2.10 Importance of epidemiology and molecular characterization of HBV

The risk that a chronically infected person will develop chronic liver disease which can further lead to liver failure is 15 to 40% (Lok, 2002). The prevalence of chronic HBV could be categorized into high, intermediate and low endemicity according to the intensity of the infection. In developed countries such as North America, Northern-Western Europe and Australia, endemicity is low with prevalence less than 2% (WHO, 2017). The Eastern and Southern Europe, the Middle East, Japan and parts of South America are classified as intermediate endemic areas with HBV prevalence of 2 to 7% occurring in all age groups (WHO, 2017). The developing countries such as South East Asia, Sub-Saharan Africa and the Amazon Basin are classified as highly endemic areas with endemicity of 8% and above (WHO, 2017).

In Sub-Saharan Africa, the prevalence of acute and chronic HBV is about 8% or more. Previously, HBV infection occurred mostly in infancy and early childhood (Hou *et al.*, 2005). This was because during the early stages of childhood, acute HBV infection is asymptomatic and not easily recognized until it progresses into the chronic stages. However, recent reports have indicated that most people of the youthful ages are becoming more vulnerable to HBV infection (WHO, 2016). Thus, the rate of HBV-related chronic liver diseases are very high. Although there is the introduction of the HBV vaccine programme, the low-income rate and ignorance to the administration of HBV vaccine are major contributing factors preventing the universal implementation of the vaccine. The epidemiology of HBV is higher in the rural settings than urban settings, there is also greater risk for males becoming chronic carriers than females and the prevalence of HBV is high in the younger age groups. Researches have established the prevailing HBV genotypes in Africa to be A, D and E (Zampino *et al.*, 2015). African countries such as Nigeria, Gabon, Cameroon, Burkina Faso and Ghana are

categorized as HBV high hyper-endemic regions with >8% HBsAg chronic carriers in the general population. Ghana, for instance, has an HBsAg chronic carrier rate of 13.8%, however molecular epidemiology study of HBV is limited. The few molecular based studies are concentrated in few regions in Ghana. In the Ashanti region of Ghana, a study of HBV done in deferred blood donors identified genotypes A, E and D as the prevailing genotypes (Candotti *et al.*, 2006). In the Greater Accra region, research on HBV chronic patients reported genotypes A, D and E being the circulating genotypes (Dongdem *et al.*, 2016). Another study in a rural setting in Ghana has also reported the presence of the HBV/E among the inhabitants, with some of the isolated strains having certain mutations (Ampah *et al.*, 2016). A more recent study by Brah and colleagues has revealed the presence of a recombinant virus, HBVA3/E, in Ghanaian patients with HBV infection (Brah *et al.*, 2016).

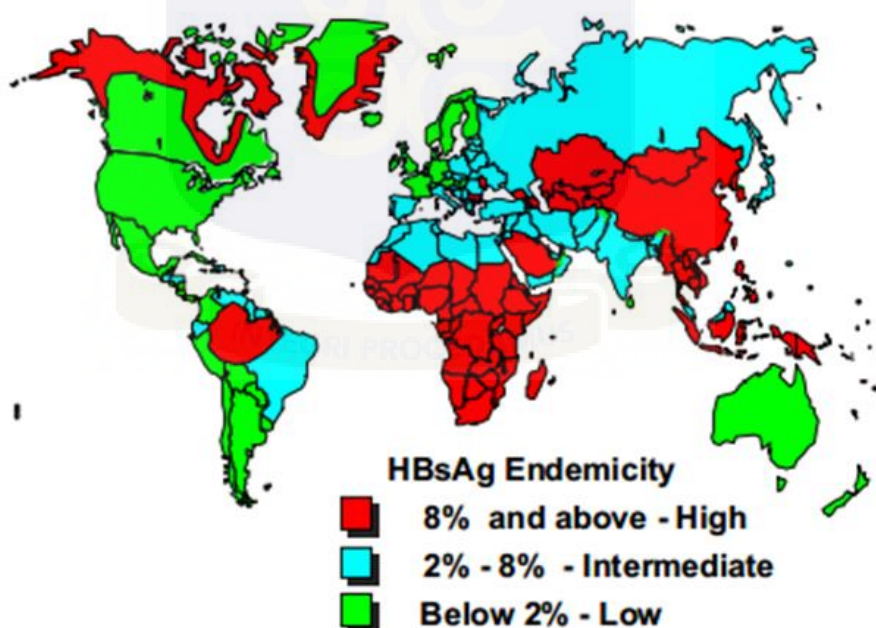


Figure 2.7: World map of HBV infection endemicity (Ott *et al.*, 2012)

2.11 Clinical outcomes of HBV genotypes

Acute and fulminant hepatitis

Many studies have shown that the development of acute forms of hepatitis B may not necessarily be based on a particular genotype. A lot of data have suggested that genotype A, B and D predominate in patients with acute forms of HBV than other genotypes such as the genotype C (Suzuki *et al.*, 2005; Wai *et al.*, 2005). However, genotype D has been shown to be more involved in Acute Liver Failure (ALF) (Wai *et al.*, 2005).

Seroconversion of HBeAg and HBsAg

In the chronic phase of HBV infection, HBeAg can persist in the replicative phase for a long period as a result of slower rate of HBeAg seroconversion. This may lead to the progression of chronic liver diseases. Most researches have proven that, HBV/C infected patients are mostly positive for HBeAg with higher serum levels of HBV DNA than those infected with HBV/B (Kao *et al.*, 2002b). To some extent, it can be concluded that HBV/C persists in the immune clearance phase thus presenting severe hepatitis outcome. Other researches have also compared the clinical differences between HBV/A and HBV/D, where HBV/D was found to be associated with significantly low HBeAg seropositivity as compared to HBV/A, however HBV/A had better prognosis of liver diseases (Sánchez-Tapias *et al.*, 2002).

HBV carriers are also at an advantage when they develop HBsAg seroclearance, they are favoured in terms of cirrhosis and hepatocellular carcinoma development (Liaw *et al.*, 1991). Many researchers have suggested that HBV/B and HBV/D infected patients have higher HBsAg seroclearance rate than patients with HBV/C and HBV/A (Yuen *et al.*, 2002).

Chronic hepatitis

The severity of HBV infection is its ability to cause chronic infection which can lead to the development of liver cirrhosis. Most studies have already established that HBV/C is involved with the persistence of HBeAg in the serum, thus presenting a more aggressive outcome as compared to other genotypes (Kao *et al.*, 2002a). A follow up study in chronic HBeAg-positive patients confirmed that disease activity increases in HBV/C infected patients in comparison to HBV/B patients (Chan *et al.*, 2003).

Cirrhosis and hepatocellular carcinoma

Since there is rapid development of chronic phase in HBV/C patients, most studies have confirmed that it may lead to severe liver diseases. Many studies done on HBV carriers have indicated that HBV/C and HBV/B infections are involved in increase development of HCC and normally dominates in cirrhosis and HCC patients (Kao *et al.*, 2000). However, the pathogenicity of HBV/C as compared to HBV/B is high, due to its association with rapid HBV DNA replication, higher viral load and high serum HBV DNA levels (Liu *et al.*, 2009).

Researches have also been done to establish the development of HCC in other HBV genotypes. HBV/F has been reported to be more predominant in liver disease related deaths than HBV/A and HBV/D (Sánchez-Tapias *et al.*, 2002). HBV/F is also associated with HCC in children often without cirrhosis (Livingston *et al.*, 2007).

Generally, molecular studies have shown that HBV/C and HBV/D are associated with more severe liver injuries with higher incidence of HCC than with HBV/A or HBV/B (Kramvis & Kew, 2005; McMahon, 2009).

Influence of HBV genotypes on antiviral therapy response

Interferon Alpha

Response rate of HBV genotypes to antiviral treatment is defined as normalization of serum aminotransferase level, loss of HBeAg and HBV DNA in 48 weeks after therapeutic administration. There have been reports that the pathogenic differences of the genotypes influence the current antiviral treatment responses. HBV/A and HBV/B infected individuals treated with interferon therapy have higher response rates than HBV/D and HBV/C individuals (Hou *et al.*, 2007). An additional study also revealed that chronic HBV carriers with HBV/C and HBV/D have a lower response rate than those infected with HBV/A or HBV/B (Erhardt *et al.*, 2005; Wiegand *et al.*, 2008).

Lamivudine

Response to Lamivudine can be sustained in HBV/B infected patients for a longer period than those with HBV/C infection, thus the likelihood of recurrence of disease in HBV/C patients is high (Liu *et al.*, 2004).

Adefovir Dipivoxil and Entacavir

Most studies have suggested no significant relation between HBV genotypes and response to entacavir therapy (Westland *et al.*, 2003).

In summary, there is a correlation between HBV genotypes response to conventional IFN but otherwise for nucleotide-based therapy. HBV genotypes and response to PEG-IFN remains inconclusive.

HBV genotype mutation

Higher viral load and naturally occurring mutation in HBV genotypes are also involved in the outcomes of HBV-related chronic liver diseases (Kao, 2007; Lin & Kao, 2008). HBV/C has been established to confer a higher frequency of basal core promoter (BCP) A1762T/G1764A mutation than other genotypes (Kao *et al.*, 2000). Yu and colleagues

established that HBV/C is associated with high viral load and has a 26-fold increased risk of development of HCC than other genotypes (Yu *et al.*, 2005). There is a high frequency of pre-S deletion in HBV/C than HBV/B.

Similarly, patients with terminable liver diseases were found to have HBV/D infection with higher frequency of BCP A1762T/G1764A mutation than HBV/A infected patients.

2.12 Pathogenesis

HBV enters the liver rather than the lymphoid tissues to escape T-cell lymphocytes recognition. It replicates in the liver cells or hepatocytes leading to the interference of the liver function. The replicative cycle of HBV poses minimal injury to the liver as seen in asymptomatic carriers despite the intrahepatic replication of HBV. This is because HBV is not cytotoxic to the hepatocytes (De Franchis *et al.*, 1993). Although HBV antigens can be presented to tolerogenic non-parenchymal liver cells or expressed on hepatocytes and directly presented to MHC molecules. The humoral and cellular responses of HBV infection is not well understood, however studies have shown a strong correlation between strength of host immunity, severity of disease and viral clearance (Ganem & Prince, 2004; Villeneuve, 2005). The immune response involves the major-histocompatibility -complex (MHC) class II-restricted, MHC class-I restricted, CD4⁺ helper T-cells and CD8⁺ cytotoxic T lymphocytes (Ganem & Prince, 2004). The antiviral cytotoxic T-lymphocytes (CTL) respond to the numerous epitopes on the HBV core, polymerase and the envelope proteins. Viral oligopeptides (viral antigens) bind to MHC class I molecules and presented on the surface of the hepatocytes. These viral oligopeptides on the surface of the hepatocytes are then recognized by virus –specific CD8⁺ cytotoxic T-cells with the CD4⁺ cells. These T-

cells can cause direct lysis of the infected hepatocytes. The T-cells can also cause the indirect release of interferon γ (IFN γ) and tumor necrosis factor alpha (TNF α), which can down regulate viral replication without cell lysis (Webster *et al.*, 2000). The hepatocyte destruction triggered by this inflammation leads to acute viral hepatitis infection. The CTL has also been demonstrated to inhibit viral replication and expression of genes in hepatocytes of a patient with acute hepatitis (Webster *et al.*, 2000). Thus HBV infection can be self-limiting when the host develops immunity against the virus. However, chronic infection can result when the HBV is not eliminated from the hepatocytes. This can be due to disturbance in the balance or up regulation of the viral population by active replication as against the host's immunodefences. Thus, CTL response to HBV is vigorous and multi-specific but does not result in the clearance of the virus, and so a patient with weakened immunity can progress to chronic HBV (Chisari, 1997). During chronic infection, the HBV genome is integrated into the genome of the host. For as long as integration exists, hepatocellular carcinoma may occur. The mechanism that results in the inability of the viral-specific T-cells to respond in chronic carriers of hepatitis B is not well understood but is thought to include deletion, energy exhaustion and dysfunction of T-cell (Chisari, 2000). The host-virus interaction is the main factor for HBV infection pathogenesis (Baumert *et al.*, 2007).

2.13 HBV Co-infections

Infections of Hepatitis B virus and hepatitis C virus account for most liver diseases worldwide. Co-infection of the two viruses are very common because both viruses have similar mode of transmission. HBV/HCV leads to severe liver disease and can increase the likelihood of liver cancer development than HBV and HCV alone (Michielsen *et al.*, 2005). Treatment of HBV/HCV represents a great challenge thus treatment is

administered individually based on the patient's DNA or RNA levels, patient's prior exposure to antiviral treatment, and based on other infections such as hepatitis D virus and human immunodeficiency virus (HIV). Since HDV can only propagate in the presence of HBV, it can lead to co-infection with HBV or super infection in individuals infected with chronic HBV (Michielsen *et al.*, 2005). Co-infection or super infection of HBV/HDV like that of HBV/HCV results in the likelihood of liver failure, liver cirrhosis or HCC which is also difficult to treat (Michielsen *et al.*, 2005). HBV/HIV co-infection can also contribute to an increased risk of progression to liver disease and liver failure (Michielsen *et al.*, 2005). In order to prevent these infections and avoid increase mortality in chronic hepatitis B carriers, constant monitoring and treatment is required.

2.14 Mode of transmission

HBV spreads through contact with infected body fluids such as blood, blood products and serous fluids. There are also lower levels of the virus found in other body fluids such as seminal and vaginal fluids, saliva, tears, sweat, urine, feces, breast milk, cerebrospinal fluids and amniotic fluids. However these fluids have not been associated with transmission except saliva. Saliva can be a source of transmission by biting but there is no documentation that it can be transmitted through kissing (Margolis *et al.*, 1991). Basically there are three modes of transmission of HBV; perinatal, sexual and parental/percutaneous transmission (Hou *et al.*, 2005). Perinatal transmission of HBV occurs when a chronically infected mother infects her infant by contact to maternal blood or mucous membranes at the time of delivery. There is an incident rate of 70-90% when mothers are both HBsAg-positive and HBeAg-positive and less, when

mothers are positive for HBsAg and negative for HBeAg (about 10-30%) (Hou *et al.*, 2005).

Sexual transmission, either homosexual or heterosexual is the main cause of HBV transmission. Homosexuals have a higher risk of acquiring HBV infection (70%) but heterosexuals account for the increased rate of HBV infections (Alter, 2003).

The parental transmission includes intravenous drug use, blood transfusion, dialysis, working in healthcare settings, tattooing and household contacts. Drug injection is the main mode of infection in drug users (Margolis *et al.*, 1991), blood and blood products recipients can also be infected although blood and its products are screened before transfusion. If the blood donor is an asymptomatic carrier and HBsAg-negative, the recipient stands a chance of being infected (Margolis *et al.*, 1991). Health workers can also be infected by prick from infected needle stick injury during medical procedures. Nosocomial spread of HBV infection normally occurs in dialysis and dental units (Margolis *et al.*, 1991). There is also person to person transmission which includes contamination of skin lesion among pre-school age children and long term household contact with a chronically infected person. There is no evidence of HBV transmission by contaminated food or water or by a vector (Hou *et al.*, 2005). The HBV vaccine was introduced in 1982 for protection against HBV during perinatal, childhood and adult HBV infections. Brands of the vaccines are Recombivax HB, Engerix-B, Elovax B, Genevac B, Shanvac B and Twinrix (CDC, 2009). There are also antiviral drugs to fight chronic infection and slow down the liver damage by suppressing viral replication to prevent liver damage (Keeffe *et al.*, 2006). These drugs, currently approved by the U.S.A FDA for treatment of chronic HBV infection includes; the nucleot(s)ide analogues (Epirir HBV) Lamivudine, (Hepsera) Adefovir, (Beraclude) Entecavir,

(Tyzeka) Telbivudine, Tenofovir, the (Intron A) interferon (IFN α) and long acting interferon (Pegasys) Pegylated Interferon alpha (PEG-IFN α).

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2.15 Diagnostics of HBV

HBV is highly infectious and responsible for about 80% of HCC cases worldwide hence better diagnostic methods are required for effective discrimination of HBV infection from other pathogen causing-infections. The most common conventional diagnostic technique for HBV is the serological test which involves measurement of several hepatitis B virus specific antigens and antibodies (example HBV surface antigen (HBsAg), hepatitis B envelope antigen (HBeAg), human antibodies against the antigens (anti-hepatitis B surface antigen (anti-HBs), hepatitis B core antibody (anti-HBc), IgM). Serological markers are used to determine whether a person has acute or chronic HBV infection, or whether the person is immune, vaccinated or susceptible to the infection, on-going or past HBV infection (Lok & McMahon, 2007). A wide range of serological techniques have been developed, which includes agar gel diffusion, immunoelectrophoresis, reverse passive haemagglutination and the more sensitive Enzyme Linked Immunosorbent assay (ELISA). ELISA can detect HBsAg concentration more than 0.1 ng/ml (Lok & McMahon, 2007) and useful in detecting other HBV markers for differentiating between acute and chronic phase of the disease. This assay can be used to detect low levels of DNA (5 pg/ml), however a more sensitive molecular diagnostic method such as PCR can detect HBV DNA as low as 10^{-3} pg/ml (Datta *et al.*, 2012). Hybridization technique can be used to detect active viral replication which is similar to HBeAg detection whiles PCR detects current infection by detection of HBsAg. Monitoring HBV DNA is necessary for determining treatment response. Other techniques such as nucleic acid sequence technique and RFLP are used

for characterization of hepatitis B strains (Lok *et al.*, 2007). Genotyping has now become the focus of most studies because of the correlation between the genotypes and disease outcome (Cao, 2009; Kao *et al.*, 2000). Also histology can be used to detect the extent of liver degeneration by taking liver biopsy.

2.15. 1 Molecular diagnostics

In the past decades, the introduction of nucleic acid amplification based method and isothermal nucleic acid amplification have greatly improved the field of HBV detection in body fluids and tissues. The conventional methods of diagnostic of HBV have been based mainly on the immune-detection of HBsAg until the issue of serologically negative HBV infections (HBeAg-negative chronic hepatitis B (CHB), occult HBV infection, emergence of escape mutants and low antigen levels), thus the introduction of molecular methods (Datta *et al.*, 2012; Gerlich, 2013; Weber, 2005). These molecular methods include nucleic acid based detection assays for HBV DNA (NAB), polymerase chain reaction (PCR), qPCR and isothermal amplification methods such as nucleic acid sequence based amplification (NASBA), transcription mediated amplification (TMA), loop mediated isothermal amplification (LAMP) and rolling circle amplification (RCA) (Craw & Balachandran, 2012). The introduction of these methods are necessary for identification of viral DNA since they are highly sensitive, specific and can tolerate and sequence variation (Datta *et al.*, 2012).

2.15.1.1 Polymerase Chain Reaction Based Strategies (PCR)

PCR uses thermostable Taq polymerase and sometimes reverse transcriptase to amplify DNA and RNA (Mullis & Faloona, 1987). The types of PCR include a fluorescent based real time PCR which allows quantification of targets, the multiplex PCR for the detection of several nucleic acids sequences and the more sensitive nested PCR which increases target specificity in a second round PCR reaction. There is also the S gene

PCR which is used for detection of serum HBV DNA and X-gene PCR for HBV DNA detection in liver tissues (Torbenenson & Thomas, 2002). PCR-restriction fragment length polymorphism (RFLP-PCR) is used for HBV genotyping and mutation (Lindh *et al.*, 1997; Mizokami *et al.*, 1999). It is simple and rapid, but lacks sensitivity to detect low HBV DNA levels because there is no second round PCR. So Naito and his colleagues designed a nested-multiplex PCR that can genotype HBV using type specific primers for genotypes A-F (Naito *et al.*, 2001).

In this study, the nested-multiplex PCR assay was adopted to genotype HBV from HBV infected patients in the Central Region of Ghana. Based on the conserved nature of the HBV nucleotide sequences, primers that amplified the HBV genome from the pre-S through to the S genes were used in the first round PCR. The second round PCR was conducted using type specific primers which identifies the HBV genotypes by giving rise to different sizes of genotype specific bands when run on an electrophoresis gel (Naito *et al.*, 2001).

2.15.2 Next Generation Sequencing (NGS)

It is the gold standard in viral diagnostics. It uses sequencing and computational tools that allows the identification of HBV variants, genotypes and sub-genotypes. It is necessary for drug resistance and epidemiological studies (Ratcliff *et al.*, 2006). Despite the fact that NGS is very expensive and requires a sophisticated computational tool for the analysis of large tons of data, it is gradually replacing the Sanger sequencing method (Radford *et al.*, 2012). Margeridon-Thermet *et al.* used ultra-deep pyrosequencing to detect co-infection and recombination among two different HBV genotypes in nucleotide reverse transcriptase inhibitor (NRTI)-treated HBV patients and NRTI- naïve HBV patients (Margeridon-Thermet *et al.*, 2009).

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study design and patients selection

The study was a cross-sectional study where ethical approval was obtained from the Noguchi Memorial Institute for Medical Research – Institutional Review Board (IRB). A total of 1,217 Individuals, (including hospital staff, visitors and patients) of all age groups who visited the three district hospitals to confirm their Hepatitis B status were investigated. Individuals who were positive for Hepatitis B infection were selected for the study. Patient recruitment started from October 2016 to March 2017.

3.2 Study sites description

Three sites were selected for the study; the Winneba Municipal Hospital (Effutu Municipal Assembly), Agona-Swedru District Hospital (Agona-West district), and Apam District Hospital (Gomoa-West district). Each of the three study sites serve an average population of 150,000 people in their respective geographical locations. They provide secondary health care and serve as the first referral hospitals. They also provide quality clinical care with skilled and competent staff than health centers and polyclinics. They normally have well-structured laboratory units. All three hospitals are in the Central Region of Ghana and operate a level two facility in the populace.

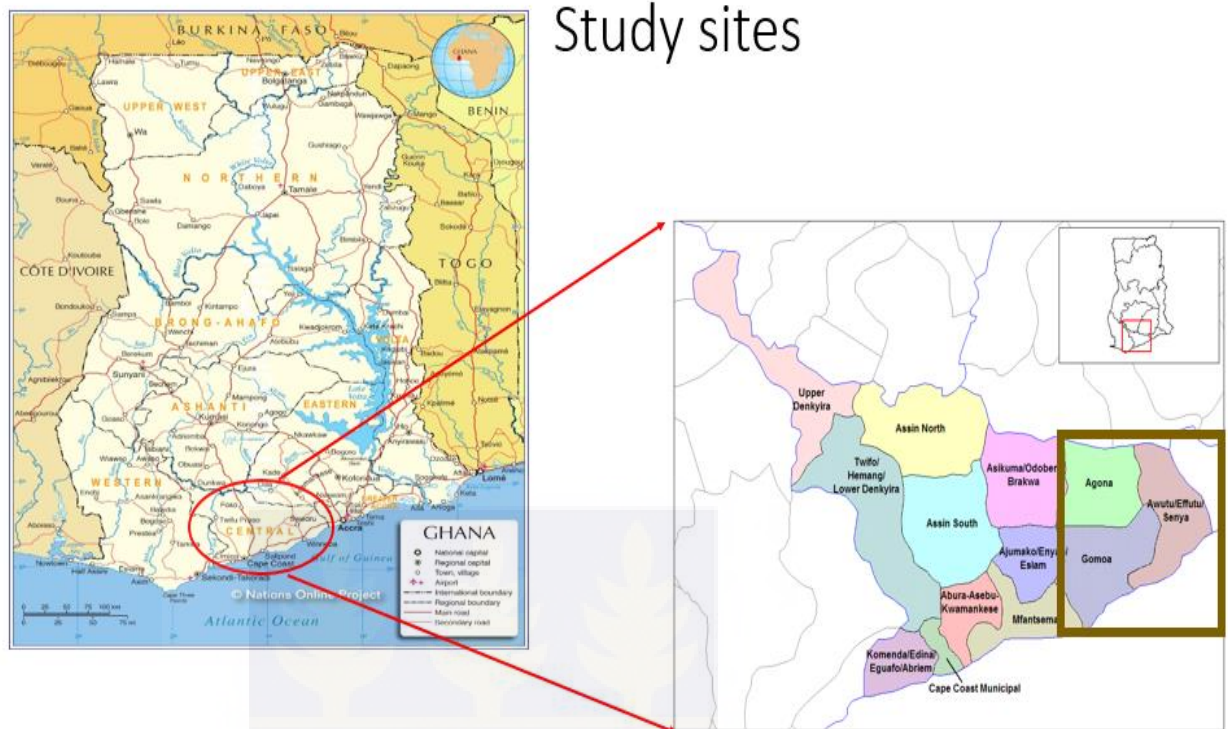


Figure 3.1: The three study sites; Effutu Municipal Assembly, Agona- West district and Gomoa-West district of the Central Region of Ghana

3.3 Population size

The sample size was calculated based on the national prevalence data for HBV of rural settings in Ghana which is 13.3% (Ofori-Asenso & Agyeman, 2016).

Sample size calculation

$$\text{Sample size (N)} = Z^2 * (p) * (1-p) / C^2$$

Z= Z-value

p= Prevalence

C= Confidence level

$$= (1.96)^2 * (0.133) * (1-0.133) / (0.05)^2$$

$$= 177.91$$

A total of 173 HBV-positive patients were recruited into the study, even though the required minimum sample size was 178. This was due to the issue of under reporting, because of lack of knowledge of HBV infection by the inhabitants of the districts.

3.4 Sampling method

A random sampling method was used, where all patients visiting the laboratory units of the three district hospitals were selected. Only the patients positive for the Hepatitis B surface antigen (HBsAg) were recruited and consent was sought from those who met the inclusion criteria to participate in the study. Questionnaires were used to collect data on the socio-demographic status of the HBsAg-seropositive patients.

3.5 Inclusion criteria

All individuals whether showing symptoms of HBV or not were included in the study.

3.6 Exclusion criteria

The exclusion criteria for this study was all patients who have been vaccinated against HBV. Institutionalized subjects were not included in the study.

3.7 Sample collection

A volume of 5 ml venous blood was collected using a 5 ml syringe. Blood was drawn and put into EDTA tubes. Blood samples were stored at a temperature of 4° C before screening.

3.8 Serological testing and storage

Blood samples were centrifuged and the sera were collected for Hepatitis B surface antigen (HBsAg) screening using the Advanced Quality™ ONE STEP HBsAg Test strip (InTec PRODUCT, Inc. China). All reagents and specimen for this procedure were brought to room temperature before the experiment proceeded. The experiments were performed according to the manufacturer's protocol. The samples that tested positive for HBsAg were transported on ice to the molecular virology laboratory (U.G), where they were stored at a temperature of -20°C prior to DNA extraction.

3.9 Deoxyribonucleic Acid (DNA) extraction

DNA was extracted from HBsAg seropositive samples using the QIAamp DNA Blood Minikit (QIAGEN, 40724 Hilden- Germany) according to the manufacturer's protocol. Microcentrifuge tubes (eppendoff tubes, 1.5 ml) were well labelled and 20 ul QIAGEN protease was pipetted into the bottom of the 1.5 ml microcentrifuge tubes. Two hundred microliters (200 ul) of the whole blood sample was added to the microcentrifuge tubes followed by the addition of 200 ul Buffer AL. The content of these tubes were mixed by pulse-vortexing for 15 seconds to yield a homogenous solution to ensure efficient lysis. The mixture was then incubated at 56°C for 10 minutes in a water bath to increase the DNA yield. After incubation, the 1.5 ml microcentrifuge tubes containing the mixture were briefly centrifuged to remove drops from inside the lid, and 200 ul of ethanol (95-100%) was added to the mixture and mixed by pulse-vortexing for 15 seconds. After mixing, the 1.5 ml microcentrifuge tubes were briefly centrifuged to remove drops from inside of the lid. The mixture was carefully transferred into QIAamp Mini Spin columns (in a 2 ml collection tube) without wetting the rim. The caps of the QIAamp Mini Spin columns were closed and span down at 6000 xg (8000 rpm) for 1

minute. The QIAamp Mini Spin columns were placed after centrifugation in clean 2 ml collection tubes and the tubes containing the filtrate were discarded. The QIAamp Mini Spin columns were carefully opened and 500 ul of Buffer AW1 was added as the wash buffer without wetting the rim and centrifuged at 6000 xg (8000 rpm) for 1 minute. The QIAamp Mini Spin columns were placed in clean 2 ml collection tubes while the collection tubes containing the filtrate were discarded. The QIAamp Mini Spin columns were carefully opened again and 500 ul of wash buffer AW2 was added without wetting the rim. The caps were closed and the mixture was centrifuged at full speed (20,000 xg; 14,000 rpm) for 3 minutes. In order to eliminate the chances of possible Buffer AW2, the QIAamp Mini Spin columns were placed in new 2 ml collection tubes, (the old collection tubes containing the filtrate were discarded) and centrifuged at full speed for 1 minute. The QIAamp Mini Spin columns were then placed in clean 1.5 ml microcentrifuge tubes and the collection tubes containing the filtrate were discarded. Finally, the QIAamp Mini Spin columns were carefully opened and 200 ul of AE buffer was added to elute the DNA. The mixture was incubated at room temperature (15-25°C) for 1 minute and then centrifuged at 6000 xg (8000 rpm) for 1 minute. The QIAamp Mini Spin columns were removed and discarded. The eluted DNA was stored at -20°C for future use.

3.10 Polymerase Chain Reaction (PCR) analysis

The detection of HBV DNA was based on the protocol proposed by Lindh *et al.* and supported by Dongdem and colleagues (Dongdem *et al.*, 2016; Lindh *et al.*, 1997). The HBV DNA was amplified using HBV primers (HBV-F: 5'GTGGTGGACTTCTCTCAATTTTC'3 and HBV-R: 5'CGGTATAAAGGGACTCACGAT 3') flanking the S region of the HBV genome.

PCR was carried out in a reaction volume of 25 µl containing; 2.5 µl of 10X PCR buffer, 1.5 µl of 25 mM MgCl₂, 0.5 µl of 10 mM dNTPs, 1.25 µl of 10 mM each oligonucleotide primer (Thermofisher Scientific Inc, U.S.A) and 1 U of DNA Taq polymerase enzyme. The Taq PCR core kit (QIAGEN, Germany) was used and 5 µl of the extracted DNA was used as template. The PCR cycling conditions for amplification were; initial denaturation at 94°C for 3 minutes followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 53°C for 1 minute, elongation at 72°C for 1.5 minutes and a final elongation of 72°C for 7 minutes using the SENSQUEST thermocycler (Dongdem *et al.*, 2016; Lindh *et al.*, 1997).

3.11 Genotyping of HBV by nested-multiplex PCR (Naito *et al.*, 2001)

This method of genotyping was adopted from Naito and colleagues to categorize HBV in strains A-F (Naito *et al.*, 2001). There are two rounds of PCR which makes use of primers designed based on the conserved nature of the nucleotide sequences in the preS/S regions of the HBV genome. Primers used are listed in table 3.1 below. The first PCR was carried out in a reaction mixture of 40 µl containing; 4 µl of 10X PCR buffer, 1.5 µl of 25 mM MgCl₂, 0.5 µl of 10 mM dNTPs, 2 µl of 10 mM each of oligonucleotide primer (P1 and S1-2), 1 U of DNA Taq polymerase enzyme and 5 µl of the extracted DNA as template. The Taq PCR core kit (QIAGEN, Germany) was used. The PCR cycling conditions for amplification were; initial denaturation at 95°C for 10 minutes followed by 40 cycles of denaturation at 94°C for 20 seconds, annealing at 55°C for 20 seconds and elongation at 72°C for 1 minute using the SENSQUEST thermocycler.

The second PCR involved two different reaction mixtures, mix A and B. The mix A contained the primers B2, BAIR, BBIR, BCIR and mix B contained the primers B2R, BD1, BE1, and BF1. An aliquot of 1 µl of the first PCR product was used as DNA

template in both tubes containing the reaction mixtures A and B. The reaction mixtures were prepared as in the first PCR reaction. The PCR cycling conditions for amplification were; initial denaturation at 95°C for 10 minutes, followed by 20 cycles of denaturation at 94°C for 20 seconds, annealing at 58°C for 20 seconds and elongation at 72°C for 30 seconds and additional 20 cycles of 94°C denaturation for 20 seconds, annealing at 60°C for 20 seconds and elongation at 72°C for 30 seconds (Naito *et al.*, 2001).

Table 3.1: Nested-multiplex PCR primers and their sequences

Primers	Sequences
First PCR P1	59-TCA CCA TAT TCT TGG GAA CAA GA-39 (nt 2823–2845, universal, sense)
S1-2	59-CGA ACC ACT GAA CAA ATG GC-39 (nt 685–704, universal, antisense)
Second PCR	
Mix A B2	5'-GGC TCM AGT TCM GGA ACA GT-3' (nt 67–86, types A to E specific, sense)
BAIR	5'-CTC GCG GAG ATT GAC GAG ATG T-3' (nt 113–134, type A specific, antisense)
BBIR	5'-CAG GTT GGT GAG TGA CTG GAG A-3' (nt 324–345, type B specific, antisense)
BCIR	5'-GGT CCT AGG AAT CCT GAT GTT G-3' (nt 165–186, type C specific, antisense)
Mix B B2R	5'-GGA GGC GGA TYT GCT GGC AA-3' (nt 3078–3097, types D to F specific, antisense)
BD1	5'-GCC AAC AAG GTA GGA GCT-3' (nt 2979–2996, type D specific, sense)
BE1	5'-CAC CAG AAA TCC AGA TTG GGA CCA-3' (nt 2955–2978, type E specific, sense)
BF1	5'-GYT ACG GTC CAG GGT TAC CA-3' (nt 3032–3051, type F specific, sense)

3.12 Agarose gel electrophoresis and visualization of HBV PCR products

The presence of the HBV DNA was detected by aliquoting 8 µl of each PCR product and mixing with 2 µl of 10X orange/green loading dye. The mixture was run on 2% agarose gel stained with 2 µl of 0.5 µg/ml ethidium bromide which contained 1X Tris Acetate EDTA as running buffer. The genotypes were determined by visualizing the gel using the gel imager600 where the genotypes were identified as bands having any of these sizes; genotype A- 68bp, genotype B- 281bp, genotype C- 122bp, genotype D- 97bp, genotype E- 167bp and genotype F- 119bp. The band sizes were determined by comparing with a 100bp to 1kbp DNA molecular ladder.

3.13 Sequencing of PCR products

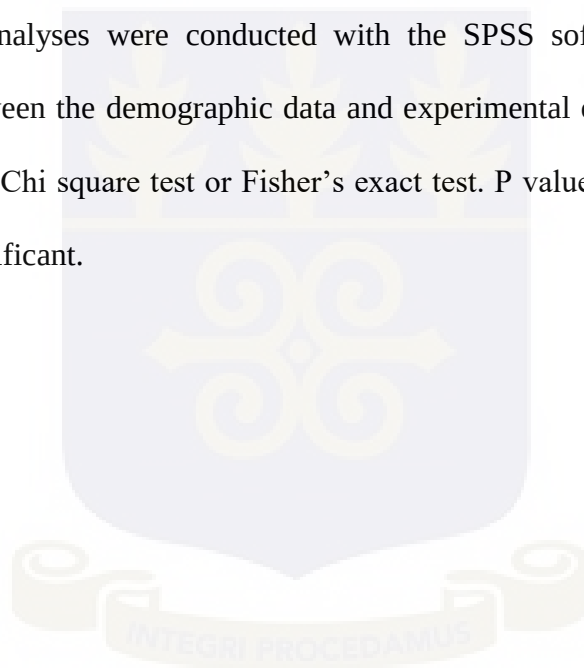
In order to establish the specificity of the nested-multiplex PCR assay, 31 products of the S gene PCR were sequenced which included samples that were typeable and untypeable by the genotype-specific PCR assay. The products of the preS/S were not sequenced because the first PCR assay did not show bands except when the second PCR assay was performed. The PCR products of the S gene were sent for Sanger sequencing (Iqaba Biotec, South Africa) in only one direction using the forward primer for the S gene of the HBV genome. HBV sequences were received in '.abi' file format where they were uploaded in the Mega 7 software and edited by certifying that the peaks corresponded to the correct base call. The chromatogram of each sequence was trimmed at the ends which contained broad peaks and the edited sequences were saved into a FASTA file format using the same Mega 7 software mentioned above.

For HBV genotyping, the FASTA file sequences were input into the HBV HepSeq software and submitted to determine the HBV genotypes.

Phylogenetic analysis was done by Mega 7 software using HBV reference strains against the isolates identified in this study. The reference strains (accession numbers: HM366311-E, AB205188-E, AB205191-E, AB106584-E, JF754615-D, AY233278-A, FJ023660-I, AF223954-C, AB073842-B, EF634480-G, AB486012-J, EU498228-H, X69798-F, AY226578- Woolly Monkey-Ancestral strain) were retrieved from the GenBank database.

3.14 Statistical analysis

All statistical analyses were conducted with the SPSS software version 20. The association between the demographic data and experimental outcomes were assessed using Pearson's Chi square test or Fisher's exact test. P value < 0.05 was considered statistically significant.



CHAPTER FOUR

4.0 RESULTS

4.1 Baseline characteristics and seroprevalence of HBsAg in patients

The study was done to identify and characterize circulating genotypes of HBV in patients attending three hospitals (Effutu Municipal, Gomoa-West and Agona-West district hospitals) in the Central region of Ghana. The prevalence of HBsAg seropositivity in the three districts was determined as 14.2% (see Appendix 5.0). Questionnaires were administered to all the 173 patients for their socio-demographic data. The questionnaires required information on the district of origin, age, gender, marital status, educational level of the patients and risk factors that may influence the transmission of the HBV. The percentage distribution of HBsAg seropositive patients from the three districts is shown in Figure 4.1; Gomoa-West district recorded the highest number of HBsAg seropositivity of 40%, followed by the Agona-West district and the Effutu Municipal Assembly with seropositivities of 31% and 29%, respectively. The total number of HBsAg seropositive males was 92 (53.2%) and the seropositivity for females was 81 (46.8%). No statistical association was observed between the distribution of HBsAg seropositivity in the districts and gender ($\chi^2 = 1.224$, P-value = 0.542) (Table 4.1).

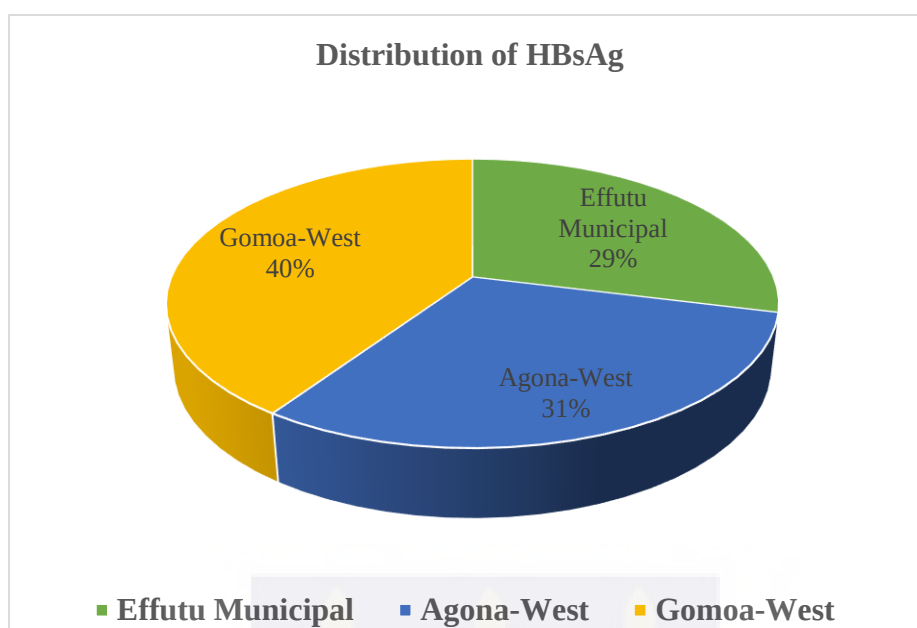


Figure 4.1: Distribution of HBsAg seropositivity in patients attending hospitals in the Gomoa-West and Agona-West districts, and the Effutu Municipal Assembly.

Table 4.1: Distribution of HBsAg seropositive patients in the districts with respect to gender

Gender	Seropositivity in districts			Total	χ^2	P-value
	Effutu Municipal	Agona-West	Gomoa-West			
Male	27(29.3)	25(27.2)	40(43.5)	92(53.2)	1.224	0.542
Female	23(28.4)	28(34.6)	30(37.0)	81(46.8)		
Total	50(28.9)	53(30.6)	70(40.5)	173(100)		

Figures in parenthesis show percentages

4.2 Amplification of S gene among HBsAg infected patients

A total of 115 (66.5%) HBsAg seropositive samples were successfully amplified for S gene with the expected amplicon size of 540bp (Figure 4.3- 4.5), and 58 (33.5%) of the samples were negative for HBV DNA (Table 4.2). Analysis of HBV infectivity (HBV DNA positivity) in the three districts showed no statistical association, suggesting that the HBV infectivities in the respective districts are not dependent on the district of origin ($\chi^2 = 1.330$, P-value = 0.514).

Table 4.2: HBV DNA positivity in HBsAg seropositive patients in the three districts

	Districts			Total	χ^2	P-value
	Effutu Municipal	Agona-West	Gomoa-West			
HBsAg seropositivity	50 (28.9)	53 (30.6)	70 (40.5)	173 (100)		
S gene amplification	32 (27.8)	33 (28.7)	50 (43.5)	115(66.5)	1.330	0.514
No amplification	18 (31.0)	20 (34.5)	20 (34.5)	58 (33.5)		

Figures in parenthesis show percentages

4.3 S gene amplification (HBV DNA positivity) and patient gender

Similarly, there was no association between HBV DNA infectivity and gender, which suggests that the infectivity was not dependent on gender ($\chi^2 = 0.074$, P-value = 0.785). Out of the 115 samples that were positive for HBV DNA amplification, 62 (53.9%) were males while 53 (46.1%) were females. Also, 30 (51.7%) males and 28 (48.3%) females were negative for the HBV DNA. The HBV DNA positivity rate among males and females HBsAg seropositive patients is shown in Table 4.3.

Table 4.3: Amplification of S gene (HBV DNA positivity) among males and females with HBsAg seropositivity

Gender	S gene amplification (HBV DNA positivity)			χ^2	P-value
	Yes	No	Total		
Males	62(53.9)	30(51.7)	92(53.2)	0.074	0.785
Females	53(46.1)	28(48.3)	81(46.8)		
Total	115 (66.5)	58(33.5)	173(100)		

Figures in parenthesis show percentages

4.4 S gene amplification (HBV DNA positivity) and age groups of patients

In assessing the PCR amplification of the S gene across different age groups, the age group 21-30 had the highest S gene amplification (HBV DNA positivity) with a percentage of 44.3%, followed by age groups 31-40, 11-20, 41-50, 51-60 and 61-70, in that order, having positivities of 33%, 10%, 9%, 3% and 1%, respectively. The

association between the S gene amplification and age groups was not statistically significant ($\chi^2 = 5.573$, P-value = 0.350) (Figure 4.2).

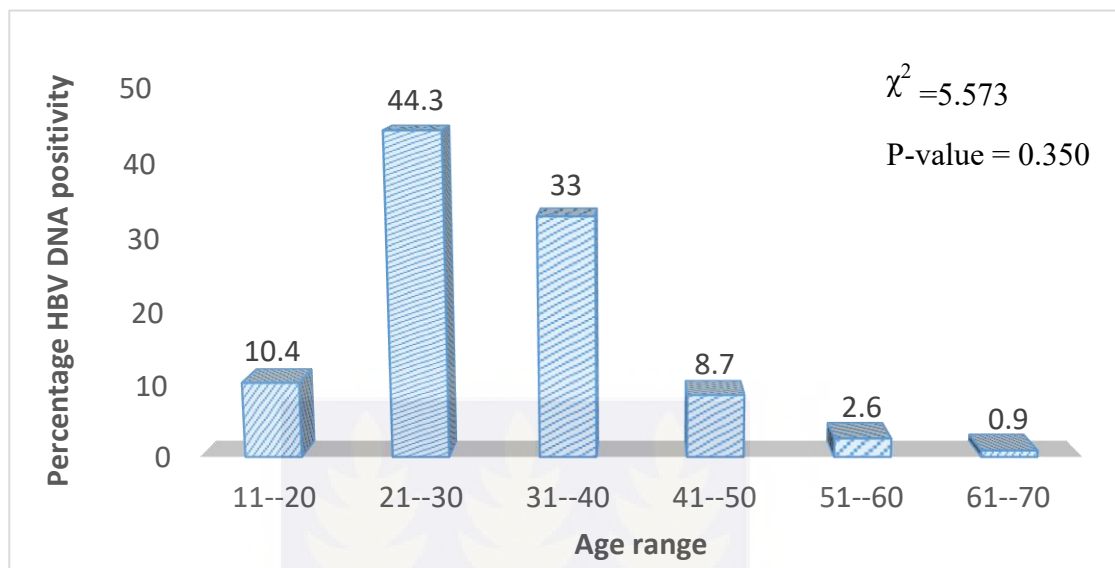


Figure 4.2: A graph of HBV DNA positivity across different age groups; the y-axis represents the percentage DNA positivity and the x-axis represents the various age groups.

4.5 Association between marital status and HBV infectivity

The association between detection of S gene amplification (HBV DNA positivity) and marital status was not statistically significant, with a Chi-square value (χ^2) of 1.291 and P-value of 0.256 (Table 4.4).

Table 4.4: Association between S gene amplification (HBV DNA positivity) and marital status

S gene amplification	Marital status		Total	χ^2	P-value
	Unmarried	Married			
Yes	66(57.4)	49(42.6)	115(66.5)	1.291	0.256
No	28(48.3)	30(51.7)	58(33.5)		
Total	94(54.3)	79(45.7)	173(100)		

Figures in parenthesis show percentages

4.6 S gene amplification (HBV DNA positivity) and educational levels of the patients

The analysis of HBV DNA positivity among the educational levels of HBsAg seropositive patients from the three districts showed that, those of basic education had the highest level of HBV DNA positivity with 44.3%, followed by those with secondary education with 28.7%, those without any educational background had 21.7% while those with tertiary education had the least with 5.2% positivity. Chi-square analysis showed a strong association between HBV DNA positivity and the educational levels of HBsAg seropositive patients, with a value (χ^2) of 10.853 and P-value of 0.013 (Table 4.5).

Table 4.5: Association between S gene amplification and educational levels of HBsAg seropositive patients

S gene amplification	Educational levels				Total	χ^2	P-value
	None	Basic education	Secondary	Tertiary			
Yes	25(21.7)	51(44.3)	33(28.7)	6(5.2)	115(66.5)	10.853	0.013
No	17(29.3)	13(22.4)	19(32.8)	9(15.5)	58(33.5)		
Total	42(24.3)	64(37.0)	52(30.1)	15(8.7)	173(100)		

Figures in parenthesis show percentages

4.7 Risk factors associated with Hepatitis B infection

Factors associated with HBV DNA positivity were also determined by comparing the amplification of S gene with risk factors such as sexual contact, dental procedure, treatment of STDs etc. There was no significant association between HBV DNA and the various factors (Table 4.6). However, sexual contact was the most common risk factor associated with HBV infectivity and transmission with a percentage of 67.5 (Table 4.6).

Table 4.6 : Association between risk factors and HBV DNA positivity

Amplification of S gene						
Risk factors	Yes(%) 115(66.5)	No(%) 58(33.5)	Total 173(100)	OR	CI	P-value
Hospitalization						
Yes	6(5.2)	4(6.9)	10(5.8)	0.743	-0.034-0.655	0.655
No	109(94.8)	54(93.1)	163(94.2)			
Treatment of STDs						
Yes	9(7.8)	4(6.9)	13(7.5)	1.146	0.017-0.827	0.827
No	106(92.2)	54(93.1)	160(92.5)			
Contact with HBV infected person						
Yes	3(2.6)	2(3.4)	5(2.9)	1.617	0.570-4.586	0.756
No	112(97.4)	56(32.4)	56(32.4)			
Health worker						
Yes	2(1.7)	0(0.0)	2(100.0)	1.513	1.359-1.685	0.312
No	113(98.3)	58(100.0)	171(98.8)			
HIV status						
Yes	3(2.6)	0(0.0)	3(1.7)	1.518	1.362-1.691	0.215
No	112(97.4)	58(100.0)	170(98.3)			
Sexual contact						
Yes	106(67.5)	51(32.5)	157(90.8)	1.617	0.570-4.586	0.363
No	9(56.2)	7(43.8)	16(9.2)			
Contact with sharp object						
Yes	5(4.3)	0(0.0)	5(2.9)	1.527	1.368-1.705	0.107
No	110(95.7)	58(100.0)	168(97.1)			
Intravenous drug use						
Yes	2(1.7)	1(1.7)	3(1.7)	1.009	0.090-11.362	0.994
No	113(98.3)	57(98.3)	170(98.3)			
Dental procedure						
Yes	4(3.5)	2(3.4)	6(3.5)	1.009	0.179-5.677	0.992
No	111(96.5)	56(96.6)	167(96.5)			
Family member diagnosed						
Yes	10(8.7)	2(3.4)	12(6.9)	2.667	0.565-12.595	0.200
No	105(91.3)	56(96.6)	161(93.1)			

Figures in parenthesis show percentages. OR is the odds ratio and CI is confidence interval.

4.8 Gel electropherogram of the presence of HBV DNA in the three districts

Figures 4.3, 4.4 and 4.5 showing gel electropherogram of HBV DNA positivity in the Gomaa-West district, Effutu Municipal Assembly and Agona-West district, respectively.

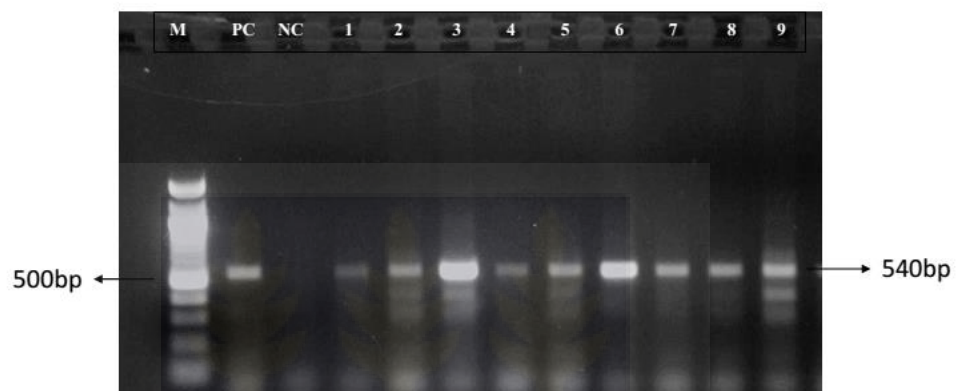


Figure 4.3: Representative gel electrophoresis pattern of HBV DNA by PCR amplification of the S gene in HBsAg seropositive samples from Gomaa-West district. The lane M represents the 100 base pair DNA molecular marker, lane PC is the positive control, lane NC is the negative control and lanes 1-9 are the HBV DNA.

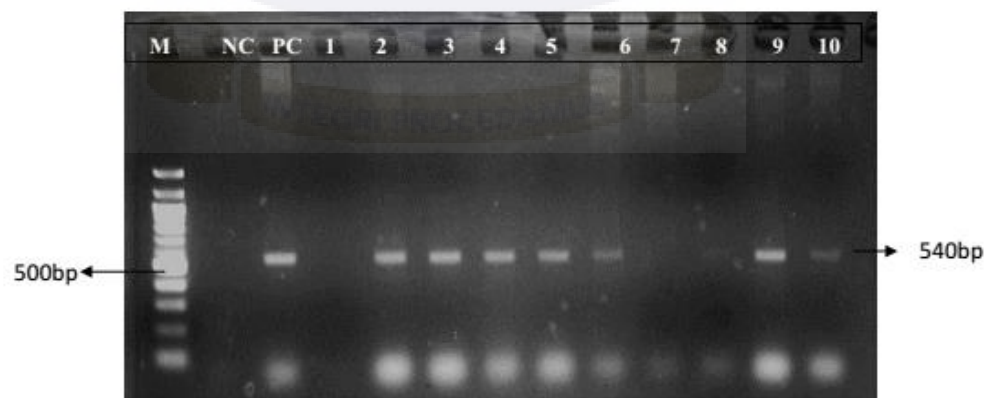


Figure 4.4: Representative gel electrophoresis pattern of HBV DNA by PCR amplification of the S gene in HBsAg seropositive samples from Effutu Municipal Assembly. The lane M represents the 100 base pair DNA molecular marker, lane NC is the negative control, lane PC is the positive control, and lanes 1-10 are the HBV DNA.

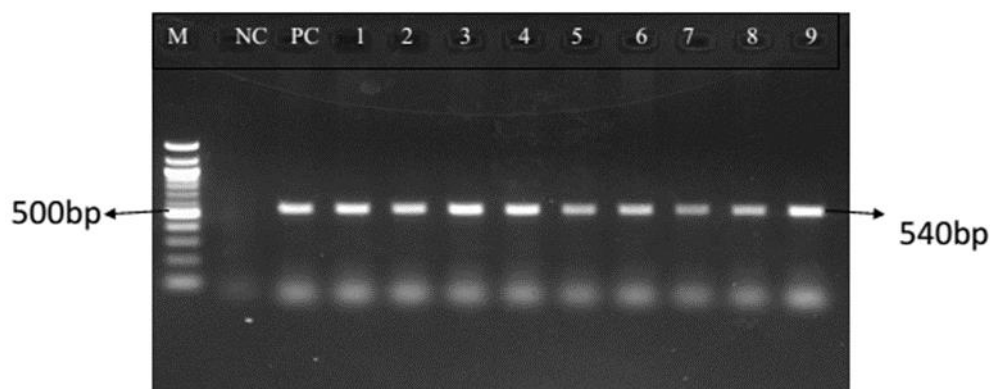


Figure 4.5: Representative gel electrophoresis pattern of HBV DNA by PCR amplification of the S gene in HBsAg seropositive samples from Agona-West district. The lane M represents the 100 base pair DNA molecular marker, lane NC is the negative control, lane PC is the positive control and lanes 1-9 are the HBV DNA.

4.9 Genotyping of HBV by nested-multiplex PCR (Naito *et al.*, 2001)

HBV was genotyped using nested-multiplex PCR adopted from Naito *et al.* Of 173 HBsAg seropositive samples subjected to genotyping, 121 (70%) showed genotype specific bands while 52 (30%) were negative. This method was quite sensitive than the S gene PCR, which gave 115 HBV DNA positive samples. Figure 4.6 shows the presence of genotype-specific bands for genotype E (167bp) in HBsAg seropositive patients.

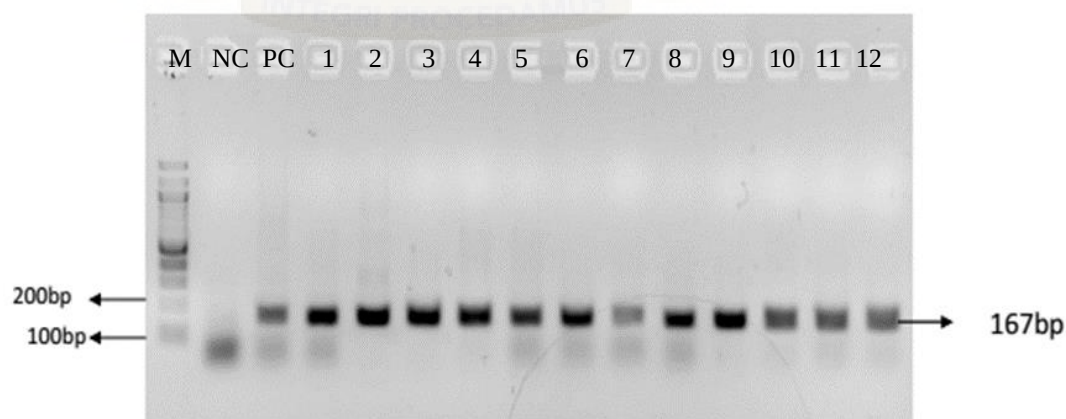


Figure 4.6: Genotyping of HBV by nested-multiplex PCR. Electrophoresis pattern of HBV Genotypes by nested-multiplex PCR in HBsAg seropositive samples from the three districts of the Central Region of Ghana. The lane M represents the 100 base pair DNA molecular marker, lane NC is the negative control, Lane PC is the positive control and lanes 1-12 are the HBV DNA showing band sizes for genotype E (167bp).

4.10 The percentage distribution of the HBV genotypes

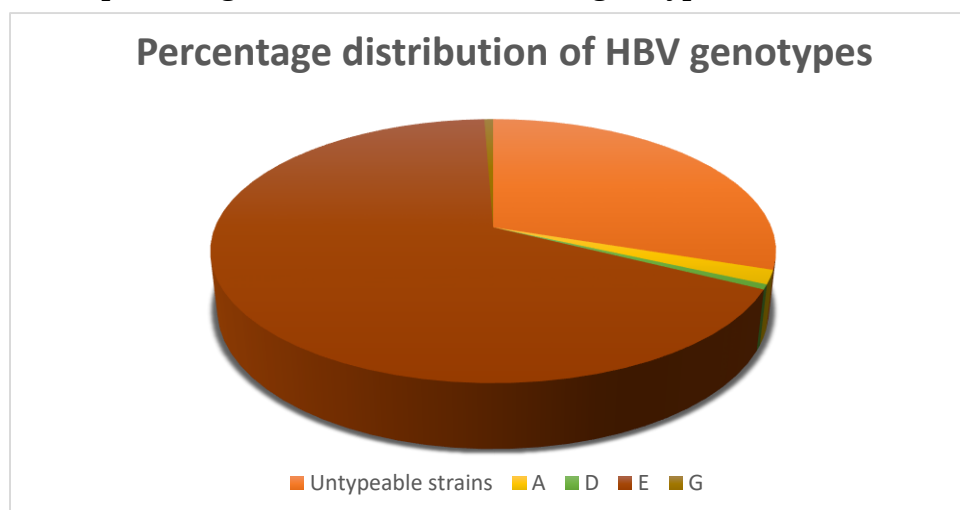


Figure 4.7: A pie chart showing the percentage distribution of HBV strains typed using the nested-multiplex PCR assay and Sequencing method. The typeable strains consisted of genotype A with a percentage of 1.7%, genotype E being the predominant genotype with 67.1% and genotypes D and G both having 0.6%. 30.1% (52) of the HBsAg seropositive samples were untypeable.

Table 4.7: Distribution of HBV genotypes in HBsAg seropositive patients in the three districts of the Central Region of Ghana

Districts	HBV genotypes				Untyped	Total
	A	D	E	G		
Effutu Municipal	1(2.0)	1(2.0)	38(76.0)	0(0.0)	10(20.0)	50(28.9)
Agona-West	0(0.0)	0(0.0)	32(60.4)	1(1.9)	20(37.7)	53(30.6)
Gomoa-West	2(2.9)	0(0.0)	46(65.7)	0(0.0)	22(31.4)	70(40.5)

Figures in parenthesis show percentages

Genotype E was found to be predominant in all three districts with percentages, 76.0, 60.4 and 65.7 in the Effutu Municipal, Agona-West and Gomoa-West districts, respectively. Genotype A was found in the Effutu Municipal (2.0%) and Gomoa-West (2.9%) but not the Agona-West district. The genotype D was found to be in only the Effutu Municipal Assembly. The rare strain G was identified in the Agona-West district.

Also, the results for distribution of the genotypes with respect to age and gender of HBsAg seropositive patients are presented in Table 4.8- 4.10.

Table 4.8: Age distribution of HBV genotypes

HBV genotypes	Age range						Total	χ^2	P-value
	11-20	21-30	31-40	41-50	51-60	61-70			
A	0(0.0)	0(0.0)	3(100)	0(0.0)	0(0.0)	0(0.0)	3(2.5)	19.569	0.485
D	1(100)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(0.8)		
E	12(10.3)	52(44.8)	38(32.8)	10(8.6)	3(2.6)	1(0.9)	116(95.9)		
G	0(0.0)	0(0.0)	1(100)	0(0.0)	0(0.0)	0(0.0)	1(0.8)		
Total	13(10.7)	52(41.9)	42(34.7)	10(8.3)	3(2.5)	1(0.8)	121(100)		

Figures in parenthesis show percentages

Genotypes E was the most prevailing genotype in all age groups whiles genotype A was found among people of age group 31-40. The genotype D was observed among age group 11-20 and the rare genotype G was observed among age groups 31-40 (Table 4.8).

Table 4.9: Distribution of HBV genotypes among males and females

Genotypes	Gender		Total	χ^2	P-value
	Male	Female			
A	2(66.6)	1(33.3)	3(2.5)	6.895	0.142
E	55(47.4)	61(52.6)	116 (95.9)		
D	0(0.0)	1(100)	1(0.8)		
G	1(100)	0(0.0)	1(0.8)		
Total	58(47.9)	63(52.1)	121(100)		

Figures in parenthesis show percentages

Genotype E was the most prevailing genotype in both males and females with 55% and 61%, respectively, followed by genotype A. However genotype D was found in only females whiles the rare genotype G was found in only males (Table 4.9).

Table 4.10: Relation between HBV genotypes and HBV DNA positivity (S gene amplification)

S gene amplification	HBV genotypes					Total	χ^2	P-value
	A	D	E	G	Untypeable strains			
Positive	3(100)	1(100)	88(75.9)	1	22(42.3)	11(66.5)	20.736	0.0001
Negative	0(0.0)	0(0.0)	28(24.1)	0	30(57.7)	58(33.5)		
Total	3(1.7)	1(0.6)	116(67.1)	1(0.6)	52(30.1)	173(100)		

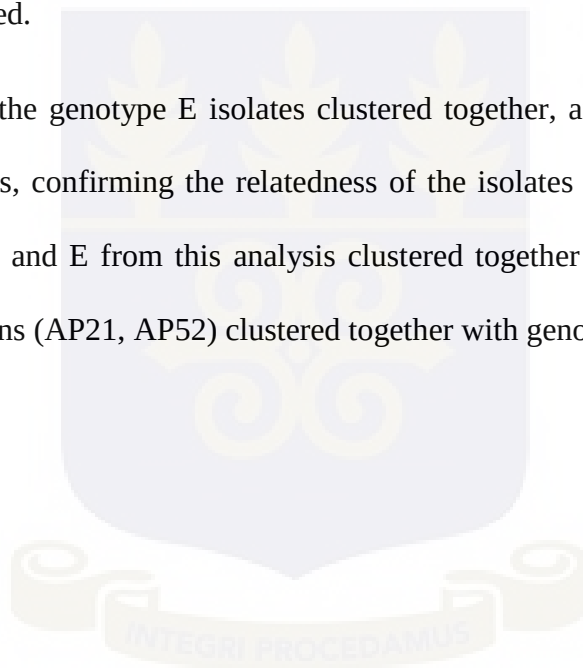
Figures in parenthesis show percentages

The relationship between HBV DNA positivity and HBV genotypes was established using the Pearson chi-square test. It was observed that the circulating HBV genotypes influenced HBV DNA positivity with a P-value of 0.0001 and $\chi^2 = 20.736$ (Table 4.10).

4.11 Phylogenetic analysis of HBV genotypes circulating in the three districts of the Central Region

The phylogenetic tree was constructed based on 540bp sequences of the S gene of the HBV genome by neighbour joining clustering method using the Mega 7 software (Figure 4.9). The phylogenetic analysis from the study showed a cluster of all the 31 isolates genotyped.

Twenty four of the genotype E isolates clustered together, and with the genotype E reference isolates, confirming the relatedness of the isolates to the reference strains. The genotype D and E from this analysis clustered together in the same clade. The genotype A strains (AP21, AP52) clustered together with genotype G (SW159).



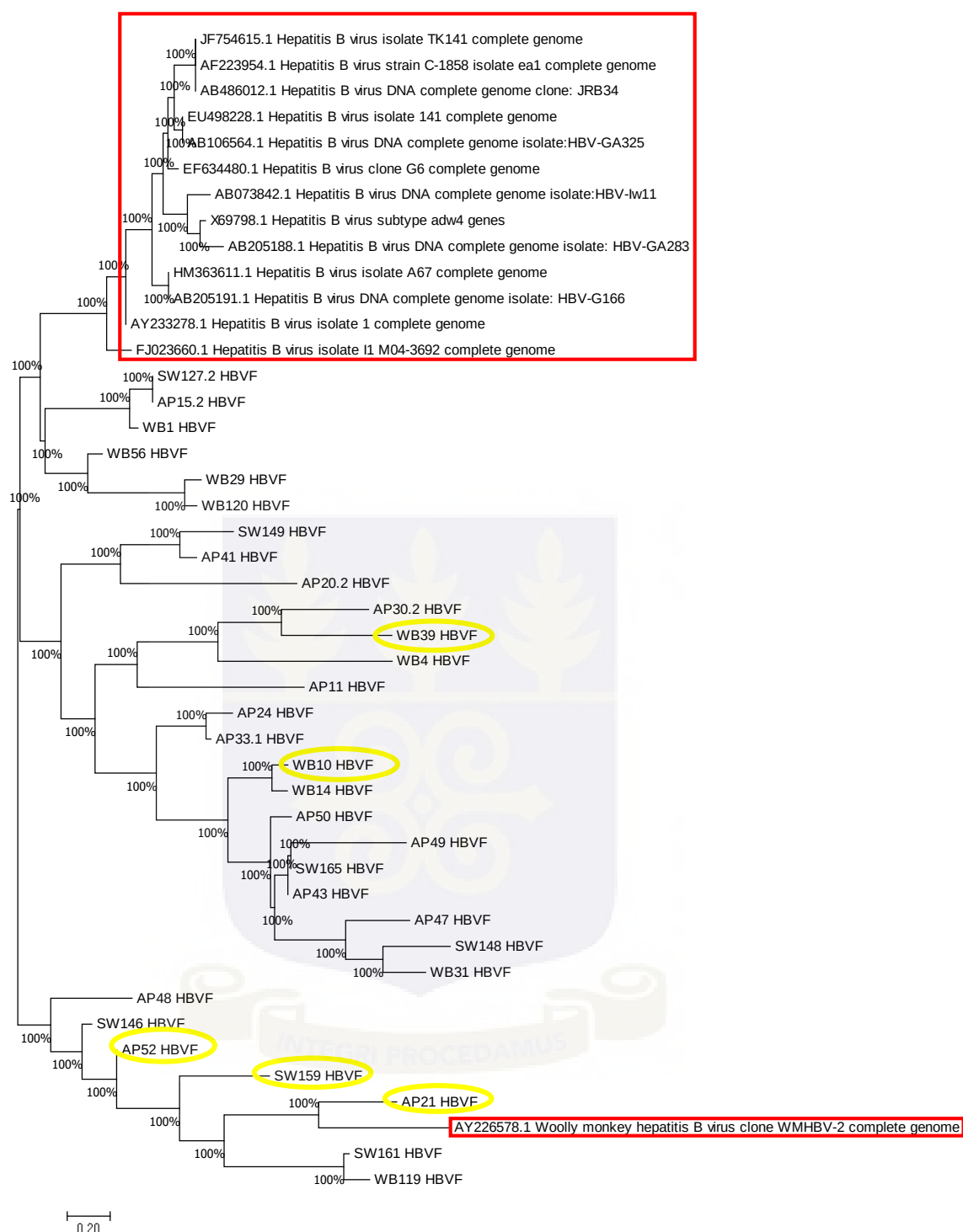


Figure 4.8: Phylogenetic tree of S gene nucleotide sequences of HBV strains in the study can be identified by SW, AP or WB. The genotypes A (AP52, AP21 and WB10), D (WB39) and genotype G (SW159) have been circled and highlighted in yellow while those not highlighted are the genotype E. Highlighted in red are Sequences of HBV References strains obtained from GenBank database, accession numbers (HM366311-E, AB205188-E, AB205191-E, AB106584-E, JF754615-D, AY233278-A, FJ023660-I, AF223954-C, AB073842-B, EF634480-G, AB486012-J, EU498228-H, X69798-F, AY226578- Woolly Monkey)

CHAPTER FIVE

5.0 DISCUSSION

Hepatitis B infection still remains a worldwide health problem, especially in developing countries such as Ghana. Ghana has been categorized as an HBV high endemic region with a prevalence rate of more than 8% (WHO, 2016, WHO, 2017). However, molecular based studies on HBV infection in Ghana are very limited, and the studies are normally concentrated in the urban settings of two populous regions (Greater Accra and Ashanti). Thus, the limited information in the other regions informed the aim of this study, which is to characterize HBV in semi-urban and rural areas in the Central Region of Ghana. To achieve this aim, 1,217 people attending three district hospitals (Effutu Municipal Assembly, Agona-West and Gomoa-West districts) were screened, and 173 were found to be positive for HBV surface antigen (HBsAg).

HBV prevalence of 14.2% was obtained from this study, which falls within the prevalence range, 5.5-22.8% in the Central Region of Ghana (Ofori-Asenso & Agyeman, 2016). The data from the study indicated that the least populated district (Gomoa-West district) had the highest number of HBsAg and HBV DNA positive patients. Even though there is no clear explanation, the low socio-economic infrastructure and educational background in the Gomoa-West district are possible contributors to the high HBV transmission rate compared to the other districts which have better socio-economic infrastructure.

HBsAg seropositivity and HBV DNA positivity from this study were independent of gender, however, the positivities were high in males than females. This result is in contrast with a study done by Dongdem and his colleagues where more females were

found to be infected with HBV than males (Dongdem *et al.*, 2016), but in agreement with a study conducted by Valente and colleagues (Valente *et al.*, 2010) where the seroprevalence of HBV was high in men as compared to women. In humans, both males and females have equal risk of HBV infection, however, researchers have associated the low rate of HBV in females to the high levels of estrogen. Estrogen is reported to inhibit stellate cells which are responsible for fibrogenesis in the liver, thus, may be causing a reduction in the risk of females getting infected with chronic hepatic diseases (Baig, 2009; Guy & Peters, 2013).

This study showed no significant association between age and HBV positivity, thus age does not appear to influence HBV infection and prevalence. The age groups 21-30 and 31-40 however, had higher prevalence of HBV infection compared to the other groups. A similar result was published by Kolou and colleagues where they reported high prevalence of HBV in people within the age 20-39 (Kolou *et al.*, 2017). Another study done in Accra reported that the vulnerable age groups of HBV infection was 20-29 and 30-39 (Dongdem *et al.*, 2016). An HBV prevalence study done in Ghana have reported that HBV is most prevalent within age group 16-39 (Ofori-Asenso & Agyeman, 2016). HBV infection may have been concentrated among age groups 21 to 40 because they represent a sexually active and the most populated age groups in the country. People of older age groups might have low HBV prevalence rate because they are hardly involved in sexual activities (Kolou *et al.*, 2017). Studies from the prevaccine era showed that children under 6 had higher prevalence of HBV infection and are more vulnerable to HBV infection (WHO, 2015). However, the strict implementation of the neonatal immunization programmes has significantly reduced HBV infection in children (Lavanchy, 2004). Recent reports indicated that most people of the youthful ages tend

to ignore the administration of HBV vaccine, making them to be more vulnerable to the infection (Ofori-Asenso & Agyeman, 2016).

The current study brought to light the fact that people who were not married had a high prevalent rate of HBV infection confirming work done by Adjei and colleagues (Adjei *et al.*, 2008). The low prevalence of HBV among married participants was probably due to their stable sexual behaviors as explained by Koopman and Lynch (Koopman & Lynch, 1999). Other studies have opposing views with the married participants being more prone to HBV infection than unmarried participants (Damale *et al.*, 2005). Therefore, the role of marital status in HBV infection remains unclear and needs further investigations.

This study has established an association between educational background and HBV prevalence in the semi-urban and rural settings. People with low educational background had high rate of HBV infection as compared to those with higher educational level. A study by Mutocheluh and his colleagues have also confirmed that there is a statistical association between educational level and HBV DNA positivity (Mutocheluh *et al.*, 2014). Researchers have reported that low educational levels lead to a huge knowledge gap about HBV infection, hence, the difficulty of the uneducated to have adequate information on the transmission dynamics of the virus (Ganczak *et al.*, 2016; Mutocheluh *et al.*, 2014; Ofori-Asenso & Agyeman, 2016). This knowledge gap may explain the increased HBV infectivity and prevalence among the uneducated patients.

Analysis of risk factors associated with HBV infection is necessary in the development of control and preventive measures. The odds of exposure to Hepatitis B infection was analyzed in this study which showed that there was no association between risk factors and HBV infection. However, as observed in this study, sexual contact was the most

common risk associated with HBV infectivity in the Central Region of Ghana. The high risk of sexual contact was supported in a study done by Abou *et al.* which suggested that unprotected sex among some African societies is the main route of HBV transmission (Abou *et al.*, 2009). The findings of this study does not support what was observed in HBV high endemic areas such as Africa where vertical transmission is the main route of HBV infection (Lavanchy, 2005). The shift in transmission route of HBV in this study might be due to the introduction of the HBV vaccine programme which has reduced HBV cases due to materno-fetal transmission, and thus resulting in a limitation in obtaining HBV data for children below 11 years of age in this study.

In Ghana, the major risk factor associated with the development of Hepatic cirrhosis leading to liver disease-associated deaths is HBV. Patients infected with HBV have an 8- fold increased risk of developing cirrhosis as compared to those infected with other factors (Blankson *et al.*, 2005). An unpublished data by Wiredu and his colleagues have suggested that HBV genotypes are responsible for chronic hepatitis B (CHB) liver cirrhosis (HC) and hepatocellular carcinoma (HCC) in Ghanaian patients (Professor E. K. Wiredu, and colleagues, unpublished data, 2011). In addition, Dongdem *et al.* further confirmed these findings when they isolated HBV in Ghanaian patients with chronic liver infections (Dongdem *et al.*, 2016). They specifically found out that, HBV genotypes are responsible for the development and progression of chronic liver diseases.

HBV genotypes are also known to influence HBeAg seroconversion rates and treatment response (Deterding *et al.*, 2008; Schaefer, 2007; Wiegand *et al.*, 2008). The circulating genotypes of HBV among the Ghanaian population have been identified as HBV/E (most predominant), HBV/A and HBV/D according to studies from the Greater-Accra

and Ashanti regions (Candotti *et al.*, 2006; Dongdem *et al.*, 2016). Till date, there is no report of HBV genotypes from the Central Region of Ghana, and therefore this study is the first to report from the Central Region. Majority of the participants in this study were living with the HBV/E, with few individuals having HBV/A and HBV/D. The HBV/G, which was discovered in this study, is rare globally and has never been reported in Africa. Researchers often report co-infection of HBV/A and HBV/G suggesting that the presence of HBV/A favours the infectivity and replication of HBV/G (Kato *et al.*, 2002). The patient with HBV/G in this study was however not infected with HBV/A, but the presence of HBV/A in the region may be a possible driving factor for the infectivity of HBV/G at the study site. The co-infectivity of HBV/A and HBV/G is evident in their ability to recombine. There is a close similarity between the genomes of HBV/G and HBV/A, with a reported genotype homology of 94.6 to 97.5% in the S gene (Kato *et al.*, 2002). Based on this, a large number of patients must be screened to authenticate the presence of HBV/G in the study area.

Clinically, HBV/E has been established to be restricted to West Africa (Norder *et al.*, 2004). About 90% of strains of HBV isolated from chronically infected patients are HBV/E (Candotti *et al.*, 2006; Suzuki *et al.*, 2003). However, at age 16, 75% of HBV/E patients might have seroconverted to anti-HBe, with a viral load less than 104IU/ml (Allain *et al.*, 2003; Candotti *et al.*, 2006). The age dependent HBeAg seroconversion to anti-HBe occurs at an early stage in patients with HBV/E infection compared to the late stage in HBV/B and HBV/C which are the common genotypes found in Asia (Allain *et al.*, 2003; Ren *et al.*, 1998). Researchers have discovered mutations in the HBsAg of the HBV/E; the Ser143leu, and the Leu216 (sLeu216*) and Trp182 (sTrp182*) substitutions, which play roles in producing false negative results during HBsAg immune-assay diagnostic and oncogenesis, respectively, thus resulting in the

development of HCC (Ampah *et al.*, 2016). Some studies have also implicated HBV/D or HBV/E to be present in HBV carriers, with HBV/E having a higher viral load (Yousif *et al.*, 2013).

A multicenter therapeutic study on four genotypes have showed that HBV/A responds better to PEG-IFN α therapy as compared to HBV/B, HBV/C, HBV/D (Janssen *et al.*, 2005). In addition, HBV/A has also been found to be more susceptible to interferon therapy than HBV/D (Erhardt *et al.*, 2005; Wiegand *et al.*, 2008). HBV/A has a 20-fold increased risk of lamivudine resistance development than HBV/D (Zöllner *et al.*, 2004). This is due to the increased emergence of YMDD mutation in HBV/A than HBV/D (Buti *et al.*, 2002). The presence of these genotypes and their clinical implication leaves HBV as a major public health problem in the study sites.

The study results suggest differential distribution of the identified genotypes across the three districts. HBV/E was distributed in all three districts while HBV/A was found in only two districts (Effutu Municipal and Gomoa-West districts). HBV/D was identified in the Effutu Municipal while the rare HBV/G was found in the Agona-West district. The distribution of these genotypes was not statistically significant, with respect to gender and age. With respect to gender, HBV/E was the most prevalent in both females and males, followed by HBV/A. These findings agree with the distribution of genotypes among chronic HBV patients in a study conducted by Dongdem *et al.* (Dongdem *et al.*, 2016). HBV/D was found in a female whereas HBV/G was found in a male.

HBV genotypes were found to be concentrated in age groups 21-30 and 31-40, however this was not statistically significant and could be due to the fact that HBsAg seropositivity was highly prevalent in people of these age groups.

Most researches have established an association between HBV genotypes and DNA levels and their effect on HCC development (Yousif *et al.*, 2013; Yu *et al.*, 2005). The HBV DNA levels were not determined, however there was a strong association between HBV genotypes and HBV DNA positivity ($\chi^2 = 20.736$; P-value = 0.0001). The strong association might suggest that the presence of the HBV genotypes influence HBV DNA positivity. The presence of the various genotypes identified, points towards the likelihood of development of liver diseases in the patients from the Central Region of Ghana.

Based on the phylogenetic analysis from this study, the presence of the HBV/D among the HBV/E isolates suggests that the HBV/D might possibly be a recombinant virus. This claim however, needs to be investigated by sequencing the whole genome or a large portion of the viral genome. The HBV/A (WB10) was also found to cluster with the HBV/E isolates suggesting a possible recombination. A research done by Brah *et al.* suggested that HBV/A can form a recombinant strain with HBV/E (Brah *et al.*, 2016). Also the clustering of the HBV/A strains with HBV/G (SW159) confirm the relatedness of these genotypes as described by researchers (Kao, 2002; Norder *et al.*, 1993).

CHAPTER SIX

6.0 CONCLUSION

Although HBV vaccination has been introduced in Ghana, this study calculated the prevalence of HBV infection to be 14.2% in the Central Region based on the presence of HBsAg in the blood of the study participants. The isolated HBV strains were genotyped and identified as A, D, E and G genotypes, with genotype E being the predominant genotype. The HBV/G identified showed as a rare strain and therefore needs to be further studied for confirmation. The genotypes identified from the study were found to cluster together, with HBV/E forming a distinct clade which is similar to the reference strains. HBV/G clustered with HBV/A because the two strains are homologous in their genetic constitution with a percentage similarity of about 95%.

6.1 RECOMMENDATIONS

Based on the results of this study, it is recommended that, large scale study and whole genome sequencing should be conducted to support the findings, taking into consideration escape mutants. The HBV genotype E is predominant and restricted to West Africa, hence further studies should be conducted on its clinical implications in relation to HBV infection. It is also suggested that the role of sex hormones in hepatic diseases should be assessed based on gender.

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APPENDIX

1.0 REAGENTS, MATERIALS AND EQUIPMENTS

1.1 Reagents for DNA extraction

QIAGEN extraction Kit - QIAamp DNA Blood Minikit (QIAGEN, 40724 Hilden-Germany)

Pure Ethyl alcohol (Sigma-Aldrich, USA).

1.2 Reagents for PCR reaction

Nuclease free sterile water (Life Science Biotechnology, Cochran Road Solon- USA)

Taq PCR Core kit (QIAGEN, Germany)

Components: Taq DNA polymerase (5 units/ul)

25 mM MgCl₂

10 mM dNTP mix (dATP, dCTP, dGTP, dTTP)

10X PCR buffer containing 15 mM MgCl₂

1.3 Reagents for gel electrophoresis

100bp DNA ladder (Promega, USA)

Loading dye (Blue/Orange G) (Promega, USA)

Electrophoresis buffer (10X Tris-acetate-EDTA) (Sigma-Aldrich, USA)

Agarose powder (BOLINE)

Ethidium Bromide (BioReagent, Sigma-Aldrich, USA)

2.0 Materials

2.2 Materials for sample collection

Vacuum Blood collection tube (EDTA tubes)

5 ml Syringes

Needles (Type 21G)

Examination gloves

Alcohol pad

Tourniquet

Phlebotomy plaster

Laboratory coat

2.3 Materials for DNA extraction, PCR, Gel electrophoresis

PCR tubes (Trefflab, Germany)

1.5 ml microcentrifuge tubes (eppendorff tubes)

0.6 ml microcentrifuge tubes (eppendorff tubes)

Sterile pipettes tips (0.1-10 ul, 2-200 ul, and 100-1000 ul) (ANACHEM, U.K.)

Falcon 50 ml conical centrifuge tubes (Fisher Scientific, U.S.A)

3.0 Equipments

3.1 Equipments for DNA extraction, PCR and Gel electrophoresis

Automatic pipettes (SCIQUIP-Scipette, U.K.)

Microcentrifuge (SCIQUIP - Scispin Mini Microfuge, Newtown-U.K.)

Mini Vortex Mixer (Fisher Scientific, Taiwan)

Thermal cycler (Labcycler SensoQuest GmbH, Goettingen-Germany)

GFL shaking water bath 1083 (ProfiLab 24, Germany)

Refrigerator (2°C-8°C)

Freezer (-20°C)

Gel casting tray (International Biotechnologies, Inc, New Haven-Connecticut)

Sample combs

Electronic balance (Mettler PE 360, U.S.A)

Gel imager (Amersham imager 600)

4.0 Reagents preparation

Qiagen protease stock solution preparation- Pipette the 5.5 ml protease solvent into the vial containing lyophilized Qiagen protease. An amount of 200 µl aliquots of the protease are stored at -20°C.

Buffer AL- Buffer AL was mixed thoroughly by shaking before use

Buffer AW1- The appropriate amount of ethanol (96-100%) is added as indicated on the bottle (130 ml).

Buffer AW2- The appropriate amount of ethanol (96-100%) is added as indicated on the bottle (160 ml)

4.1 Preparation of agarose gel for PCR

4.1.1 Preparation of 1X Tris acetate EDTA (TAE) buffer from 10X TAE buffer

Initial concentration (C1) - 10X

Final concentration (C2)-1X

Final volume (V2) - (1000 ml or 1 L)

Initial volume (V1)-?

$$V1 = C2V2/C1 = 1X * 1000 \text{ ml} / 10X = 100 \text{ ml}$$

A volume of 100 ml 10X TAE was added to 900 ml of distilled water

4.1.2 Preparation of 2% agarose gel

Prepare 2% agarose gel by adding 2 grams of agarose powder to 100 ml 1X TAE buffer.

Microwave at high temperature for 2 minutes.

Leave mixture to cool for a while and add 2 ul ethidium bromide.

Insert combs in tray, pour out the molten agarose into the gel casting tray and allow to set.

5.0 Calculation of seroprevalence of HBsAg seropositive patients

Total number patients screened = 1,217

Total number of HBsAg seropositive patients = 173

Seroprevalence = $173/1217 * 100$

= 14.2%

INFORMED CONSENT FORMS AND DATA COLLECTION TOOLS

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH INSTITUTIONAL REVIEW BOARD (NMIMR-IRB)

NMIMR-IRB CONSENT FORM TEMPLATE

Title: Molecular characterization of Hepatitis B virus in Rural and Semi- Urban Areas in the Central Region of Ghana.

Principal Investigator: Caroline Agyare Boatemaa

Address: Department of Biochemistry, Cell and Molecular Biology, University of Ghana. P. O. Box LG 54 Volta Road Legon,

General Information about Research

Viral hepatitis is an inflammation of the liver caused by a virus (hepatitis B virus). There are 10 groups of the virus and each respond to treatment differently. This liver inflammation can be less severe and show symptoms such as yellow eyes and skin, dark urine, fever, abdominal pain etc. It can also show no or mild symptoms where the individual can recover fully or progress into liver disease such as liver cancer. Liver cancer is difficult and expensive to treat. However when the disease is detected early, it can be treated and managed. There are vaccines to protect those tested negative for the virus. It affects more children than adult. Every year about 780,000 die worldwide from the liver cancer caused by hepatitis B virus. Hepatitis B infection is 100 times more infectious than HIV/AIDS. In Ghana at least one out of 5 people is infected with HBV (WHO, 2015). This study seeks to determine the presence of the HBV, and to know the type of HBV groups in the Central Region. The study will inform policy makers to know the type of treatment regimen to be given to patients within this area. This will be done by taking blood samples from individuals with or without the signs and symptoms of hepatitis B infection.

You qualified for the research whether you healthy or not, shows signs of the infection or not. You must also not be immunized against the infection.

If you agree to these terms to participate in this study, your blood sample will be collected, you will also be required to give your socio-demographic information for this research. This procedure is going to take about 20 minutes.

Possible Risks and Discomforts

You will encounter no risk as the safety protocols for laboratory sample collection will be followed. The needle stick may hurt, there will be some bruising and pain at the site of venipuncture. There will be no risk of infection since sample collection will be done by qualified personnel.

Possible Benefits

It is very difficult to know if a person is infected with HBV since the virus can remain

hidden for a long time and gradually cause severe liver diseases. To know your HBV status can help you receive early and appropriate treatment. And in the society, it will help improve drug treatment and prevent the progression of liver diseases and eventually prevent death.

Alternatives to Participation

If we find you to be positive for the virus, we will recommend that you go and see the doctor who will refer you to do further test to check for any signs of liver diseases and then the necessary treatment will be given to you.

Confidentiality

We will protect information about you to the best of our ability. The information of your medical records will be protected. Information that identifies you such as consent form, questionnaires will be kept locked and secured. If information from the research is published or presented at scientific meetings, your identifiers will not be included. Information about you will be destroyed after research is completed. You will not be named in any reports. Some of the staff of this hospital and the University of Ghana, Department of Biochemistry, Cell and Molecular Biology faculty members may sometimes look at your research records.

Compensation

There is not going to be any compensation being it monetary or any type given to you for your participation in this study.

Additional Cost

There is not going to be any additional cost to you for participating in the research.

Voluntary Participation and Right to Leave the Research

This research is purely voluntary and you can withdraw from the study without any penalty and compensation to the researchers involved.

Termination of Participation by the Researcher

Your participation in the research will be terminated if we later find out that you have been immunized against the HBV and also when the blood sample collected is not enough for the study.

Contacts for Additional Information

Project supervisor: Dr. Osbourne Quaye, Ph.D.; Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon. Tel: +233-277-459566. Email: OQuaye@ug.edu.gh

Your rights as a Participant

This research has been reviewed and approved by the Institutional Review Board of

Noguchi Memorial Institute for Medical Research (NMIMR-IRB). If you have any questions about your rights as a research participant you can contact the IRB Office between the hours of 8am-5pm through the landline 0302916438 or email addresses: nirb@noguchi.mimcom.org

VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research title **(Molecular characterisation of Hepatitis B virus in Rural and Semi- Urban Areas in three Districts of the Central Region of Ghana)** has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate as a volunteer.

Date

Name and signature or mark of volunteer

If volunteers cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer has agreed to take part in the research.

Date

Name and signature of witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

Date

Name Signature of Person Who Obtained Consent

Hepatitis B Questionnaires
Department of Biochemistry, Cell and Molecular biology
School of Biological Sciences
University of Ghana, Legon.

Type of specimen: serum/plasma/blood
(specify):.....

Date specimen collected:...../...../.....

Patient Information

Medical Record #.....Last name:.....

First name:.....

Names of Parents/ Guardian (if child):

Father.....

Mother

Location of birth.....

Type of location (town or village).....

Occupation.....

Educational level (Primary/ Secondary/
University).....

District Settlement.....
Area.....

DOB...../...../..... or Age (Years) Sex: Male ☐
Female ☐

Ethnicity (look for ethnic groups in the districts)

Hepatitis B vaccination: ☐Yes ☐No ☐Not known, If vaccinated, approximate year:
.....

Results from viral hepatitis serological testing

	HBsAg (positive/negative)
Result	
Sample date	

Details of current illness

Date of onset of illness:/...../.....

Signs and Symptoms (check all that apply):

☐ Scleral icterus ☐ Dark urine ☐ Hepatomegaly ☐ Abdominal pain ☐ Diarrhoea
☐ Fever ☐ Arthralgia ☐ Rash ☐ Other
(specify).....

Has patient been previously diagnosed with hepatitis B: ☐ Yes ☐ No ☐ Not known *If yes: date of diagnosis? / /*

Additional Patient History

Have any family members been diagnosed with chronic hepatitis B and/or hepatitis D? ☐ Yes ☐ No ☐ Not known

During the **3 months** prior to illness, did patient receive any:

Prescribed Injection ☐ Yes ☐ No ☐ Not known Surgery ☐ Yes ☐ No ☐ Not known

Hemodialysis ☐ Yes ☐ No ☐ Not known Dental procedure ☐ Yes ☐ No
☐ Not known

During the **3 months** prior to illness, did patient inject drugs not prescribed by a doctor?

☐ Yes ☐ No ☐ Not known

If Yes: was needle sharing involved? ☐ Yes ☐ No ☐ Not known

During the **patient's lifetime**, did patient inject drugs not prescribed by a doctor?

☐ Yes ☐ No ☐ Not known

What is the marital status of patients.....

Was the Patient Pregnant? ☐ Yes ☐ No ☐ Unknown

If yes, Date of Delivery:/...../.....

Is/Was the Patient Hospitalized? ☐ Yes ☐ No ☐ Unknown

If yes – Admission Date:/...../.....; Discharge Date:/...../.....

How Many Sex Partners has the Patient Had in a Lifetime?.....

Did the Patient Receive an Organ Transplant Prior to 1992? ☐ Yes ☐ No ☐ Unknown
Was the Patient Ever

Treated for Sexually Transmitted Disease? ☐ Yes ☐ No ☐ Unknown

What is the HIV status of patient.....

Did the Patient Ever Work in the Medical Field and Have a Blood Exposure?
Ever had Contact to a Person who had Hepatitis?
☐ Yes ☐ No ☐ Unknown

Person Completing This Form:

.....Date:.....

6.0 ETHICAL CLEARANCE FROM

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH
Established 1979A Constituent of the College of Health Sciences

INSTITUTIONAL REVIEW BOARD

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Telex No: 2556 UGL GH

My Ref. No: DF-23
Your Ref. No:

University of Ghana
Post Office Box LG 581
Legon, Accra
Ghana



13th July, 2016

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824

IRB 00001276

NMIMR-IRB CPN 107/15-16

IORG 0000908

On 13th July 2016, the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB) at a full board meeting reviewed and approved your protocol titled:

TITLE OF PROTOCOL : Molecular characterization of Hepatitis B Virus (HBV) in Rural and Semi-Urban Areas in three Districts of the Central Region of Ghana

PRINCIPAL INVESTIGATOR : Caroline Agyare Boatemaa, MPhil Cand.

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to NMIMR-IRB within seven days verbally and fourteen days in writing.

This certificate is valid till 12th July, 2017. You are to submit annual reports for continuing review.

Signature of Chair:
Mrs. Chris Dadzie
(NMIMR – IRB, Chair)