Epidemiological Surveillance of HBV and HEV among Pregnant Women Attending Antenatal in Selected Hospitals in Ibadan, Oyo State, Nigeria

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Certification

This is to certify that **Ibukun Akinwumi AKINDELE** with matriculation number **LCU/PG/001705,** carried out this research titled "Epidemiological Surveillance of HBV and HEV among Pregnant Women Attending Antenatal in Selected Hospitals in Ibadan, Oyo State, Nigeria'' in the Department of Biological Science, Faculty of Applied Sciences, Lead City University, Ibadan, Oyo state, for the award of Doctor of Philosophy Degree (PhD) in Medical Virology and thatthis has not been previously submitted.

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Dedication

This research work is dedicated to God Almighty, my darling wife Oluwafunmike and children, Peter, Paul and Peace Akindele.

Acknowledgement

My utmost thanks goes to God Almighty, who bestowed grace and favor beyond my comprehension upon me during my PhD years. It was by His power that this thesis was completed. All that I am, to Him I owe.

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Even though the above-mentioned persons and institutions have assisted in the process of this research work, I alone stand responsible for any error or omission, if any, found in the work.

Abstract

Hepatitis B virus (HBV) infection in pregnancy is associated with potential viral transmission from mother to newborn which often makes the newborn a chronic carrier of HBV. Hepatitis E virus (HEV) infection causes acute viral hepatitis with high mortality rate among pregnant women. The overlap of HBV and HEV can have more adverse effects which frequently leads to severe complications and poor outcomes in pregnancy. This study aimed to investigate the prevalence rate of HBV and HEV infections among pregnant women attending antenatal in selected hospitals in Ibadan. A total of 297 pregnant women attending Adeoyo Maternity Teaching Hospital and Akinyele Primary Health Centre, Ibadan were enrolled into the study. From each participant, 5ml of blood was collected and Alanineaminotransferase (ALT) was measured. The plasma samples were screened for HBV serological markers (HBsAg, HBsAb, HBeAg, HBeAb, HBcAb) and HEV antibodies (IgM and IgG) using ELISA assays. Data were analyzed using SPSS version 16. Significance level set at $P<0.05$. Samples with serological evidence of HBV infection were screened for HBV DNA using the Real time-PCR technique. Samples positive for HBV DNA were subjected to genome sequencing. The seroprevalence of HBsAg was 7.4%, HBsAb (12.1%), HBeAg (0.7%), HBeAb (6.7%) and HBcAb (7.1%). Thirty (10.1%) women tested positive for anti-HEV IgG while none had detectable anti-HEV IgM. HBV-DNA prevalence was 21/22(95.0%). No significant relationship was identified among HBV and HEV seropositivity in the pregnant women in terms of age, trimester, source of water supply, education, animal contact, previous history of Jaundice, previous histories of blood transfusion and previous surgical operation. HBV and HEV IgG co-positivity rate of 1.7% was observed, with significantly high HBV prevalence among pregnant women with elevated ALT level compared with those with normal ALT level. Following molecular characterization of the HBV gene, the viral isolate was genotype E, typical of the dominant HBV genotype in Nigeria; which maintains susceptibility to current antiviral drugs and also shows low-frequency mutations and polymorphisms which were not considered to have a significant effect on immune escape for genotype E within the study population. There was high prevalence of HBV infection among pregnant women which could increase risk of mother-to-child transmission. Also, a limited number of pregnant women with increased infectivity with HBV had high viral load which may lead to increase maternal and childbirth morbidity and mortality. Hence, the need to increase access to vaccination in pregnant women. Similarly, a significant number of pregnant women had been previously exposed to HEV, demonstrating the endemicity of HEV infection in Ibadan, warranting the need to intensify personal hygiene enlightenment campaigns.

Keywords: Pregnancy, HBsAg, HEV IgM, HEV IgG, ALT
Word Count: 425

Table of Contents

Chapter Three: Methodology

List of Tables

List of Figures

List of Acronyms

Abbreviation	Meaning
AIH	Autoimmune Hepatitis
HAV	Hepatitis A Virus
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HDV	Hepatitis D Virus
HEV	Hepatitis E Virus
HCC	Hepatocellular Carcinoma
WHO	World Health Organization
WHA	World Health Assembly
EPI	Extended Program for Immunization
HBsAg	Hepatitis B Surface Antigen
NPI-NG	Nigeria National Program on Immunization
Anti-HBs	Hepatitis B Surface Antibody
HBcAb	Hepatitis B Core Antibody
HBeAg	Hepatitis B Envelope Antigen
HBeAb	Hepatitis B Envelope Antibody

HEV IgM	Hepatitis E Virus Immunoglobulin M
HEV IgG	Hepatitis E Virus Immunoglobulin G

Chapter One

Introduction

1.1 Background to the Study

Hepatitis B infection is an infectious disease caused by the hepatitis B virus (HBV) that affects the liver. It can cause both acute and chronic infections. Many people have no symptoms during the initial infection. Some develop a rapid onset of sickness with vomiting, yellowish skin, tiredness, dark urine and abdominal pain. Often these symptoms last a few weeks and rarely does the initial infection result in death. It may take 30 to 180 days for symptoms to begin[1]. In those who get infected around the time of birth 90% develop chronic hepatitis B while less than 10% of those infected after the age of 5 years do. Most of those with chronic disease have no symptoms; however, cirrhosis and liver cancer may eventually develop. These complications result in the death of 15 to 25% of those with chronic disease[2]. HBV is a double-stranded DNA virus. It is one of the smallest enveloped animal viruses, and the 40 nm virions, capable of infecting liver cells known as hepatocytes[2].

The virus is transmitted by exposure to infectious blood or body fluids. Infection around the time of birth or from contact with other people's blood during childhood is the most frequent method by which hepatitis B is acquired in areas where the disease is common[3]. In areas where the disease is rare, intravenous drug use and sexual intercourse are the most frequent routes of infection[3]. Other risk factors include working in healthcare, blood transfusions, dialysis, living with an infected person and travel in countries where the infection rate is high[3]. Globally, tattooing and acupuncture led to a significant number of cases in the 1980s; however, this has become less common with

improved sterility[4]. The infection can be diagnosed 30 to 60 days after exposure. The diagnosis is usually confirmed by testing the blood for parts of the virus and for antibodies against the virus[3].

The infection has been preventable by vaccination since 1982. Vaccination is recommended by the World Health Organization in the first day of life. Two or three more doses are required at a later time in life. This vaccine efficacy is about 95%. According to WHO recommendations, 190 countries in the world have introduced hepatitis B vaccination into their national childhood immunization programs with an excellent profile of safety, immunogenicity, and effectiveness[5]. It is also recommended that all blood be tested for hepatitis B before transfusion and condoms be used to prevent infection. During an initial infection, care is based on the symptoms that a person has. In those who develop chronic disease, antiviral medication such as tenofovir disoproxil fumarate, entacavir and interferon alafenamide fumarate may be useful; however, these drugs are expensive[6].

About a third of the world population has been infected at one point in their lives, including 240 million to 350 million who have chronic infections[7]. Approximately 1.5 million people become newly infected each year[6]. Over 750,000 people die of hepatitis B each year and about 300,000 of these are due to liver cancer[6]. The disease is now only common in East Asia and sub-Saharan Africa where between 5 and 10% of adults are chronically infected. Rates in Europe and North America are less than 1%[8]. HBV was originally known as "serum hepatitis"[9].

Hepatitis E virus (HEV) infection is the most frequent cause of acute viral hepatitis (AVH) in developing countries[10]. The disease was first recognized in the Indian subcontinent in the 1950s. In the industrialized countries, hepatitis E is considered as an emerging disease of global importance and has been reported in a number of developed countries. A seroprevalence study of hepatitis E carried out in Romanian population highlighted that hepatitis E clearly is an emerging disease in

Romania as in other developed countries[11]. Also, a study from India has shown that post-transplant patients who are immunocompromised progressed to chronic HEV infection[12].

In addition to this peculiar trait of progressing to chronic hepatitis E in immunocompromised patients, HEV has an interesting course in pregnant women in certain geographical regions of the world[13]. Studies from various developing countries have shown that the incidence of HEV infection in pregnancy is high and a significant proportion of pregnant women can progress to fulminant hepatitis with a mortality rate varying from 30−100%[13].

Hepatitis E virus (HEV) is a single-stranded RNA virus that causes large-scale epidemics of acute viral hepatitis, particularly in developing countries. In men and non-pregnant women, the disease is usually self-limited and has a case-fatality rate of less than 0.1%. However in pregnant women particularly from certain geographic areas in India and Nigeria, HEV infection is more severe, often leading to fulminant hepatic failure and death in a significant proportion of patients[14,24]. In contrast, reports from Egypt, Europe and the USA have shown that the course and severity of hepatitis E virus during pregnancy is not different from that in non-pregnant women[15]. The reasons for these geographical differences are not clear. The high mortality rate in pregnancy has been thought to be secondary to the associated hormonal (estrogen and progesterone) changes during pregnancy and consequent immunological changes. These immunological changes include down regulation of p65 component of NF κB with a predominant Th2 bias in the T-cell response along with host susceptibility factors, mediated by HLA expression[16].

1.2 Statement of the Problem

Despite the existence of effective vaccines against hepatitis A and B, viral hepatitis is still a major global public health concern[17]. Hepatitis B virus (HBV) is a global life-threatening disease with

hundreds of millions of individuals chronically infected[17]. The WHO estimates that 296 million people were living with chronic hepatitis B infection in 2018[17]. Hepatitis B virus (HBV), an hepadnavirus that infects liver cells, was responsible for an estimated 1.1 million deaths in 2019, mostly from the subsequent development of hepatocellular carcinomas (HCC)[19]. In Africa, approximately 60 million people live with chronic HBV infection with an estimated prevalence of 6.2%[20]. Nigeria has been ranked as one of the hyper-endemic countries for HBV infection with a prevalence rate of 8.1%[18]. Approximately nine in ten Nigerians living with chronic HBV are ignorant of their infection status, and are missing from the global public health statistics due to lack of resources, awareness, and political will for addressing Nigeria's HBV plight[18,21].

Generally, pregnant women have depressed immunity, thus infection with HBV is of clinical importance[22]. Hepatitis B virus (HBV) causes fulminant hepatitis in pregnant women and in regions where HBV incidence is high, there is vertical transmission from an infected mother, either prepartum or perinatally, with the child having greater than 60% risk of acquiring HBV infection[22].

Hepatitis E virus (HEV) infection is a major public health problem in Africa, especially in resource limited countries[23]. About 20 million cases and 3.3 million acute cases of HEV occur globally[20,24]. In developed countries, genetic similarities between human HEV strains and those isolated from pigs, cows, chickens, rabbits, rats, and fish have been observed[18]. In African countries, some HEV outbreaks were reported in Ethiopia, Somalia, Uganda, Democratic Republic of Congo, Sudan, South Sudan, and Nigeria[25]. In Nigeria, only a few HEV seroprevalence studies have been performed until today which detected a seroprevalence (IgG and total antibodies) between 7.0%–66.7% in different populations[26, 27]. Two outbreaks of HEV have been documented in Nigeria; one small outbreak in Port Harcourt, South-South Nigeria involving ten people between November 1997–June 1998 and another outbreak in 2017 with about 1,815 individuals including pregnant

women and a case fatality rate of 0.6% in Borno State, Northeast Nigeria[27,28]. While during the first outbreak, only HEV-2 was reported, the second outbreak was caused by both genotypes 1 and 2[29, 31].

Hepatitis E virus (HEV) is an enteric virus which is mainly transmitted via the fecal-oral route especially through contaminated water and it is associated with large water-borne outbreaks[30]. HEV can also be transmitted parenterally by blood transfusion or direct contact with infected animals[31]. The infection is self-limiting with a mortality rate of about 1.0 to 2.0% in the general population. However, the mortality rate can increase up to 45.0% in high risk populations such as pregnant women[32]. In immunodeficient or immunocompromised patients HEV infection may result in chronic infections[32].

Hepatitis B and E viruses cause a devastating disease in immunosuppressed individuals such as pregnant women[32]. The risk for a pregnant woman who is positive for HBV acquired through perinatal, sexual or blood contact, to be infected with HEV is high[35]. This can be as a result of the consumption of contaminated water or lack of proper hygiene, leading to infections with the two viruses in a pregnant woman, which can present additional risks, which frequently lead to severe complications and poor outcomes in pregnancy[35]. In patients with chronic HBV infection, HEV is a common cause of liver failure, accounting for 20% of cases in regions endemic for HEV[32]. Furthermore, in some studies, a higher HEV seroprevalence in individuals with HBV-related liver disease in comparison to healthy individuals has been observed[33,35]. HBV and HEV are transmitted vertically from infected mother to infant, thereby resulting in both maternal and fetal complications[35].

1.3 Justification of the Study

Co-infection of HBV with other viral infections has been reported by several researchers[36]. Studies on HBV infection with other viruses in different group have been carried out. Hepatitis B infection with HCV and HIV are known to affect the progression therapy management and clinical outcomes of these infections. Also, tuberculosis (TB) and viral hepatitis infections in the same patient poses a unique challenge to such a patient[37]. Hepatitis B infection with malaria also leads to a public health challenges[38]. There is paucity of information on HBV infection with HEV, especially among pregnant women. Therefore, epidemiological study in this population group seems to be necessary to reveal the real prevalence and to estimate their true burden.

1.4 Aim and Objectives of the Study

This study aimed to determine the prevalence rate of HBV and HEV infections among pregnant women attending antenatal clinics in selected hospitals in Ibadan, Oyo State, Nigeria.

The specific objectives were to:

i. Determine the seroprevalence of HBV and HEV among pregnant women attending antenatal clinics.

ii. Investigate the co-infection rate of HBV and HEV among pregnant women in the study population.

iii. Identify the risk factors associated with HBV and HEV infections among pregnant women attending antenatal clinics.

iv. Conduct molecular analysis on serologically positive samples using real time-PCR to detect and quantify HBV DNA and HEV RNA.

v. Perform genome sequencing on HBV DNA and HEV RNA positive samples and compare sequences with reference strains to identify potential mutations.

1.5 Significance of the Study

Elimination of HBV is still a challenge, though vaccination and effective management strategies appear to be a progress towards achieving success in the eradication of the virus[18]**.** Due to the abundance of asymptomatic or unreported cases of HEV in Nigeria, prevalence of such diseases is underestimated even under the integrated disease surveillance and response (IDSR) system[34]**.** Therefore, epidemiological study among pregnant women attending antenatal clinics in Ibadan is necessary to reveal the real prevalence and to estimate their true burden for early diagnosis and proper management**.**

This research will provide valuable data for increasing the overall understanding of the devastating diseases caused by HBV and HEV infections and will facilitate the development of future HEV vaccination programs to precisely target the high risk populations who are prone to having the worst outcome from the infections[35].

1.6 Scope of the Study

HIV and HCV screening of plasma samples using RDT, ALT measurement using Colorimetric Method, HBV and HEV serological screening of plasma samples using ELISA method, HBV and HEV molecular analysis using real time-PCR and Genome sequencing using Nested PCR and Sanger techniques.

1.7 Limitation of the Study

Due to financial constraints, screening for HBV DNA using real time PCR (qPCR) could not be performed in all HBsAg negative study samples. Thus, the prevalence of occult HBV infection

within the entire study population could not be established.

1.8 Operational Definition of Terms

Acute Hepatitis Infection: A new, suddenly occurring infection. It occurs with a person's first exposure to the hepatitis B virus.

Antibody: A protein molecule produced by the immune system in response to a foreign body, such as the hepatitis B virus and its antigens.

Antigen: A protein on the surface of a virus, bacteria or cell that can stimulate the immune system to produce antibodies as a defense mechanism.

Chronic Hepatitis B Infection: A patient who tests positive for the hepatitis B virus for more than six months is considered to have a chronic hepatitis B infection.

Hepatitis B Surface Antigen (HBsAg): The surface protein of the hepatitis B virus that is used as a marker to detect infection.

Hepatitis B Surface Antibody (HBsAb or anti-HBs): The antibody formed in response to the surface protein of the hepatitis B virus. It can be produced in response to vaccination or recovery from an actual hepatitis B infection.

Hepatitis B Core Antibody (HBcAb or anti-HBc): This antibody only refers to a part of the virus itself.

Hepatitis B "e" Antigen (HBeAg): A marker of a high degree of hepatitis B infectivity, it correlates with a high level of viral replication. It is primarily used to help determine the clinical management of patients with chronic hepatitis B infection.

Hepatitis E Virus Immunoglobulin M (Anti-HEV IgM): This antibody indicates recent infection with hepatitis E virus.

Hepatitis E Virus Immunoglobulin G (Anti-HEV IgG): This antibody indicates past infection

with hepatitis E virus.

HBV DNA: A marker of viral replication. It indicates how much viral load or hepatitis B virus (HBV) is in a patient.

Alanine aminotransferase: It is an enzyme that helps the liver convert food into energy. High enzymes levels can be a sign that the liver is injured or irritated and the enzymes are leaking out of the liver cells.

Hepatocellular Carcinoma (HCC): A malignant tumor of the liver, otherwise known as liver cancer. Chronic hepatitis B and C infections may increase the risk of developing liver cancer.

PCR (polymerase chain reaction): A highly sophisticated scientific method of detecting the presence of hepatitis B virus DNA in the blood.

Perinatal Transmission (vertical transmission): The transmission of an infectious disease, such as hepatitis B, from a mother to her newborn. The most important mode of HBV transmission globally is from the mother to her newborn baby.

Seroconversion: A change in status from antigen positive/antibody negative to antigen negative/antibody positive.

Vaccine: A medication that stimulates the production of antibodies to protect against a specific disease.

Viral Load: Measurement of the actual amount of virus in the bloodstream such as hepatitis B and C.

Virus: A tiny microorganism, smaller than bacteria, which can invade the body and cause disease.

Pregnancy: This is the term used to describe the period in which a fetus develops inside a woman's womb or uterus.

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Chapter Two

Literature Review

2.1 Structure of Hepatitis B Virus

Hepatitis B virus (HBV) is a member of the hepadnaviridae family. The virus particle (virion) consists of an outer lipid envelope and an icosahedral nucleocapsid core composed of protein. These virions are 30–40 nm in diameter. The nucleocapsid encloses the viral DNA and a DNA polymerase that has reverse transcriptase activity. The outer envelope contains embedded proteins that are involved in viral binding of, and entry into, susceptible cells. The virus is one of the smallest enveloped animal viruses, and the 40 nm virions, capable of infecting liver cells known as hepatocytes, are referred to as "Dane particles". In addition to the Dane particles, filamentous and spherical bodies lacking a core can be found in the serum of infected individuals (Figure 2.1). These particles are not infectious and are composed of the lipid and protein that forms part of the surface of the virion, which are called the surface antigens (HBsAg), and is produced in excess during the life cycle of the virus[1].

The genome of HBV is made of circular DNA, but it is unusual because the DNA is not fully double-stranded. One end of the full length strand is linked to the viral DNA polymerase (Figure 2.2). The genome is 3020–3320 nucleotides long (for the full-length strand) and 1700–2800 nucleotides long (for the short length-strand)[2]. The negative-sense (non-coding) is complementary to the viral mRNA. The viral DNA is found in the nucleus soon after infection of the cell. The partially double-stranded DNA is rendered fully double-stranded by completion of the (+) sense strand and removal of a protein molecule from the (−) sense strand and a short sequence of RNA

from the (+) sense strand. Non-coding bases are removed from the ends of the (−) sense strand and the ends are rejoined. There are four known genes encoded by the genome, called C, X, P, and S. The core protein is coded for by gene C (HBcAg), and its start codon is preceded by an upstream in-frame AUG start codon from which the pre-core protein is produced. HBeAg is produced by proteolytic processing of the pre-core protein (Figure 2.3).

In some rare strains of the virus known as Hepatitis B virus precore mutants, no HBeAg is present[3]. The DNA polymerase is encoded by gene P. Gene S is the gene that codes for the surface antigen (HBsAg). The HBsAg gene is one long open reading frame but contains three in frame "start" (ATG) codons that divide the gene into three sections, pre-S1, pre-S2, and S. Because of the multiple start codons, polypeptides of three different sizes called large (the order from surface to the inside: pre-S1, pre-S2, and S), middle (pre-S2, S), and small (S) are produced. The function of the protein coded for by gene X is not fully understood but it is associated with the development of liver cancer (Figure 2.4). It stimulates genes that promote cell growth and inactivates growth regulating molecules[4].

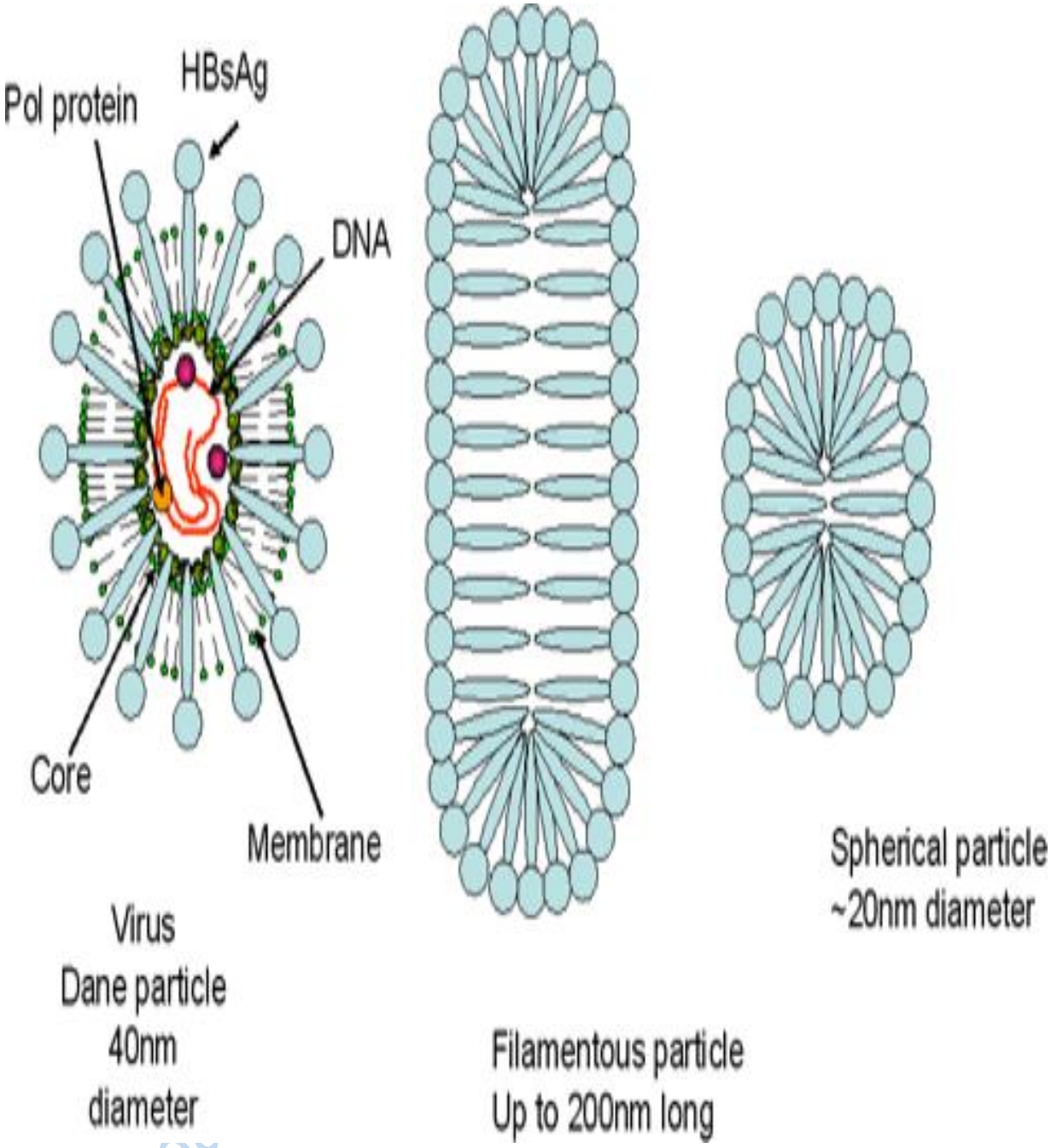

Figure 2.1: Diagrammatic Representation of the Three HBV Morphologies

Source[1]

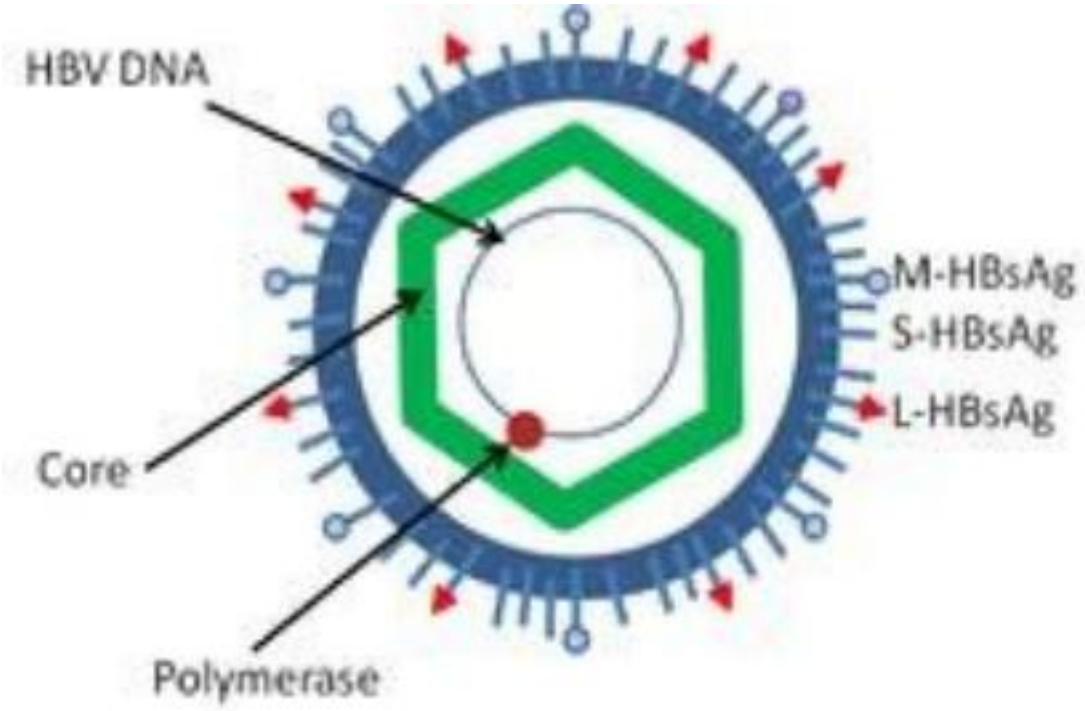

Figure 2.2: The Structure of Hepatitis B Virus

Source[2]

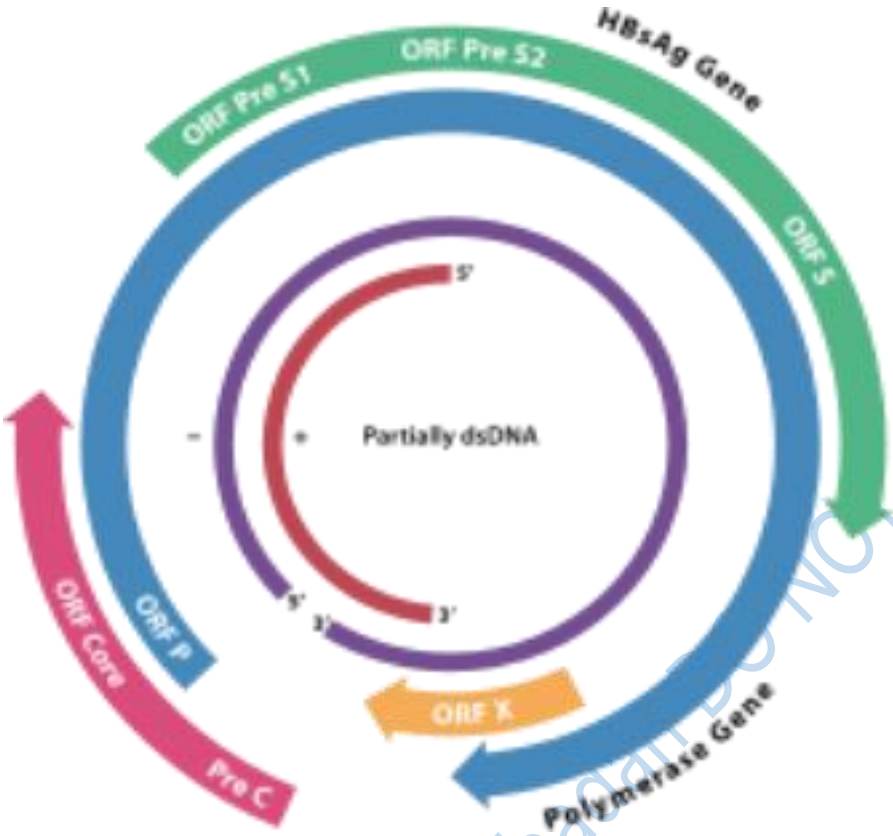

Figure 2.3: The Genome Organization of HBV

Source[4]

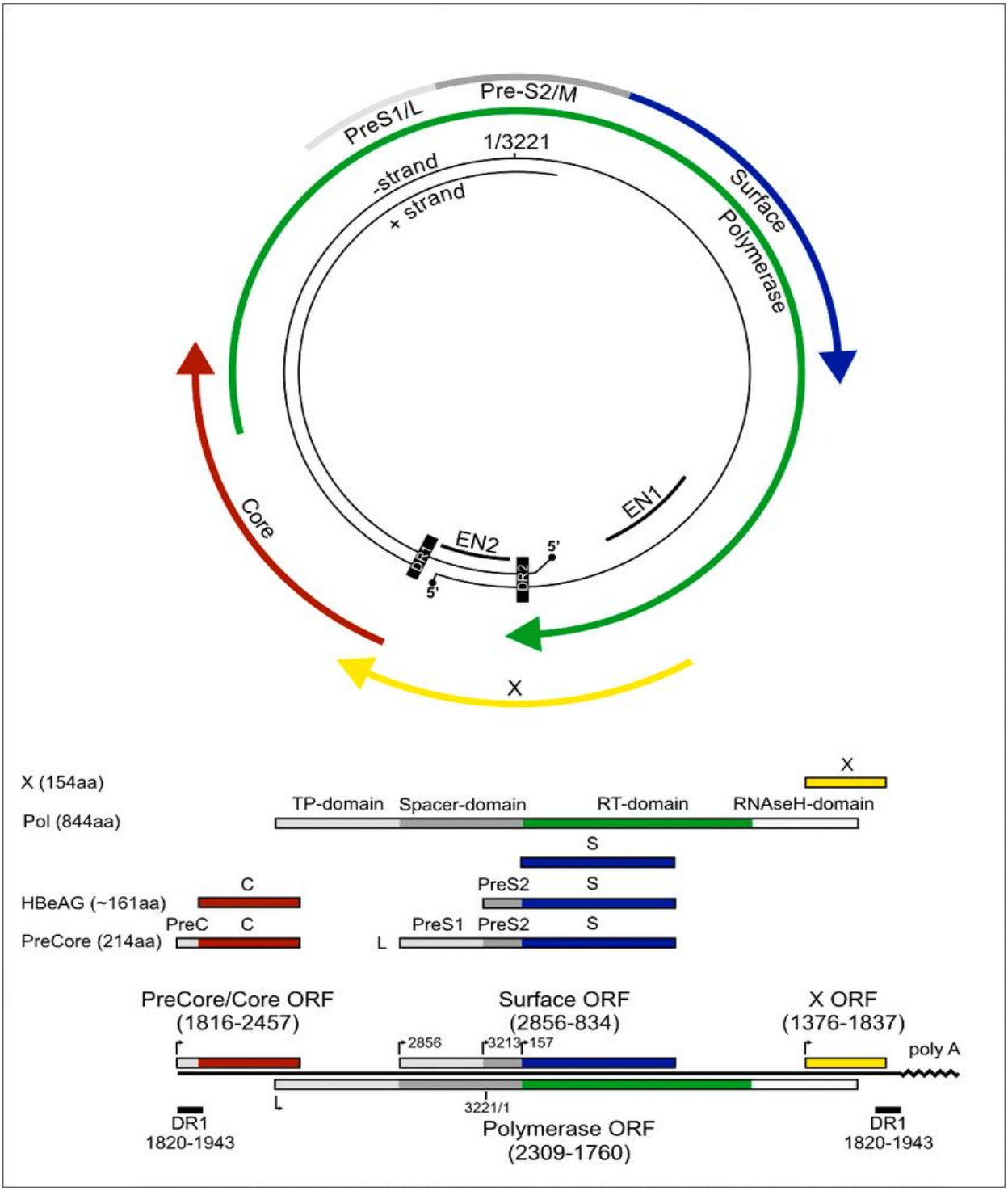

Fig 2.4: Diagrammatic Representation of the Genome Organization and Transcripts of HBV

Source[4]

2.2 Modes of Transmission

Transmission of hepatitis B virus results from exposure to infectious blood or body fluids containing blood. It is 50 to 100 times more infectious than HIV. Possible forms of transmission include sexual contact, blood transfusions and transfusion with other human blood products, re-use of contaminated needles and syringes, and vertical transmission from mother to child (MTCT) during childbirth. Without intervention, a mother who is positive for HBsAg has a 20% risk of passing the infection to her offspring at the time of birth. This risk is as high as 90% if the mother is also positive for HBeAg[5].

HBV can be transmitted between family members within households, possibly by contact of non-intact skin or mucous membrane with secretions or saliva containing HBV. However, at least 30% of reported hepatitis B among adults cannot be associated with an identifiable risk factor[6]. Breastfeeding after proper immunoprophylaxis does not appear to contribute to mother-to-child-transmission (MTCT) of HBV. The virus may be detected within 30 to 60 days after infection and can persist and develop into chronic hepatitis B. The incubation period of the hepatitis B virus is 75 days on average but can vary from 30 to 180 days[5].

2.3 Life Cycle and Pathogenesis

The life cycle of hepatitis B virus is complex. Hepatitis B is one of a few known pararetroviruses: non-retroviruses that still use reverse transcription in their replication process. The virus gains entry into the cell by binding to NTCP on the surface and being endocytosed. Because the virus multiplies via RNA made by a host enzyme, the viral genomic DNA has to be transferred to the cell nucleus by host proteins called chaperones. The partially double-stranded viral DNA is then made fully

double stranded by a viral polymerase and transformed into covalently closed circular DNA (cccDNA) (Figure 2.5)[7].

This cccDNA serves as a template for transcription of four viral mRNAs by host RNA polymerase. The largest mRNA, (which is longer than the viral genome), is used to make the new copies of the genome and to make the capsid core protein and the viral DNA polymerase. These four viral transcripts undergo additional processing and go on to form progeny virions that are released from the cell or returned to the nucleus and re-cycled to produce even more copies. The long mRNA is then transported back to the cytoplasm where the virion P protein (the DNA polymerase) synthesizes DNA via its reverse transcriptase activity (Figure 2.6)[7].

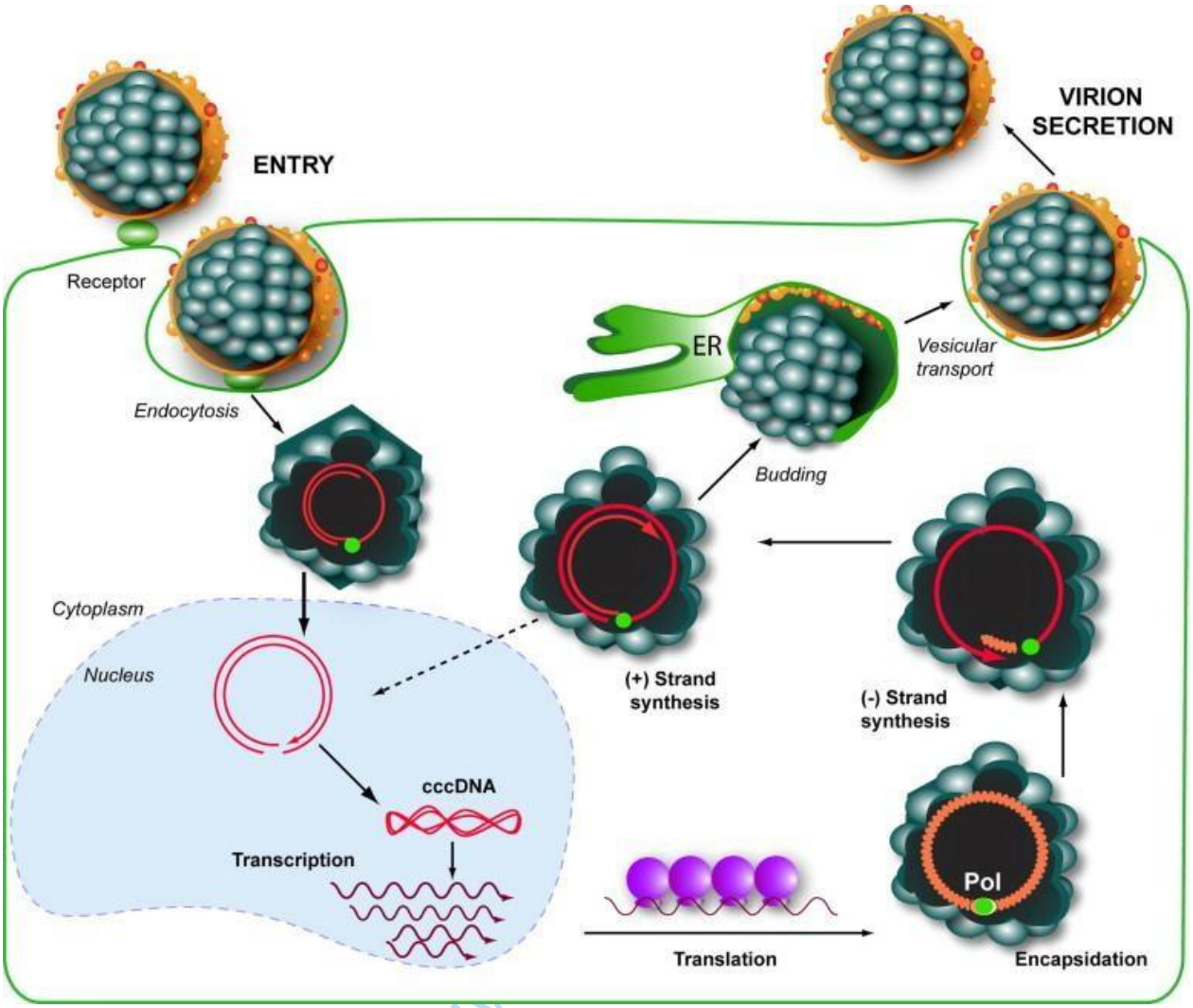

Fig. 2.5: Schematic Representation of the HBV Life Cycle One

Source[7]

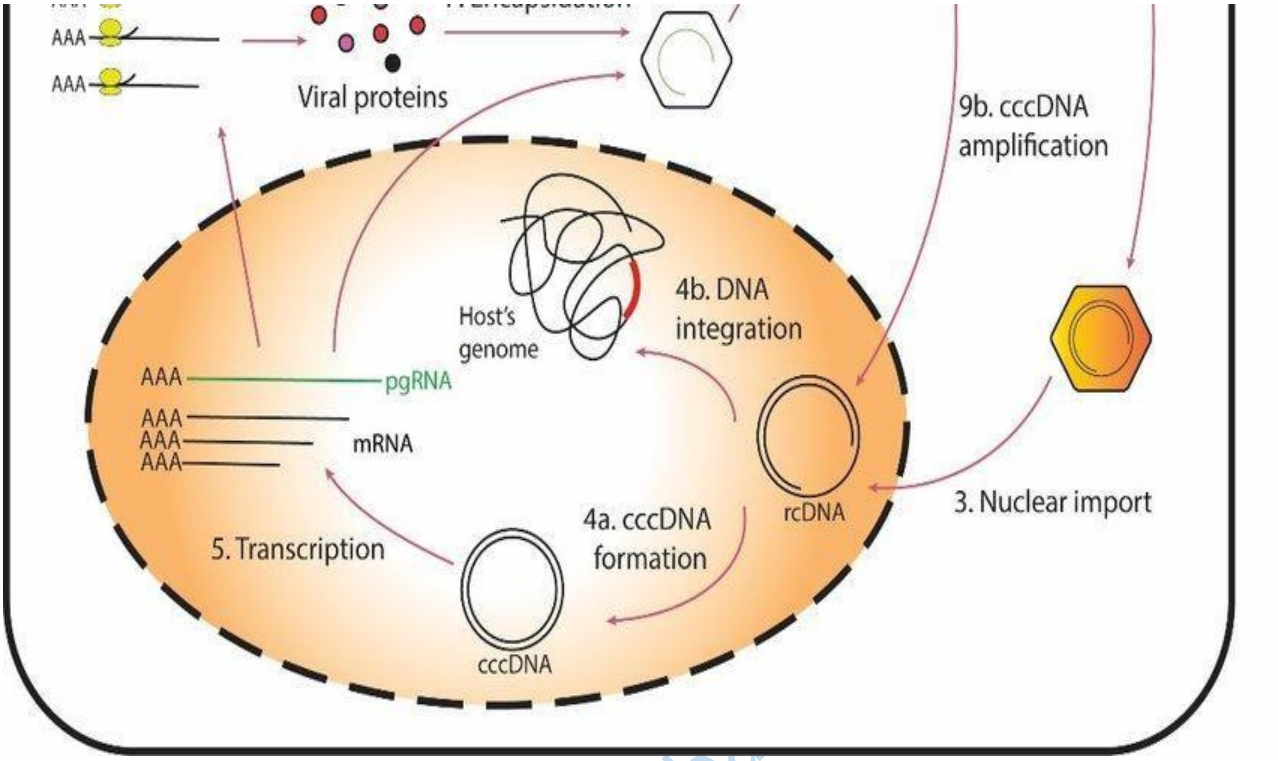

Figure 2.6: Schematic Representation of the HBV Life Cycle Two

Source[7]

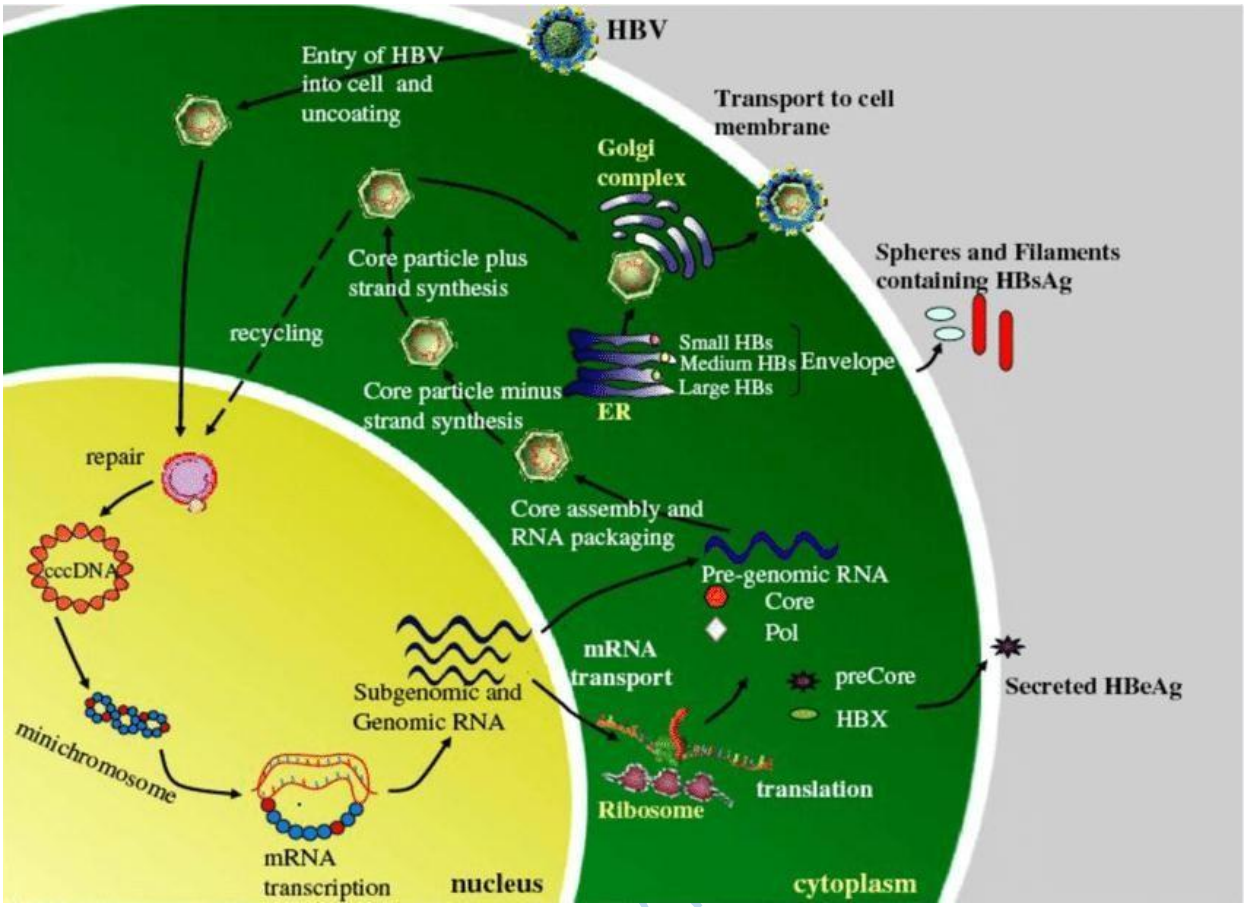

Figure 2.7: Schematic Representation of the HBV Life Cycle Three

Source[7]

2.4 HBV in Pregnancy

Viral hepatitis during pregnancy is associated with high risk of maternal complications such as premature labour, intra-ventricular haemorrhage, intra-partum and post-partum haemorrhage from coagulation failure due to lack of production of vitamin K dependent clotting factors[8].

There is a high rate of vertical transmission causing fetal and neonatal hepatitis which can have serious effects which may lead to impaired mental and physical health later in life and it is also said to be the most familiar cause of jaundice in pregnancy[9]. Perinatal transmission of this disease occurs if the mother has had acute Hepatitis B infection during late pregnancy, in the first postpartum or if the mother is a chronic hepatitis B surface antigen (HBsAg) carrier[9]. In patients with acute hepatitis B, vertical transmission occurs in up to 10% of neonates when infection occurs in the first trimester and in 80%–90% of neonates when the infection occurs in the third trimester[9].

Ten to twenty percent of neonates born to HBsAg-positive mothers and 90% of those born to both HBsAg and HBeAg-positive mothers will be infected with HBV[8].

2.5 Immunology

Hepatitis B virus primarily interferes with the functions of the liver by replicating in hepatocytes. A functional receptor is sodium taurocholate cotransporting polypeptide (NTCP). There is evidence that the receptor in the human hepatitis B virus is carboxypeptidase D. The virions bind to the host cell via the preS domain of the viral surface antigen and are subsequently internalized by endocytosis[10]. HBV-preS-specific receptors are expressed primarily on hepatocytes; however, viral DNA and proteins have also been detected in extrahepatic sites, suggesting that cellular receptors for HBV may also exist on extrahepatic cells[10].

During HBV infection, the host immune response causes both hepatocellular damage and viral clearance. Although the innate immune response does not play a significant role in these processes, the adaptive immune response, in particular virus-specific cytotoxic T lymphocytes (CTLs), contributes to most of the liver injury associated with HBV infection[11]. CTLs eliminate HBV infection by killing infected cells and producing antiviral cytokines, which are then used to purge HBV from viable hepatocytes. Although liver damage is initiated and mediated by the CTLs, antigen-nonspecific inflammatory cells can worsen CTL-induced immunopathology, and platelets activated at the site of infection may facilitate the accumulation of CTLs in the liver[11].

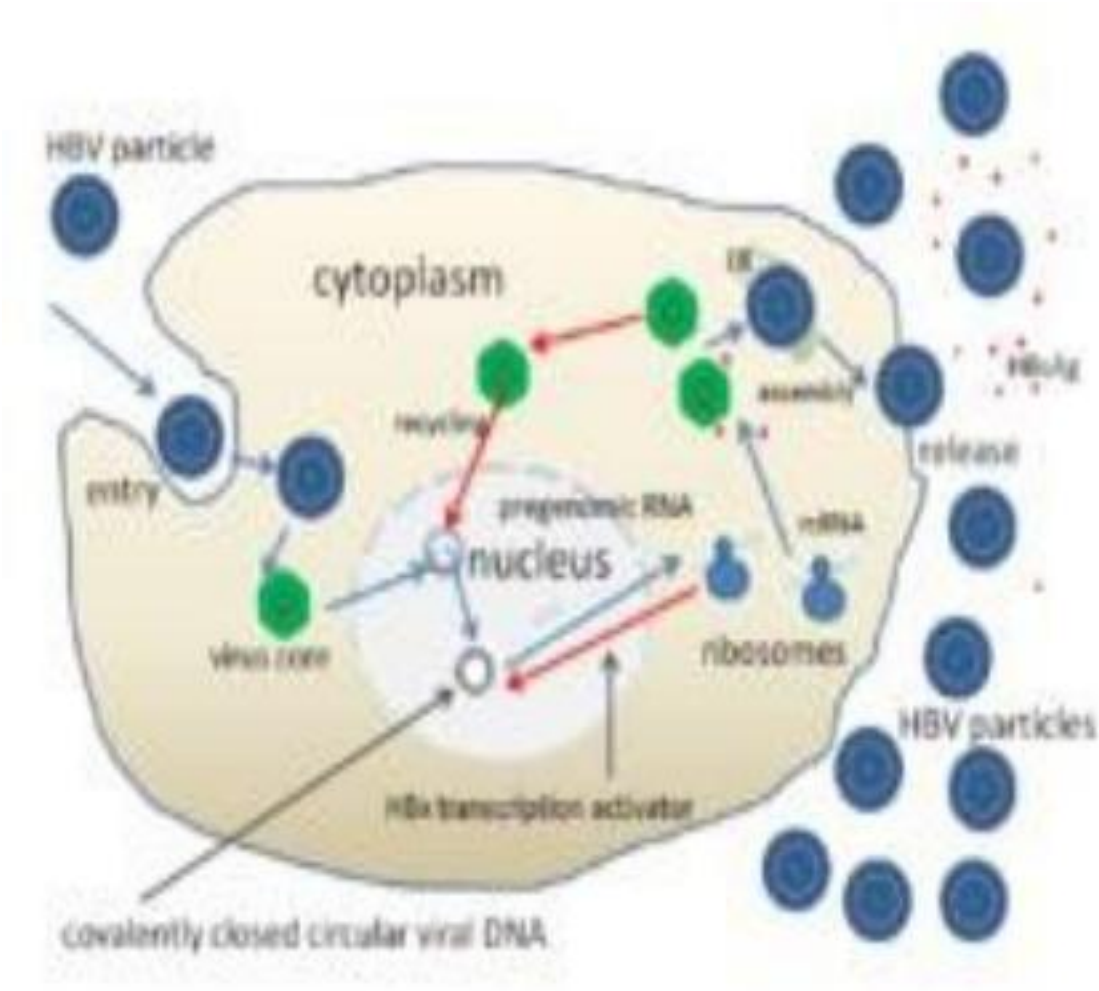

Figure 2.8: Hepatitis B Virus Replication

Source[7]

2.6 Signs and Symptoms

Acute infection with hepatitis B virus is associated with acute viral hepatitis, an illness that begins with general ill-health, loss of appetite, nausea, vomiting, body aches, mild fever, and dark urine, and then progresses to development of jaundice[12]. It has been noted that itchy skin has been an indication as a possible symptom of all hepatitis virus types. The illness lasts for a few weeks and then gradually improves in most affected people. A few people may have a more severe form of liver disease known as (fulminant hepatic failure) and may die as a result. The infection may be entirely asymptomatic and may go unrecognized[12].

Chronic infection with hepatitis B virus either may be asymptomatic or may be associated with a chronic inflammation of the liver (chronic hepatitis), leading to cirrhosis over a period of several years. This type of infection dramatically increases the incidence of hepatocellular carcinoma (HCC; liver cancer). Across Europe, hepatitis B and C cause approximately 50% of hepatocellular carcinomas. Chronic carriers are encouraged to avoid consuming alcohol as it increases their risk for cirrhosis and liver cancer. Hepatitis B virus has been linked to the development of membranous glomerulonephritis (MGN) [12].

Symptoms outside of the liver are present in 1–10% of HBV-infected people and include serum-sickness–like syndrome, acute necrotizing vasculitis (polyarteritis nodosa), membranous glomerulonephritis, and papular acrodermatitis of childhood (Gianotti–Crosti syndrome). The serum-sickness–like syndrome occurs in the setting of acute hepatitis B, often preceding the onset of jaundice. The clinical features are fever, skin rash, and polyarteritis. The symptoms often subside shortly after the onset of jaundice but can persist throughout the duration of acute hepatitis B. About 30–50% of people with acute necrotizing vasculitis (polyarteritis nodosa) are HBV carriers. HBV-

associated nephropathy has been described in adults but is more common in children[13]. Membranous glomerulonephritis is the most common form. Other immune-mediated hematological disorders, such as essential mixed cryoglobulinemia and aplastic anemia[14].

2.7 Prevalence of Hepatitis B Virus

In 2004, an estimated 350 million individuals were infected worldwide. National and regional prevalences range from over 10% in Asia to under 0.5% in the United States and Northern Europe[15]. Routes of infection include vertical transmission (such as through childbirth), early life horizontal transmission (bites, lesions, and sanitary habits), and adult horizontal transmission (sexual contact, intravenous drug use)[15].

The primary method of transmission reflects the prevalence of chronic HBV infection in a given area. In low prevalence areas such as the continental United States and Western Europe, injection drug abuse and unprotected sex are the primary methods, although other factors may also be important[16]. In moderate prevalence areas, which include Eastern Europe, Russia, and Japan, where 2–7% of the population is chronically infected, the disease is predominantly spread among children[16]. In high-prevalence areas such as China and South East Asia, transmission during childbirth is most common, although in other areas of high endemicity such as Africa, transmission during childhood is a significant factor[16]. The prevalence of chronic HBV infection in areas of high endemicity is at least 8% with 10–15% prevalence in Africa/Far East (Figure 2.9)[17]. Current study reported a prevalence of 8.8% in the average risk Nigerian population[18].

As of 2010, China has 120 million infected people, followed by India and Indonesia with 40 million and 12 million, respectively[6]. According to World Health Organization (WHO), an estimated 600,000 people die every year related to the infection[6]. In the United States about 19,000 new cases occurred in 2011 down nearly 90% from 1990[15]. In Nigeria, hepatitis B remained a hyper-endemic area with an estimate of 12% of the total population being chronic carriers, despite series of viral screening assays, various generations of HBV vaccines, and effective treatment options, HBV still remain a risky, life-threatening illness and significant public health problem[18]. The Society for Gastroenterology and Hepatology in Nigeria also reported that about 20 million Nigerians are infected with Hepatitis B virus[18].

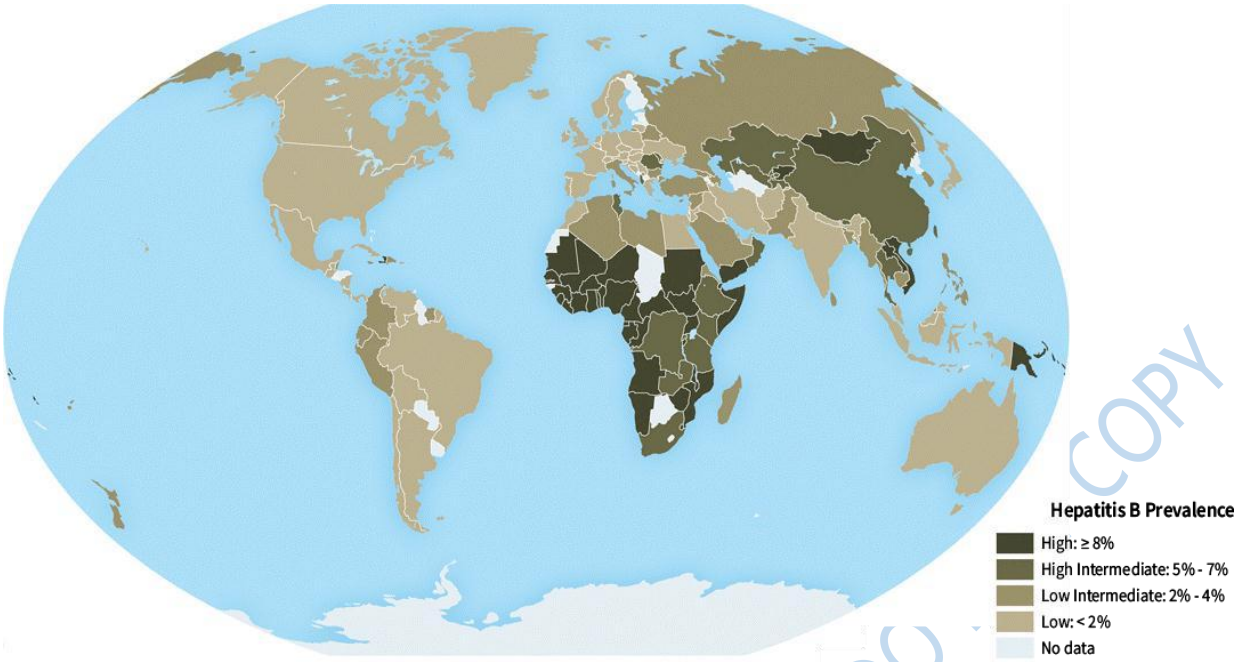

Figure 2.9: Diagrammatic Representation of the Global Distribution of Hepatitis B

Source[15]

2.8 History of HBV Infection

The earliest record of an epidemic caused by hepatitis B virus was made by Lurman in 1885. An outbreak of smallpox occurred in Bremen in 1883 and 1,289 shipyard employees were vaccinated with lymph from other people. After several weeks, and up to eight months later, 191 of the vaccinated workers became ill with jaundice and were diagnosed as suffering from serum hepatitis. Other employees who had been inoculated with different batches of lymph remained healthy. Lurman's paper, now regarded as a classical example of an epidemiological study, proved that contaminated lymph was the source of the outbreak. Later, numerous similar outbreaks were reported following the introduction, in 1909, of hypodermic needles that were used, and, more importantly, reused, for administering Salvarsan for the treatment of syphilis. The virus was not discovered until 1966 when Baruch Blumberg, then working at the National Institutes of Health (NIH), discovered the Australia antigen (later known to be hepatitis B surface antigen, or HBsAg) in the blood of Australian aboriginal people[19].

Although a virus had been suspected since the research published by Frederick MacCallum in 1947, David Dane and others discovered the virus particle in 1970 by electron microscopy[20]. By the early 1980s the genome of the virus had been sequenced, and the first vaccines were being tested[21].

2.9 Serotypes and Genotypes

The virus is divided into four major serotypes (adr, adw, ayr, ayw) based on antigenic epitopes presented on its envelope proteins[22]. There are several genotypes of hepatitis viruses with different clinical implications and distinct geographic distributions. HBV is classified into 10 genotypes (A-J) and about 40 subgenotypes with a correlation between genotypes and their modes of

transmission[22]. In fact, HBV genotype A is found in North America, Europe, South-East Africa and India; genotypes B and C in Asia and Oceania while genotype D is the most common in North America, North Africa, Europe, the Middle-East and Oceania. HBV genotype E is hyperendemic in West Africa; genotype F is found in South America; and genotypes G and H are in Central and South America. Available literature have proclaimed genotype E to be the predominant genotype in Nigeria (Figure 2.10)[22]. Differences between genotypes affect the disease severity, course and likelihood of complications, and response to treatment and possibly vaccination[22].

Figure 2.10: The Global Distribution of HBV Genotypes and Sub-genotypes. Aa/A1 („a‟ for Africa/Asia), Ae/A2 („e‟ for Europe), Ba/B2 („a‟ for Asia), Bj/B1 („j‟ for Japan), Ce/C1 („e‟ for east) and Cs/C2 („s‟ for south)

Source[22]

2.10 Pathology of Hepatitis B Infection

Exposure to HBV trigger a cascade of complex interactions between the virus and the non-immune host which leads to a diverse range of clinical manifestations ranging from an asymptomatic state to an acute or fulminant hepatitis and eventually to a chronic disease with progression to several sequelae. HBV infection pathogenesis is therefore a function of viral replication and immune responses from the host cell to the invasion of the virus[28,33]. Several other factors also influence the natural history of an HBV infection, such as host factors (immune status and genetics), viral factors (the size of the inoculum, the virulence of the HBV strain causing the infection and co-infection with other pathogens) as well as environmental factors (alcohol use and exposure to toxins)[30]. HBV being a hepatotropic virus and as such its major target organ will be the liver which made up of a diversity of cells including bile ductule epithelia, Kupffer cells and hepatocytes, all of which bear the greater part of the liver's functionality.

The liver is crucial for various reasons including its nutrients processing and energy storing ability, body detoxification, blood homeostasis and its ability to assist in immune response to microbial infection. All these roles therefore make any injury or infection to this organ to automatically interfere with any of its important roles, and thereby impairing the overall state of wellbeing. Of all the cells of the liver, HBV has an affinity for hepatocytes which constitute about 70% of the liver's cell mass[34]. The virus can also infect extrahepatic cells such as peripheral blood lymphocytes (PBL) and monocytes especially those that may share the same HBV receptor with the hepatocytes. Following HBV Invasion, HBV replicates within the host target cells and in so doing alter the normal functioning of the cell. A process that does not induce cytopathic effects, and this mean the virus by itself is not the direct cause of cell and liver damage. It is reported that the immune response from the host cell to viral antigens released on the surface of infected cells is the main contributing

factor to liver injury[29,32].

2.10.1 Acute HBV Infection

After primary exposure to the virus, asymptomatic incubation period kickstarts, and often last between one to four months. At this period no effective innate immune reaction to the presence of the virus is detectable and it is this delayed response that accounts for the numerous viral replication and consequential widespread infection of the hepatocyte population (Figure 2.11)[34]. Active viral replication is marked by the presence of HBeAg (a tolerogen and indicator of infectivity) in serum, accompanied by a high titre viremia and HBsAg which may persist over time but finally becomes negative after 2-4 months of the infection.

In an attempt to confine the rapid spread of the virus, the host put up an adaptive humoral immune response. High levels of antibodies against the viral core antigen (initially IgM antiHBc and later total anti-HBc; IgM+IgG) and HBeAg (anti-HBe) are secreted but these do not provide any recognized immune protection[23]. This is followed jointly by production of neutralizing antibodies to HBsAg (anti-HBs) which bind to the large amounts of HBsAg and eliminate cell-free virions. While at this process however, large amounts of immune complex (HBsAg/anti-HBs) deposits are produced, and these infiltrate in the blood stream to cause a type III hypersensitivity reaction characterized by skin rash, athralgia and in some cases nephritis[23,77].

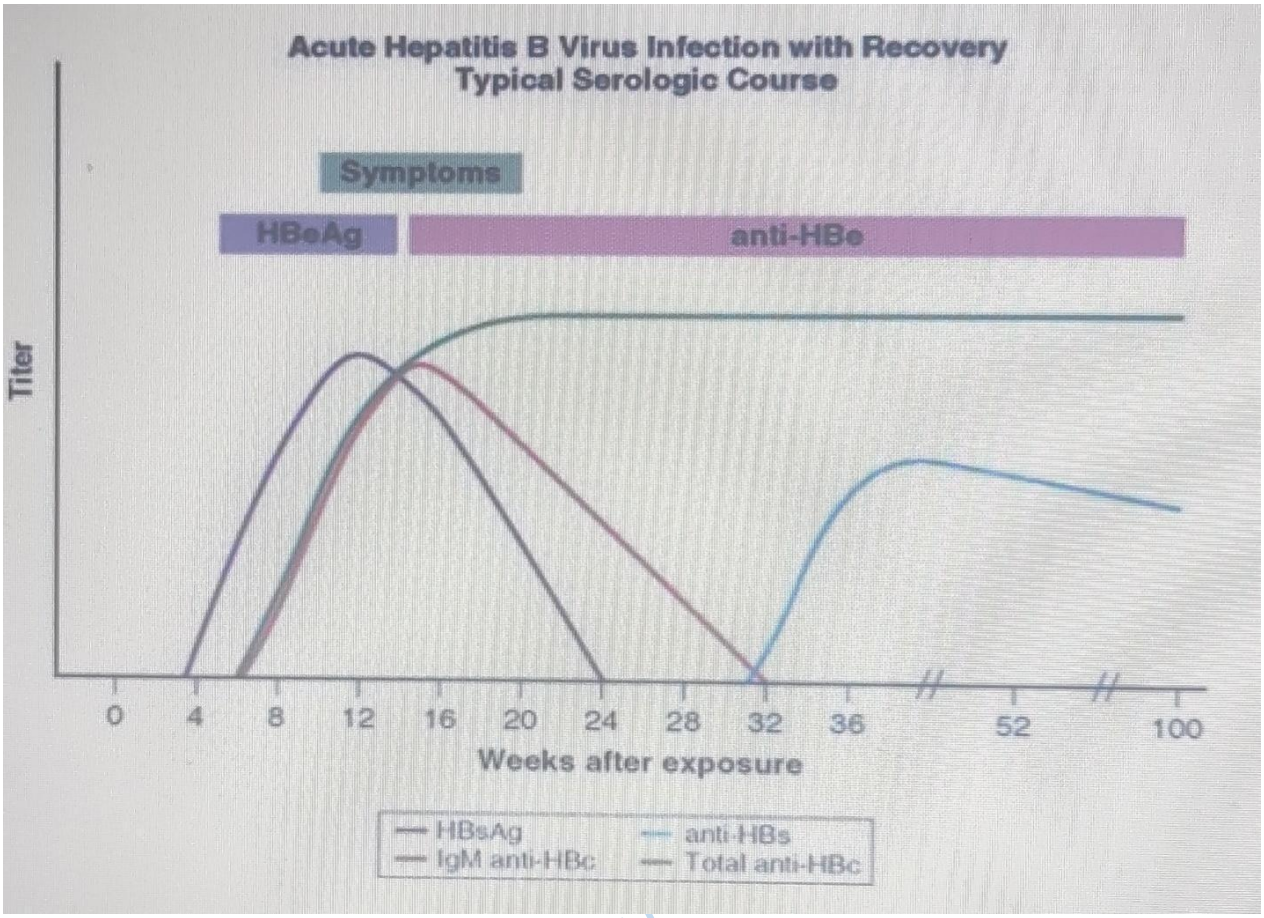

Figure 2.11: Graphical Representation of the Typical Serologic Course During an Acute HBV Infection

Source[34]

A cell-mediated immune response to the infection is also initiated, which typically involves virus specific cytotoxic T lymphocytes (CTL; CD8+ T cells) against viral antigens presented by major histocompatibility complex molecules (MHC class I) on infected cell surfaces. These CD8+ T cells act to effect clearance of cell-bound virions by both cytolytic and non-cytolytic means, essentially reducing the viral load[77]. Lysis of infected hepatocytes leads to leakage of liver aminotranferases (ALT and AST) into the blood stream, suggesting damage to the liver cells[78.79]. The damage then marks the onset of hepatitis associated with clinical symptoms such as right upper quadrant discomfort, nausea and jaundice among other non-specific symptoms. The symptoms may persist for up to three months after which they resolve with hepatocyte regeneration and undetectable viremia[79]. Viral DNA (cccDNA) may not be outrightly cleared after recovery as its presence within intact hepatocytes can still be seen; a source of hepatitis B flares in the event of an immune-suppressed state. Complete recovery from acute hepatitis B is almost always certain but death may result in rare case (0.1-1%) from liver failure due to onset of fulminant hepatitis. Acute HBV infection may also progress to a state of chronicity[78,79].

2.10.2 Chronic HBV Infection

Natural History of Chronic HBV Infection

The natural history of chronic hepatitis B infection is a unique and complex process that can present a variable course, which is dependent on a balance between viral parameters and host immune response[80]. Almost 90% of infected newborns and 30% of infected children develop emphasizing the primary role of the patient's immune system. Recently, consensus statements have schematically grouped chronic hepatitis B into five phases: the "immune tolerant phase", the "immune active phase", the "inactive HBV carrier state", the "HBeAg-negative chronic hepatitis

B" and finally the "HBsAg-negative phase"[81,82]. Typically, the initial phase of chronic HBV infection is marked by HBeAg-positivity, high levels of HBV DNA in serum and variable elevation of serum alanine aminotransferase (ALT). However, in a percentage of HBeAg-positive patients, ALT levels are normal and liver damage is minimal despite high levels of HBV replication. This state called the "immune tolerant phase" is frequent after perinatal infections and it can persist for years[82].The "immune active phase" also known as "chronic hepatitis B phase" is typified by elevated ALT levels, low levels of HBV DNA in serum, and moderate to severe liver necroinflammation that may lead to fibrosis. While this phase can occur many years after experiencing the "immune tolerant phase" in perinatally-infected patients, it can be detected shortly after infection during adulthood. Large cell dysplasia has been frequently found in advanced chronic hepatitis B with increased activity, this involves hepatocytes with senescent features and no sign of proliferative or apoptotic activity, and results during periods of frequent neoplastic transformation, thus raising the possibility that it might occur as a safeguard against the development of hepatocellular carcinoma[83].

Senescence in tumourigenesis is said to be a defense mechanism and evasion of senescence is said to lead to malignancy[84]. Among patients in the "immune active phase", many eventually graduate into the "inactive HBV carrier phase", marked by loss of HBeAg, seroconversion to anti-HBe antibodies and decline of serum HBV DNA to barely detectable levels, alongside improvement of fibrosis and inflammation over time[81,85]. It is however reported that up to one-third of patients will undergo seroconversion to anti-HBe antibodies and transition to the "HBeAg-negative chronic hepatitis B state", marked by periodic reactivation associated with fluctuating levels of ALT and HBV DNA, and active hepatitis with variable degree of fibrosis. This transition has been attributed to the occurrence of nucleotide substitutions in the pre-core and/or the basal core promoter region

that preclude expression of the e antigen[86]. Emergence of these HBV variants is associated with active liver disease, and with a high tendency of developing cirrhosis and HCC. In contrast, loss of HBsAg in patients in the "HBsAg-negative phase" has a favorable prognosis, although HCC incidence is still higher than in non-infected populations[87]. Observation of such may be linked to the findings that persistent traces of HBV DNA are mostly detectable in the blood for many years after clinical recovery from acute hepatitis, even with the presence of serum antibodies and HBV-specific CTLs[86]. While a most of liver cancers develop in cirrhotic livers, a certain fraction of HBV-related HCCs occurs in a background of chronic hepatitis B in the absence of liver cirrhosis. The incidence of cirrhosis often appears to be about 2-fold higher in HBeAg-negative compared to HBeAg-positive chronic hepatitis[88]. The lower rate of underlying cirrhosis in HBV-related HCCs compared to other etiologies argues for a far more direct role of HBV in the tumoural process. In additional, distinctive gene expression profiles have been detected in the non-tumoural livers of chronic HBV carriers, such as activated expression of genes implicated in pro-apoptotic, inflammatory and DNA repair responses, connoting specific pathways activated by chronic hepatitis B[89]. Thus, besides the effects of host immune responses, HBV replication might trigger various signaling cascades in the infected hepatocyte[89].

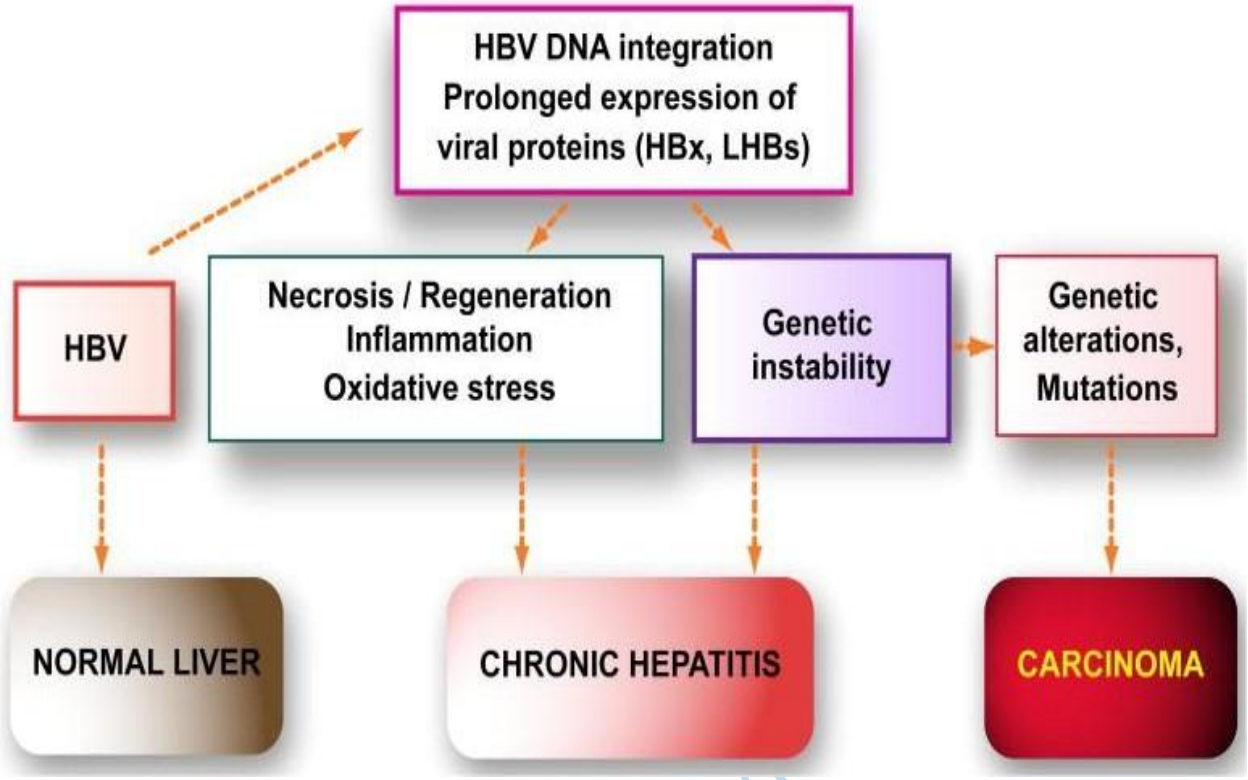

Figure 2.12: Chronic HBV Infection and Hepatocarcinogenesis

Source[90]

Integration of viral, host and environmental parameters with molecular changes might help to identify novel targeted strategies in improving the management of chronic hepatitis B. Risk scores have been confirmed to estimate the risk of developing HCC in less than 10 years post presentation. Scores such as age, gender, HBV DNA levels, core promoter mutations and cirrhosis, has been used to identify high-risk patients for treatment and screening of HCC (Figure 2.12)[90].

Chronic hepatitis B is defined by persistent HBV infection with high titre serum HBsAg positivity for more than 6 months (Figure 2.13)[91]. These results following failure of the host immune system to effectively clear an acute HBV infection after primary exposure or to protect against subsequent infection. Which then results to viral replication persistence and is accompanied by HBeAg positivity for a long period of time with eventual seroconversion to anti-HBe after years of infection.

The eventual course of a chronic HBV infection is variable, another school of thought reported it to specifically involve four dynamic phases: "the immune tolerance phase", "the immune clearance phase", "the inactive carrier phase" and "the reactivation phase". Making the ultimate outcome of chronic HBV infection to be varied, usually involving sequelae such as liver cirrhosis, rare cases of fulminant hepatitis and in some cases HCC, all of which can be fatal[92,93].

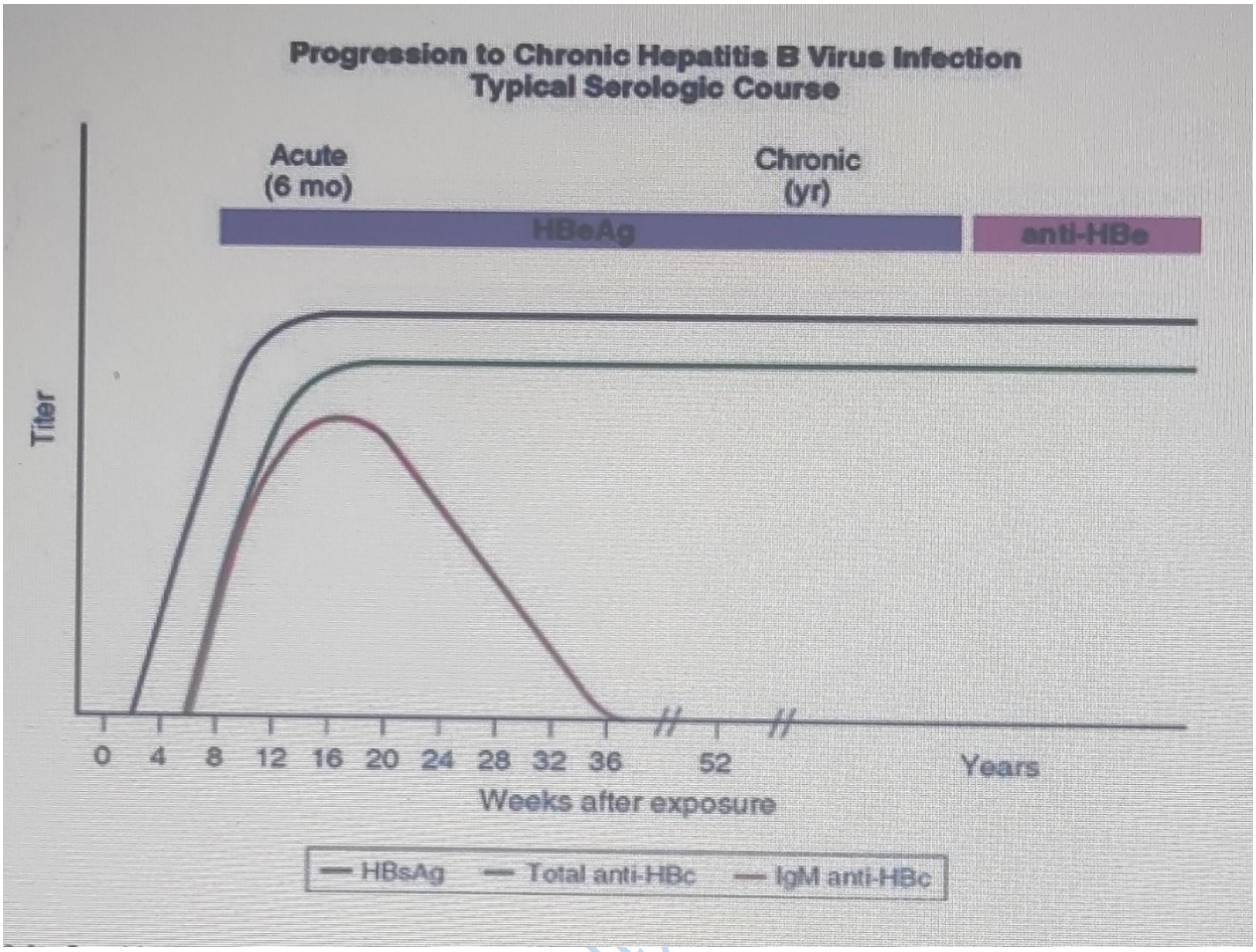

Figure 2.13: Graphical Representation of the Typical Serologic Course During the Progression from Acute HBV Infection to Chronic Hepatitis B

Source[91]

2.10.3 Immune Tolerance Phase

The immune tolerance phase is clinically explained as persistent HBeAg positive with viral DNA levels ≥20 000 IU/mL with no productive immunological reaction to the presence of the virus[13]. This is generally said to be the first phase of chronic infection in perinatally- and childhood-acquired HBV infections but is rarely observed in those infected at adulthood. Those in this phase has a high infectivity rate due to the high level of viremia. Nevertheless, even with extensive intrahepatic viral replication coupled with the absence of an efficient immune response, no liver injury is noticed and thus infected hepatocytes stay intact and blood alanine transaminase (ALT) levels also remain continuously normal[94-96].

This state of immune inactivity is largely as a result of the tolerogenic property of viral HBeAg that is reported to induce T helper (Th) cell tolerance to HBeAg and to render the CTL response to HBcAg ineffective[95,96]. The extent of viral replication and high levels of viral antigens released have also been reported to exhaust HBV specific CD8+ T cell response. It is however proposed that regulatory T cells (Treg) also play a role in immune tolerance to infection via the suppression of HBV-specific T cell response[95]. Among those with perinatally-acquired infections, it has been suggested that maternal HBeAg is transferred by means of the placenta to the fetus where the viral antigen prompt clonal deletion or ignorance of fetal HBV-specific T cells[77,95]. These mechanisms produce a quiescent disease state which usually last up to several decades after which immune reactivity may be returned[93,96].

2.10.4 Immune Clearance Phase

Once the host immune response is brought back, an immune reaction is launched in an effort to clear the infection during a phase called “the immune reactive” or “clearance phase of chronic

hepatitis B". Those HBV infections acquired in adulthood that goes on to achieve a state of chronicity move directly to this phase shortly after infection. The host immune response causes serum HBV DNA levels to decline, reaching up to 2000 IU/ml while blood ALT titre elevates as infected hepatocytes are destroyed[79,92]. This leads to previously asymptomatic patients begin to experience bouts of clinical manifestations consistent with liver injury, identify as acute exacerbation of chronic hepatitis B. The symptoms usually mimic acute HBV infection and are mostly self-limiting following hepatic inflammation which in certain cases could be come with liver fibrosis. Cases of mortality during this phase are mainly due to liver failure. In most immunologically responsive patients however, remission of liver disease is the usual outcome and is accompany by spontaneous seroconvertion to anti-HBe with fluctuating levels of serum HBeAg[93].

2.10.5 Inactive Carrier Phase

It's been reported that the of success of the immune clearance phase in initiating viral clearance is evident in the reduction of viral DNA levels to 105 copies/mL, serum ALT level (above ULN) and in some cases liver histological grade and stage. The age of the patient, severity of hepatic disease, chances of response to therapy, the risk that accompany adverse effects and complications is required before considering making the decision to treat[97]. Two separate therapeutic agents are available for the treatment of chronic HBV infection, both immunomodulators and antiviral drugs[98]. Immunomodulators used in the treatment of chronic HBV infection, such as interferon alpha (IFNα) and pegylated interferon α (peginterferon α; PEG-IFNα), usually initiate an antiviral response by eliciting host cellular immune responses against cell-bound virions[99,100]. Advantages that accompany PEG-IFNα therapy includes define duration of treatment, no resistance selection and also durable response to the virus. Although PEG-IFNα is also used in hepatitis C therapy, it is very

effective in the treatment of chronic HBV infection in HBV and HCV co-infected individuals[97]. Nonetheless, PEG-IFNα has a number of disadvantages, such as its high cost, the associated need for frequent subcutaneous administration and risk of adverse effects[100]. Nucleos(t)ide reverse transcriptase inhibitors (NRTIs; antiviral drugs) on the other hand are easily administered orally and are usually free of significant adverse effects, which made them the therapeutic agents of choice for chronic hepatitis B treatment[101,102]. Example of the NRTIs available for treatment of chronic HBV infection include Lamivudine (3TC), Tenofovir disoproxil fumarate (TDF), Adefovir dipivoxil (ADV), Telbivudine (LdT), Entecavir (ETV) and Emtricitabine (FTC). In effect, these NRTIs act by inhibiting the viral reverse transcriptase activity that then interrupts HBV DNA replication and consequently suppresses synthesis of infectious virions[102,103].

The larger part of these NRTIs also form part of the highly active antiretroviral therapy (HAART) regimen used in the treatment of HIV infection and as such ADV, LdT and 3TC+TDF/Truvada (Truvada, a combination of FTC and TDF) for example, have been used in the treatment of chronic HBV infection in HBV/HIV co-infected individuals[104,105]. A major blow to the success of antiviral treatment was the development of drug resistance following long-term exposure to some of the NRTIs[102,106]. First line use of the most potent NRTIs which have a high barrier to resistance such as ETV or TDF as well as combination therapy with 3TC+TDF or Truvada have been reported to be some of the best strategies for preventing resistance development to NRTIs[106,107].

While ensuring successful therapeutic effect, it is essential to monitor response to therapy, which involves laboratory evaluation of serum HBV DNA level, serological markers (HBsAg, HBeAg and anti-HBe) and serum ALT levels during the course of treatment[104]. Ideal duration of therapy is not standardized but is dependent on maintained virologic (<2000 copies/mL) and serologic (loss of HBsAg and HBeAg/anti-HBe seroconversion) response to treatment with normalization of

serum ALT levels post treatment cessation[108].

2.11 Prevention of HBV Infection

Several strategies have been described in the prevention of HBV infection, namely; behavioral modification, passive immune-prophylaxis and active immunization[109]. Behavior modification targeted at reducing the risk of transmission of HBV infection includes the practice of risk-reduction measures such as safe sex and non-sharing of sharp objects (razors and syringes) as well as strict compliance to universal precautions in the health care setting in order to minimize nosocomial and occupational HBV exposures. These measures have been reported to be more effective in regions where the hepatitis B epidemic is chiefly driven by adult-acquired HBV infections when compared to regions such as Asia and Sub-Saharan Africa where prevalence of infant- and childhood-acquired HBV infections are reportedly higher respectively[110]. In Asia and Sub-Saharan Africa regions, passive immune-prophylaxis offers a better means of reducing the incidence of primary HBV infection as compared to behavior modification.

Immune-prophylaxis such as hepatitis B immunoglobulin (HBIG) is administered as a precautionary measure in neonates of mother positive to HBsAg and is also recommended for individuals following needle stick and sexual exposures or after liver transplantation[109]. To a great extent, active immunization (vaccination) remains the single most effective preventive measure (100%) against primary HBV infection and consequently, chronic HBV infection and its fatal sequelae[27].

A safe and highly effective hepatitis B vaccines are globally available, conventionally in the form of plasma-derived vaccines (containing highly purified viral surface protein from plasma of chronic

HBsAg carriers) and currently in the form of recombinant DNA vaccines (containing the viral surface protein produced via recombinant DNA technology)[111]. The vaccine has been administered as a monovalent formulation or in a fixed combination with other vaccines (diphtheria-tetanus-pertussis, Heamophilus influenzae type b, hepatitis A and inactivated polio vaccines). In most cases, the hepatitis B vaccine is administered in 3 or 4 doses to initiate release of anti-HBs (optimum protective titre ≥10 mIU/mL) and to prime immune memory cells (B and Th cells) against the HBsAg component which confers long-lasting (~20 years) immunity to HBV infection[111]. Prompt administration of the HB vaccine in combination with HBIG as a post-exposure prophylaxis is also strongly effective (>90%) in preventing neonatal HBV infections. Hepatitis B vaccination is also recommended for individuals at a high risk for HBV infection, such as health care workers, familial contacts of chronically infected individuals and persons with high-risk behaviors[109,110].

About two million health care workers has been reported by WHO to be at risk of occupational exposure to HBV each year with a low vaccination coverage among them. The United state of America centers for disease control and prevention (CDC) reported the risk of being infected to be dependent on the prevalence of the HBV carriers alongside frequency of exposure of HCWs to blood and other body fluids and tissue and the virulence of the virus[24]. HCWs in Nigeria are at a higher risk because Nigeria is a holoendemic area[26]. The mode of transmission of HBV in health care settings is most often by needle prick injuries and poor adherence to universal precautions, the risk of acquiring HBV in HCWs has been estimated to be four times greater than that of the general population[26]. HBV vaccination is therefore strongly recommended for HCWs as part of the universal precautions policy for protection of HCWs. HCWs who are HBV negative after screening should take hepatitis B vaccination, while those who are HBV positive should be treated. However, vaccination among HCWs continue to be a challenge for many countries[109]. Some studies reported

that all HCWs including administrative staff within health care delivery settings should receive the hepatitis B vaccine[26]. Prevention of occupational hazards among HCWs requires a thorough knowledge of the risks and practical measures to be taken, and the need for HCWs workers to familiarize themselves with universal work precautions as it has been reported that about a million HCWs had cut and puncture injuries per year[27].

National guideline for prevention, care and treatment of HBV and hepatitis C virus (HCV) infections was developed in 2016, with vaccination of HCWs being one of the preventive methods in health care settings[27].

2.12 Diagnosis of HBV Infection

Diagnosis of HBV infection can be done either at the acute or chronic stages of the disease. Meanwhile, presentation of certain clinical symptoms such as jaundice, right upper quadrant discomfort and nausea may be suggestive of an acute HBV infection, majority of acute HBV infections are subclinical and as such most diagnoses are made during the active stages of chronic disease. Conclusive diagnosis of HBV infection is made with the aid of laboratory tests that assess serological, molecular, biochemical and histological markers of infection, also putting into consideration patient history (risk factors predisposing to an HBV infection)[67]. Laboratory diagnosis of an HBV infection relies absolutely on ability to distinguish the virus form all other possible etiologies of hepatitis[58]. In the absence of a reliable in vitro tissue culture systems for the cultivation of HBV, differential diagnosis of the virus is dependent on other laboratory tests. Serological tests, has been a major first line diagnostic tool used in determining the presence of specific HBV antigens and their corresponding antibodies using antigen-antibody reaction[58]. Other diagnostic tools include molecular methods which detect the presence of viral nucleic acids. Also

carting out an assessment of liver function with biochemical tests also assists in differentiating viral hepatitis from non-viral causes of hepatic injury and helps in monitoring of the prognosis of the disease. In the laboratory diagnosis of HBV infection, of all the specimens used, blood takes the first place as it is a relatively non-invasive specimen and also circulates throughout the body, harbors an abundance of viral particles and secreted proteins during an infection[58,67]. Other specimens of importance in the diagnosis of HBV infections, includes liver biopsies which are used both in histopathology (grading and staging of liver disease) and immunohistochemical staining (detection of viral antigen in liver tissue). But because liver biopsies involve surgery, these methods are rarely used during clinical diagnosis of an HBV infection[67].

2.12.1 Serological Testing

Serological markers screening (host antibodies and viral antigen) helps in establishing a suspected HBV infection and help to distinguish acute self-limiting infections from the different phases of chronic disease. A number of automated systems exist for quantitative and qualitative determinations of serological markers in clinical specimens (serum or plasma), employing the enzyme immunoassay (EIA) principle together with colorimetric or chemiluminescence signal measurement of results[67]. The diagnostic algorithm in depicts the interpretation of results for the primary serological markers; HBsAg, anti-HBc, anti-HBs, and IgM anti-HBc. Secondary prognostic markers including HBeAg which gives an indication of persistent viral replication as well as infectivity and anti-HBe which denotes seroconversion and a less infectious state, may also be tested (Figure 2.14)[58].

2.12.2 Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a serological assay usually used to quantify

antibodies, antigens, proteins, hormones and glycoproteins in biological samples through antigen-antibody complex system. ELISA is usually carried out on a 96-well polystyrene plates, coated for very strong protein binding. ELISA requires primary and/or secondary detection antibodies or analyte/antigen or antibody/antigen coatings, buffers, wash solutions, and substrate/chromogen depending on the type employed. The primary antibody is specific and only binds to the protein or antigen of interest, while the secondary antibody is an enzyme-bound antibody that binds to a non-enzyme-bound primary antibody[67].

Four basic steps are involved during ELISA process:

1. Coating (with antigen or antibody)
2. Blocking (usually addition of bovine serum albumin [BSA])
3. Detection
4. Optical density reading

Detection is accomplished by adding a substrate capable of producing color. Many substrates are available for ELISA detection, however, the most usually used ones are horseradish peroxidase (HRP) and alkaline phosphatase (ALP). The substrate for HRP is hydrogen peroxide, which turns blue.

ELISA techniques can be divided into four types, namely

1. Direct ELISA (plate costed with antigen, screening antibody)
2. Indirect ELISA (antigen coated plate, antigen antibody screening)
3. Sandwich ELISA (antibody coated plate, antigen screening)

4. Competitive ELISA (antibody screening)

2.12.3 Direct ELISA

Both direct and indirect ELISA begin by applying the antigen to the wells of an ELISA plate. For the first binding step, antigen is added to the plate and incubated at 37^{O}C for 1 hour or optionally at 4^{O}C overnight. After the incubation step is complete, the next step is to wash potentially unbound antibodies from the plate and use agents such as BSA, ovalbumin aprotinin, or other proteins from animals for blocking to remove unbound antibodies from portions of the ELISA plate. This step prevents non-specific antibodies from binding to the plate and minimizes false positive results. After adding the buffer, wash the plate again and add the enzyme-bound primary detection antibody of choice. The plates are incubated for an additional hour[68].

In direct ELISA, the primary detection antibody binds directly to the protein of interest. The plate is then washed again to remove unbound antibody and a substrate/chromophore such as alkaline phosphatase (AP) or Horseradish Peroxidase (HRP) is added to the plate resulting in a color change. The color change of the sample is caused by the hydrolysis of the phosphate groups of the AP substrate or the oxidation of the HRP substrate. Direct ELISA helps to eliminate secondary antibody cross-reactivity and it is also fast due to fewer steps compared to indirect ELISA. Its disadvantages include lower sensitivity and higher reaction cost compared to other types of ELISA[67,68].

2.12.4 Indirect ELISA

The procedure for the indirect ELISA is the same as for the direct ELISA, except for the additional washing step and the type of antibodies added after the removal of the buffer. Indirect ELISA technique requires a primary antibody that binds to the protein of interest, and a secondary antibody

that is complementary to the primary antibody. At first, the primary antibody is added, followed by a washing step and then secondary antibody before incubation. The subsequent steps are the same as for direct ELISA, including washing steps, adding substrate, and detecting color change.

Indirect ELISA is more sensitive than direct ELISA. It is also inexpensive and flexible due to the large number of primary antibodies available. The major disadvantage of indirect ELISA is the risk of cross-reactivity between secondary detection antibodies[68].

2.12.5 ELISA Sandwich

Sandwich ELISA begins with a capture antibody coated on the wells of the plate unlike direct and indirect ELISA. This is called a "sandwich" because the antigen is sandwiched between two layers of antibodies (a capture antibody and a detection antibody). After the addition of the capture antibody to the plate, the plate is covered and incubated overnight at $4^{O}C$. After completion of the coating step, the plate is washed with PBS, the wells are blocked with BSA. Buffer washings are carried out at room temperature for at least 1-2 hours. Finally, before adding the antigen, the plate is washed once more with PBS[68].

The antigen of choice is then added to the plate to bind to the capture antibody and incubated at $37^{O}C$ for 90 minutes. The plate is washed again, then primary antibodies added and incubated for another 1-2 hours at room temperature followed by washing with buffer. Thereafter, a secondary antibody is added and incubated for another 1-2 hours Wash the plate and add the substrate to produce a colour change.

Sandwich ELISA is more sensitive than all the other types of ELISA. However, its main disadvantages are time consumption and cost, as well as the need to use a "matched pair" (bivalent/polyvalent antigen) and secondary antibodies[67,68].

2.12.6 Competitive ELISA

Competitive ELISA verifies the presence of antigen-specific antibodies in the test serum. It uses two specific antibodies; an enzyme-conjugated antibody and another antibody present in the test serum for positive sera. The combination of two antibodies in wells will allow competition for antigen binding. The test is negative if there is a colour change while no colour change indicates a positive result. Competitive ELISA has low specificity and cannot be used on diluted samples. However, the advantages are that it requires less sample purification, can measure a wide range of antigens in a given sample, can be used for small antigens, and has low variability[67,68].

TEST PROFILE	RESULTS	INTERPRETATION
HBsAg Anti-HBc Anti-HBs	Negative Negative Negative	Susceptible, never been infected
HBsAg Anti-HBc Anti-HBs	Negative Positive positive	Immune due to natural infection
HBsAg Anti-HBc Anti-HBs	Negative Negative Positive	Immune due to hepatitis B vaccination
HBsAg Anti-HBc IgM anti-HBc Anti-HBs	Positive Positive Positive Negative	Acutely infected
HBsAg Anti-HBc IgM anti-HBc Anti-HBs	Positive Positive Negative Negative	Chronically infected
HBsAg Anti-HBc Anti-HBs	Negative Positive Negative	Varied interpretation*

*1. Resolved infection (most common)
2. False-positive anti-HBc, thus susceptible
3."low level" chronic infection
4. Resolving acute infection

Figure 2.14: HBV Serological Results Interpretation

Source[58]

2.12.7 Biochemical Testing

The moment an HBV infection has been established using serological method, routine liver biochemical tests is usually the next line of action in determining the presence of hepatic injury and give an indication of the prognosis of disease[67]. Different measures of certain biochemical markers (aminotransferases, alkaline phosphatase, gamma-glutamyl transferase, bilirubin and albumin) aids in assessing liver viability and functioning. These biochemical markers are released into circulation once there is a damage to liver cells or during an alteration in liver metabolism. Although they are highly variable as they depend on many other variables including age, gender, physical exercise, stress, time of day as well as food ingestion, and may also vary from one person to another. A usually tested biochemical marker in the diagnosis of hepatitis B infection is serum ALT, an aminotransferase enzyme which is measured against a standard normal range (upper limit of normal; ULN) to determine damage to the liver[58,67]. Sustained and elevated (above the ULN) levels of serum ALT are suggestive of hepatic injury or liver inflammation and correlate with progressive liver disease. In some cases, however, individuals with severe liver disease may not show elevated serum ALT levels at all, thereby requiring other laboratory tests to arrive at a diagnosis[58,67].

2.13 Molecular Testing

A number of nucleic acid-based tests for the direct detection of viral DNA are now widely available. These molecular methods uses either signal (HBV Hybrid-Capture I, II) or target (conventional nested and real-time PCRs) amplification-based assays specific for the qualitative and quantitative (sensitivities reaching up to 101 -102 copies/mL for some PCR assays) detection of HBV DNA[73]. Determining the presence of HBV DNA has proving to be a viable alternative to serological tests for HBeAg as it correlates with the presence of active HBV infection with high viral loads which indicates infectivity. Molecular diagnostic methods therefore provide

supplementary information regarding viral replication during the course of an HBV infection, especially in cases where serological profiles are not enough to establish infection.

2.13.1 Molecular Techniques

As an advancement over traditional techniques such as filtering, staining, microscopy and serology, a more sensitive and specific methods such as nucleic acid-based testing comprising PCR methods and DNA sequencing have been employed in the analysis of viruses[25].

2.13.2 Polymerase Chain Reaction (PCR)

This method is used to amplify a small fragment of the nucleic acid (DNA or RNA) to produce many copies of the fragment which will in turn help to easily identify the specific pathogen. At the beginning of the process, the target DNA or RNA is extracted from the respective sample, and then a master mix is prepared containing all the components that we necessary for an effective amplification process. The PCR process goes through three established phases: Denaturation, Annealing and Elongation.

At the denaturation stage, al high temperature of up to 95^0C, the two strands of the double stranded DNA (dsDNA) are separated (denatured) into single stranded DNA (ssDNA). The heat helps to break up the bonds between the strands exposing the nitrogenous bases. The primers (present in the master mix) then anneal to the exposed nitrogenous bases from the 3'end of the open reading frame to the S' end if they are complementary. This is done at a lower temperature of 40-700C. This stage takes place over 30-45 cycles. The third period which is the elongation step is carried out at of 72^0C. At this stage, the DNA polymerase enzyme (Taq polymerase) attaches to the primer and drags it along the length of the ssDNA thereby synthesizing a new dsDNA of the virus.

There are various forms of PCR: Conventional PCR, Nested PCR, Reverse transcriptase PCR (RT-

PCR), Real Time PCR (RT-qPCR) etc. Conventional PCR also called end-point PCR makes use of a pair of primers (forward and reverse) to anneal to its template. The process runs through its entire duration and the amplicons generated are processed using gel electrophoresis to be able to visualize the DNA bands and their sizes. This is then correlated with the expected size to interpret the result. An advancement over this type of PCR is the real time PCR which produces result in real time while the process is still in progress. Realtime PCR takes advantage of a fluorescent reporter (probes), which florescence proportionate to the amount of PCR amplicon. In addition to detection, it gives room for quantification of the number of copies in the amplicon. RT-qPCR is fast and has higher sample throughput than the conventional PCR. It is also more sensitive and specific[25].

PCR has transformed laboratory investigations because of its exquisite sensitivity and specificity, and is clearly the method of choice. PCR has now become the gold standard for the detection of several pathogens. It permits, especially in a few hours, the "acellular cloning" of a DNA fragment through an automated system, which usually takes several days with standard techniques of molecular cloning. It is also used to make genetic fingerprints, diagnostics, or varietal selection. PCR is still essential for performing sequencing or site directed mutagenesis[112].

2.13.3 DNA Sequencing

Since the advent of sequencing in the 1970s, some methods have become obsolete, and some newer and more efficient methods have become of interest to many researchers today seeking to understand genetic variation worldwide. Sequencing of viral genomes has helped scientists to better understand viruses in terms of their virulence, pathogenesis, transmission, mutations, and even virus-host relationships. It has also helped accelerate the development of drugs, vaccines, and therapies with high sensitivity and specificity. Some types and generations of sequencing have been

established between the start and the present. Industries have also developed sequencing platforms with various advantages and disadvantages. Examples of generations of sequencing are: Sanger sequencing; Capillary electrophoresis and fragment analysis, and next generation sequencing (NGS).

2.13.4 Sanger Sequencing

Researchers choose Sanger sequencing for low-throughput, targeted or short-read sequencing Because of its sensitivity and relative simplicity in terms of workflow and technique. Sanger sequencing remains the gold standard in sequencing technology today and is used in applications ranging from targeted sequencing to confirmation of variants identified by orthogonal methods. It uses the chain termination method to determine the identity and order of nucleotide bases in a given DNA strand. This method uses chemical analogues of four nucleotide bases. These analogs, called ddNTPs, lack the hydroxyl group needed to extend the polynucleotide chains that make up the DNA molecule. By mixing radiolabeled ddNTPs with template DNA, strands of any possible length are obtained when the ddNTPs are inserted randomly, terminating the chain. In the mid-1990s, researchers performed Sanger sequencing using capillary electrophoresis. Using this technology, labeled DNA fragments are separated by size in long thin acrylic fiber capillaries filled with a gel matrix. A sample containing labeled fragments is electrokinetically injected into a capillary and an electric field is applied to pull the fragments upward. As they pass through the detector laser inside the instrument, the labels are detected and sequenced[112].

2.13.5 Next Generation Sequencing (NGS)

While several improvements have been made to Sanger sequencing over the years, new high throughput methods, called Next Generation Sequencing (NGS) technologies, have also emerged. The spectrum of NGS analysis can range from a small number of genes to the entire genome, depending on the goal. Whole genome sequencing (WGS) and whole exome sequencing (WES) provide DNA base sequencing in the genome and exome, respectively. Whole-transcriptome sequencing provides information about the sequence of coding and multiple non-coding forms of RNA to assess variation and gene expression levels throughout the entire transcriptome. Platforms such as Illumina, PacBio, MGI, Oxford Nanopore, etc. use the NGS principle for their work.

Targeted sequencing covers a relatively small set of specific genes or regions of interest. Fast processing time, low cost, small sample input requirements, and relative ease of interpretation make targeted sequencing particularly suitable for clinical and translational studies. Sanger sequencing is often used to confirm variants identified by NGS[112].

Several serological methods have been used in the past to study HBV infections in Nigeria, however, few molecular surveils have been conducted. Undoubtedly, molecular methods are useful for accurately diagnosing patients presenting with suspected symptoms, while serological investigations are informative in understanding previous exposures and levels of immunities developed by individuals with respect to HBV infections.

2.14 Treatment of Chronic HBV Infection

Secondary to preventing HBV infection, is lowering HBV-related morbidity and mortality. Which can only be is achieved through therapeutic measures with the sole purpose of improving quality of life by interjecting the development of potentially fatal sequelae[108]. For this purpose, chronic HBV

infection treatment is aimed at sustaining viral replication suppression thereby preventing hepatic flares and bringing about remission of liver disease long before the development of liver cirrhosis and HCC. A good response to therapy is defined to be a very low serum HBV DNA level (lower than the limit that real-time PCR assays can detect) alongside maintained loss of HBeAg or anti-HBe seroconversion, and improvement of liver disease via normalization of biochemical markers[97,98,108].

Indication for therapeutic intervention of chronic HBV infection is based on a combination of three basic criteria; serum HBV DNA level (>105 copies/mL or >3.7E+5 IU/mL), serum ALT level (above ULN) and in rare cases liver histological grade and stage. It is therefore of note that patient's age, severity of liver disease, possibility of response to therapy, and the risk of adverse effects and complications must be put into consideration before making the decision to treat HBV infection[97]. Two distinct types therapeutic regimens are available for the treatment of chronic HBV infection namely, immunomodulators and antiviral drugs[97]. Examples of Immunomodulators reported for the treatment of chronic HBV infection, are interferon alpha (IFNα) and pegylated interferon α (peginterferon α; PEG-IFNα), they effect an antiviral response by initiating host cellular immune responses against cell-bound virions[99,100]. The therapeutic advantages of PEG-IFNα therapy include definite duration of treatment, no resistance selection as well as long lasting response to the virus. While PEG-IFNα is also used in hepatitis C therapy, it is said to be effective in the treatment of chronic HBV infection in HBV/HCV co-infected individuals[98]. PEG-IFNα has a number of disadvantages however, these includes its high cost, the need for frequent subcutaneous administration and risk of adverse effects[100]. Contrary to immunomodulator, nucleos(t)ide reverse transcriptase inhibitors (NRTIs; antiviral drugs) can be easily administered orally and are generally void of significant adverse effects, making them the therapeutic agents of choice for chronic

hepatitis B treatment[101,102]. The NRTIs available for treatment of chronic HBV infection include Lamivudine (3TC), Adefovir dipivoxil (ADV), Telbivudine (LdT), Entecavir (ETV), Emtricitabine (FTC) and Tenofovir disoproxil fumarate (TDF). Therapeutically, these NRTIs act by inhibiting the viral reverse transcriptase activity which then alter HBV DNA replication and as a result suppresses synthesis of infectious virions[102,103].

Interestingly, most of these NRTIs also form part of the most effective antiretroviral therapy (HAART) regimen for treatment of HIV infection and as such ADV, LdT and 3TC+TDF/Truvada (Truvada is a combination of FTC and TDF) are a good combination used in the treatment of chronic HBV infection in HBV/HIV co-infected individuals[104,105]. A major setback to the success of HBV antiviral treatment was reported, which is the development of drug resistance which follows long-term exposure to some of the NRTIs as discussed previously[101,102]. First line use of the most potent NRTIs which have a high barrier to resistance such as ETV or TDF as well as combination therapy with 3TC+TDF or Truvada have been reported to be some of the best strategies for preventing resistance development to NRTIs[106,107]. To ensure successful treatment outcome, it is necessary to monitor response to therapy, which involves an evaluation of serum HBV DNA level, serological markers (HBsAg, HBeAg and anti-HBe) and serum ALT levels over the course of treatment. Optimal duration of therapy is not standardized but is dependent on sustained virologic (<2000 copies/mL) and serologic (loss of HBsAg and HBeAg/anti-HBe seroconversion) response to treatment with normalization of serum ALT levels post treatment cessation[99].

2.15 Hepatitis B Vaccination

The World Health Assembly (WHA) as part of her recommendations, recommended in 1992 that the hepatitis B vaccine be incorporated universally into national routine immunization programmes

or national Expanded Programmes on Immunization (EPI) in order to curb the global burden of hepatitis B[24]. The most recent coverage report showed that the hepatitis B vaccine had so far been introduced into national EPIs in 179 of the 193 WHO member states. As a result of this, global hepatitis B vaccination coverage is currently estimated at 84%, reaching as high as 91% in the Western Pacific region while that in the South East Asian region is at 90%[111]. In Sub-Saharan Africa, vaccination coverage was reported at 75% as at the end of 2022[111].

2.16 Structure of Hepatitis E Virus

HEV is a small, nonenveloped, RNA virus belonging to the *Hepevirus* genus in the *Hepeviridae* family. The virion is 27-34 nm in diameter with a positive-sense, single-stranded, 7.2 kb genome (Figure 2.15). The virus genome is organized into three open reading frames (ORF1, ORF2, and ORF3). ORF1 is involved in viral replication and protein processing through RNA-dependent RNA polymerase. ORF2 encodes the viral capsid protein, which is involved in attachment to host cells and induction of neutralizing antibodies. Finally, ORF3 encodes for a small immunogenic phosphorylated protein (pORF3) involved in virion morphogenesis and release (Figure 2.16)[113].

The virus has short 5 and 3 noncoding regions and three overlapping open reading frames (ORFs). ORF1 encodes the nonstructural proteins, including a methyl transferase (MT), cysteine protease (P), helicase (Hel), and RNA polymerase (RdRp), as well as three regions of unknown function (Y, PPR, and X). The 5 end of the RNA genome is capped with 7-methylguanosine (7 mG), and the 3 end is polyadenylated (poly A)[113]. Characterization of HEV genomes from geographically distinct locations has identified at least four major genotypes that may differ up to 20% at the nucleotide level. HEV genotypes are further classified into subtypes: genotype 1, five (1a–1e); genotype 2, two (2a and 2b); genotype 3, ten (3a–3j); and genotype 4, seven (4a–4g)[114].

2.17 Modes of Transmission

HEV is classically transmitted feco-orally, although person-to-person transmission has also been reported (Figure 2.17)[116]. On the other hand, HEV have been found in animals and are transmitted zoonotically via the ingestion of raw or undercooked meat and close contact to infected animals[41]. HEV has been occasionally linked to nosocomial spread[117]. Vertical transmission from mother to infant is also known to occur[115]. It is infrequently transmitted by transfusion of blood or blood products[116].

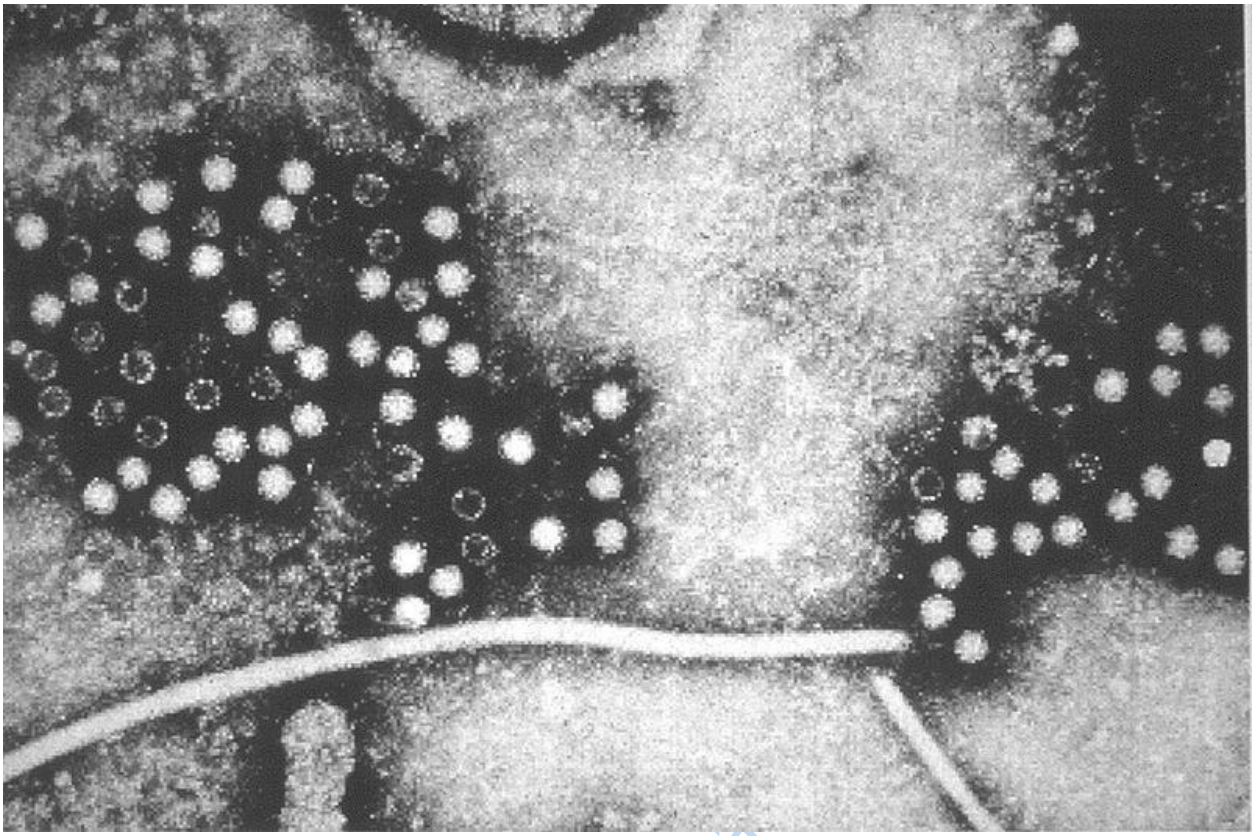

Figure 2.15: Electron Microscopy of HEV

Source[113]

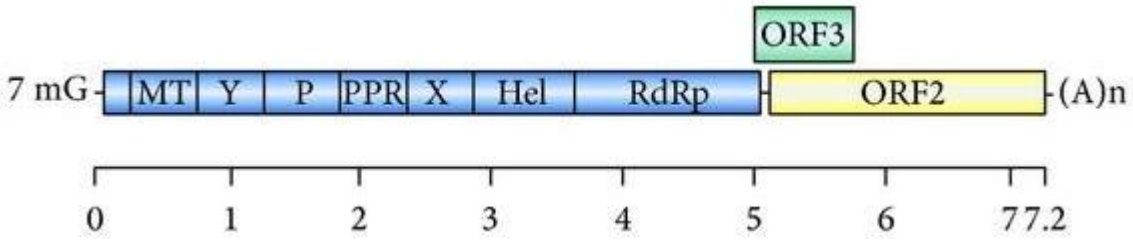

Figure 2.16: The structure of the Hepatitis E Virus Genome

Source[113]

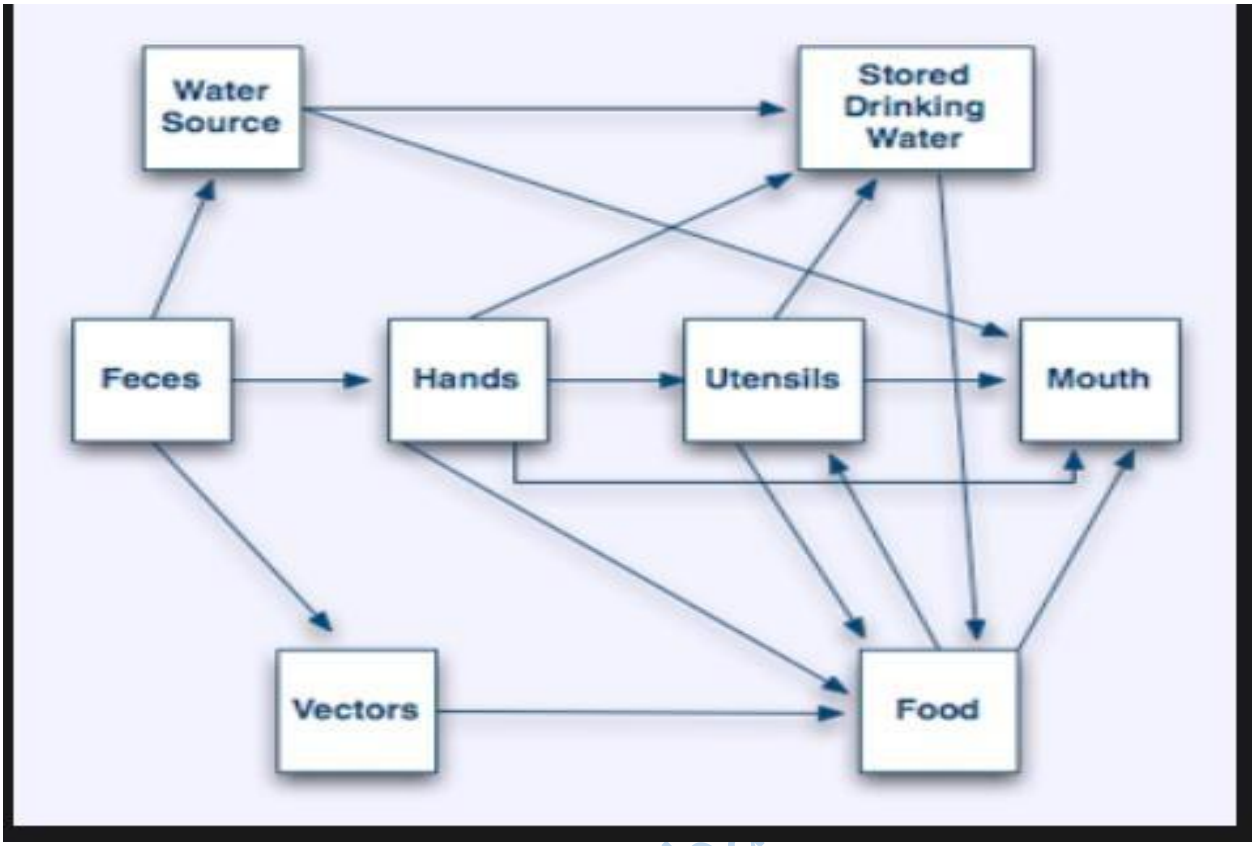

Figure 2.17: Faecal Oral Route of Transmission

Source[116]

2.18 Geographical Distribution of HEV Genotypes

HEV genotypes 1 and 2 only infect humans and are transmitted by contaminated water in developing countries. While HEV genotypes 3 and 4 infect humans, domestic swine and wild boar, deer and other animals and are responsible for sporadic cases of autochthonous HEV in both developing and developed countries. Genotype 1 includes isolates from Asia, the Middle East, and North Africa. Genotype 2 has been found in Mexico and Nigeria. Genotype 3 was recovered from swine in North America, Europe, Egypt, Asia and New Zealand and from humans in North and South America, Europe, Japan and China. Genotype 4 was found in humans and swine in Asia (Figure 2.18)[114].

HEV genotypes are also important as they correlate with the severity of infection. Accumulating evidence suggests that genotypes 3 and 4 are less pathogenic in humans, while genotype 1 isolated has been shown to be more pathogenic. This explains the high severity of infection in India where genotype 1 is the commonest subtype in comparison to US, where genotype 3 is the commonest type seen. Hepatitis E can occur either in large epidemics or in the form of sporadic cases. Although hepatitis E infection is endemic in Southeast and Central Asian countries, outbreaks have also been reported from several parts of the Middle East, Africa and Mexico (Figure 2.20)[114].

The outbreaks of Hepatitis E are large and the overall attack rates ranges from 1 to 15%, varying from 3−30% in adults to 0.2−10% in children[114.] Children have a high rate of sub clinical infection. In the US and Western Europe, less than 1% of patients of acute viral hepatitis have hepatitis E as the etiology of their infection and was thought to be associated with their travel to HEV-endemic regions. However the recent paper from France highlighting that 90% of acute hepatitis E patients

acquired by the indigenous route by contaminated water supplies and uncooked shellfish may change this current perception of hepatitis E epidemiology[114].

2.19 Prevalence of HEV in the World

The global annual disease burden for HEV genotypes 1 and 2 in 2005 was estimated to be 20.1 million incident infections resulting in 3.4 million symptomatic cases, 70,000 deaths and 3000 still births[114]. The study model represented 71% of the world's population with incidence rates greater for younger individuals (aged 15–20 years)[114]. The burden for HEV genotypes 3 and 4 is not known. HEV seroprevalence ranges from less than 5% to 5a2.5% in different countries[114]. In Nigeria, only a few HEV seroprevalence studies have been performed until today which detected a seroprevalence (IgG and total antibodies) between 7.0%–66.7% in different populations (Figure 2.19)[115].

Outbreaks and sporadic cases of hepatitis E occur around the world. These outbreaks frequently occur in resource-limited countries with limited access to essential water, sanitation, hygiene and health services, and may affect several hundred to several thousand persons[118]. In recent years, some of these outbreaks have occurred in areas of conflict and humanitarian emergencies, such as war zones, and in camps for refugees or internally displaced populations (IDP)[118]. An estimated 20 million infections and 3.3 million acute cases occur annually worldwide with an estimated 70,000 deaths[118].

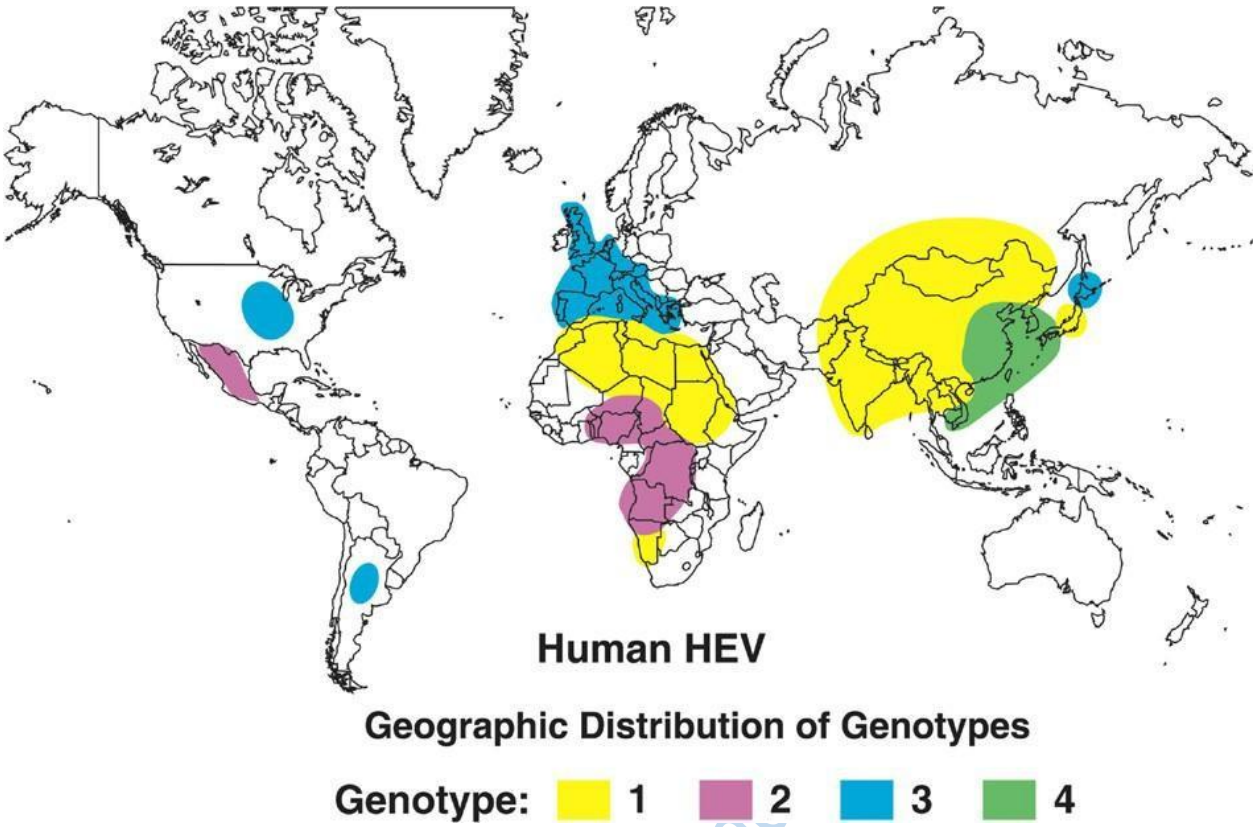

Figure 2.18: Geographical Distribution of HEV Genotypes

Source[117]

Table 2.1: Comparison of the Four Hepatitis E Virus Genotypes by Select Characteristics

Characteristics	Genotype 1	Genotype 2	Genotype3	Genotype 4
Viral discovery	1983	1986	1995	2003
Geographic distribution	Developing countries	Mexico, West Africa	Developed countries	China, Taiwan, Japan
Food-borne transmission	No	No	Yes	Yes
Fecal-oral transmission	Yes	Yes	?	No
Water-borne transmission	Yes	Yes	?	No
Person-to-person transmission	Yes	Unknown	Yes	Unknown
Zoonotic transmission	No	No	Yes	Yes
Occurrence of epidemics	Common	Smaller scale epidemics	No epidemics	Uncommon
Highest attack rate	Young adults	Young adults	Persons ≥ 40 yr of age	Young adults
Gender	Male preponderance	Not discriminatory	Mostly male	Not discriminatory
Mortality rate	0.5%-3%	0.5%-3%	Not determined	0.5%-3%
Mortality among pregnant women	High	High	Not determined	High
Chronic infection	None	None	Yes	None
Severe disease among immuno-compromised	Not reported	Not reported	Yes	Not reported
Interspecies transmission	Only humans and non-human primates	Only humans and non-human primates	Humans	Humans
			Pigs	Pigs

Osur dun 1

Source[39]

Hepatitis E virus accounts for both sporadic and epidemic outbreaks in developing countries that leads to self-limiting diseases. The overall attack rate, based on the global burden of HEV, ranges from 1 to 15%, being higher among adult (3 to 30%) than children (0.2 to 10%)[119]. Males are usually more frequently infected[119]. There was an outbreak in Chad in which there were distinctive cases and fatalities also in Sudan where several fatal cases occurred[119]. In October 2007, an epidemic of hepatitis E was reported in Kitgum District of northern Uganda where no earlier epidemics had been recorded. It was reported to be one of the largest hepatitis E outbreaks in the world[120]. In 2009, the epidemic had led to illness in 10,196 persons and 160 deaths[120]. In 2011, Tangail a neighbourhood of Dhaka Bangladesh was reported with a small outbreak[121]. In 2012 an outbreak was also reported in South Sudanese refugee camps in Maban County near the Sudan border.

South Sudan's Ministry of Health documented over 400 cases and 16 fatalities as of September 13, 2012. In 2013, 88 people died due to the outbreak. Almost 4,000 patients had been treated by Medical charity Medecins Sans Frontieres (MSF)[122]. In April 2014, Nepal, Biratnagar Municipality recorded over 6,000 outbreak of local infection and at least 9 death[122] . There was an outbreak reported in Damasak, Niger Republic[119]. Later there were some reported cases in Ngala Borno northeast Nigeria that border with Cameroon[119]. One hundred and forty-six (146) were confirmed and some suspected cases out of which 21 were confirmed in July 2017[119]. The outbreak was reported in three local government areas; Ngala, Mobbar and Monguno in Borno state in which Ngala had the highest cases with 25 pregnant women (21%) infected 2 deaths and a fatality rate of 8%[119].

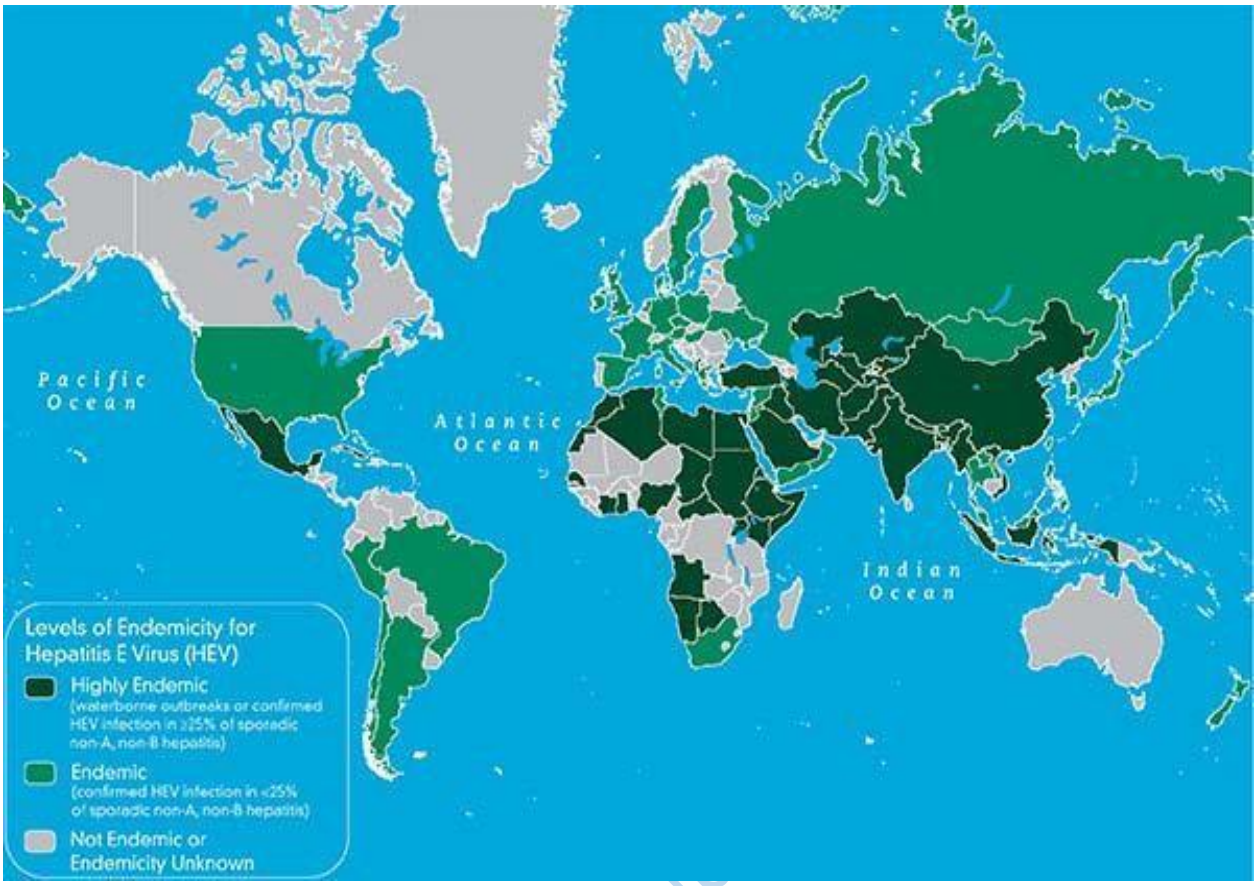

Figure 2.19: A Map Showing Prevalence of HEV in the World[114].

Source[119]

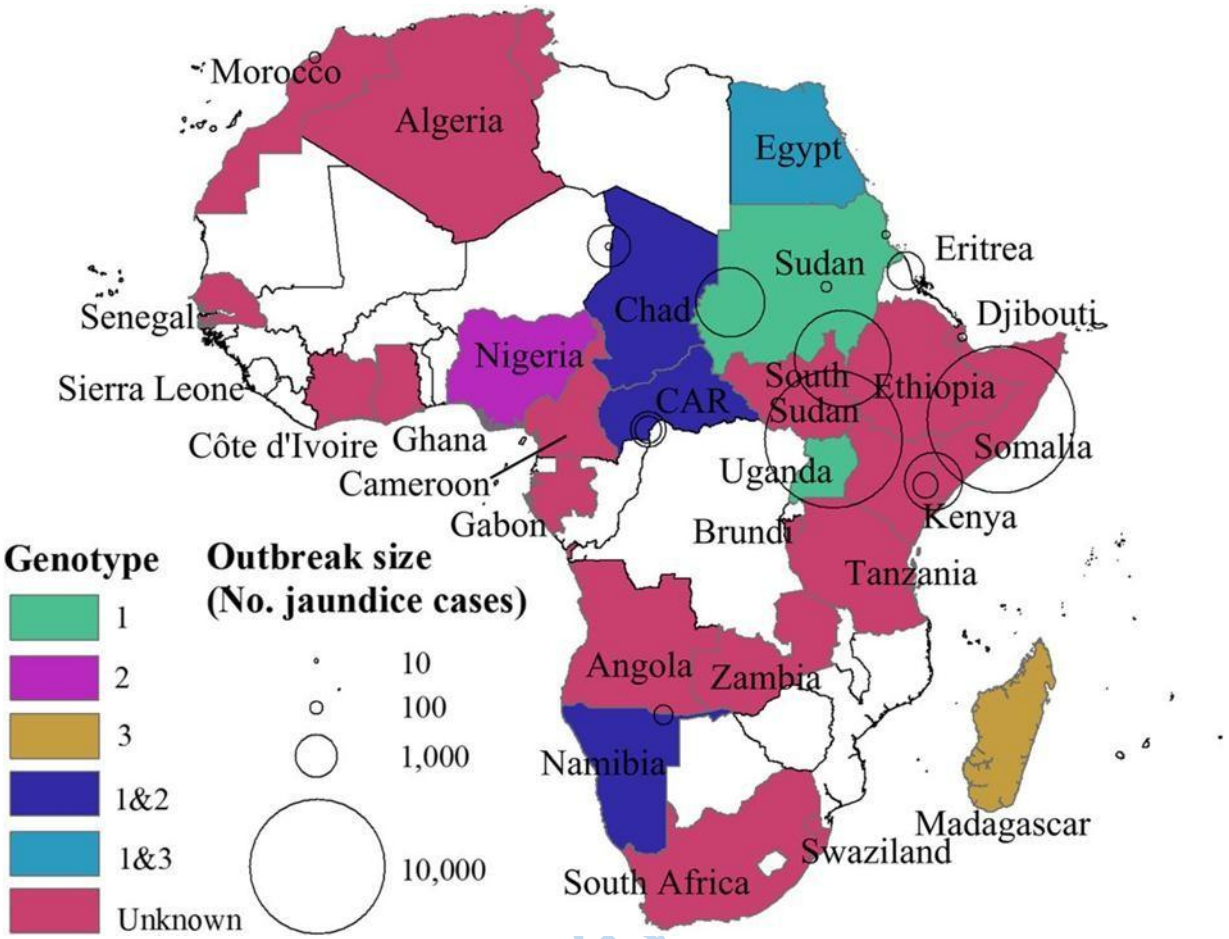

Figure 2.20: African Map Showing Genotypes of HEV in the World

Source[114]

2.20 Pathogenesis

HEV is difficult to culture in cell lines, therefore its pathogenesis and method of replication are not well understood. It is known that when the virus is faeco-orally transmitted, it replicates in the intestinal cells and reaches the liver through the portal vein. The virus attaches to hepatocytes through the binding of truncated peptide p239 to heparin sulphate proteoglycans on the cell wall, and enters the target cell by endocytosis[123].

The virus has three open reading frames (ORFs): ORF1 encodes for non-structural proteins (such as RNA dependent RNA polymerase, methyltransferase, and RNA helicase); ORF2 encodes for the viral capsid; and ORF3 encodes phosphoproteins involved in viral morphogenesis and release[118]. Both viral (such as the infecting genotype and infectious dose) and host factors (including age, pre-existing hepatic disease, pregnancy) influence the severity of disease (Figure 2.21)[123].

2.21 Clinical Presentation of Hepatitis E in Pregnancy

Acute hepatitis caused by HEV resembles that caused by other hepatotropic viruses [hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), Epstein–Barr virus or cytomegalovirus], or due to drug induced liver injury (DILI). The average incubation period is 30–40 days (range 2–9 weeks), with the majority of HEV infections being asymptomatic (67–93%)[124]. Features of symptomatic infections include anorexia, epigastric pain, discoloured urine, nausea, vomiting, diarrhoea, fever, jaundice, elevation of serum transaminase and hepatomegaly[125]. During outbreaks in developing countries, a case fatality rate of 0.5–4% has been reported[125]. A higher mortality rate of 8–11% in non-endemic countries is possibly due to infection in individuals with pre-existing liver disease. Incidence of disease and fulminant hepatitis is even higher in pregnant

women during the third trimester. Mortality in pregnancy also varies with geographical region, and reaches 10–25% in developing countries.

Chronic HEV infection refers to a condition following the acute phase, in which HEV replication persists for more than 3 months. Cases of chronic HEV infection have been documented in solid-organ transplant (kidney, liver and pancreas) recipients and in immunosuppressive conditions like infection with human immunodeficiency virus. Only genotype 3 has been shown to be involved in chronic infections[126]. Non-hepatic manifestations of HEV can include pancreatitis, haematological manifestations (thrombocytopenia, haemolysis) or neurological syndromes (Guillain–Barre syndrome, meningoencephalitis, pseudotumour cerebri, nerve palsies). The severe liver injury due to HEV infection during pregnancy may be related to one of several possible host factors, such as differences in immune and hormonal factors occurring during pregnancy[127].

2.22 HEV Infection during Pregnancy

It was found that HEV infected pregnant women with fulminant hepatic failure had lower CD4 count and higher CD8 counts, they also observed that the levels of estrogens, progesterone and beta-HCG were significantly higher in the above-mentioned group when compared to HEV negative patients or control healthy pregnant females[128].

Although the levels of hormones were physiologically high in the normal control population; patients with HEV infection seemed to have significantly higher levels than controls, which probably explain the direct interaction of HEV with the immune system. In another interesting study on the cellular immune response in both pregnant and non-pregnant women with acute hepatitis E and the control population, they found that pregnant women with HEV had generalized immune suppression characterized by decrease in lymphocyte response to phytohemagglutinin (PHA) with

a predominant Th2 bias as compared to non-pregnant women with hepatitis E and normal healthy controls (Figure 2.22)[129].

Hepatitis E virus infection during pregnancy is stormy because Th1 response is required. This elevated Th1 immune response if still remains insufficient to fight with such a high HEV load, there is a possibility that Th1 response goes on increasing but in the due process, the cytotoxic immune response may result in reduced fetal protection and eventually fetal death[129].

2.23 HEV Antibodies (IgG and IgM) in Pregnancy

Both IgM and IgG antibody to HEV (anti-HEV) are produced following infection. The titer of IgM anti-HEV declines rapidly during early convalescence; IgG anti-HEV persists and appears to provide at least short-term protection against disease. Detection of anti-HEV IgM is indicative of acute infection with HEV. The presence of IgG antibodies points out to previous exposure to HEV. Anti-HEV IgM is detectable 4 days after the onset of jaundice and persists for up to 3–5 months. Shortly after the appearance of IgM, IgG antibodies develop and peak at about 4 weeks after the onset of symptoms and persist for a variable period of 1 to 14 years after infection (Figure 2.23)[130].

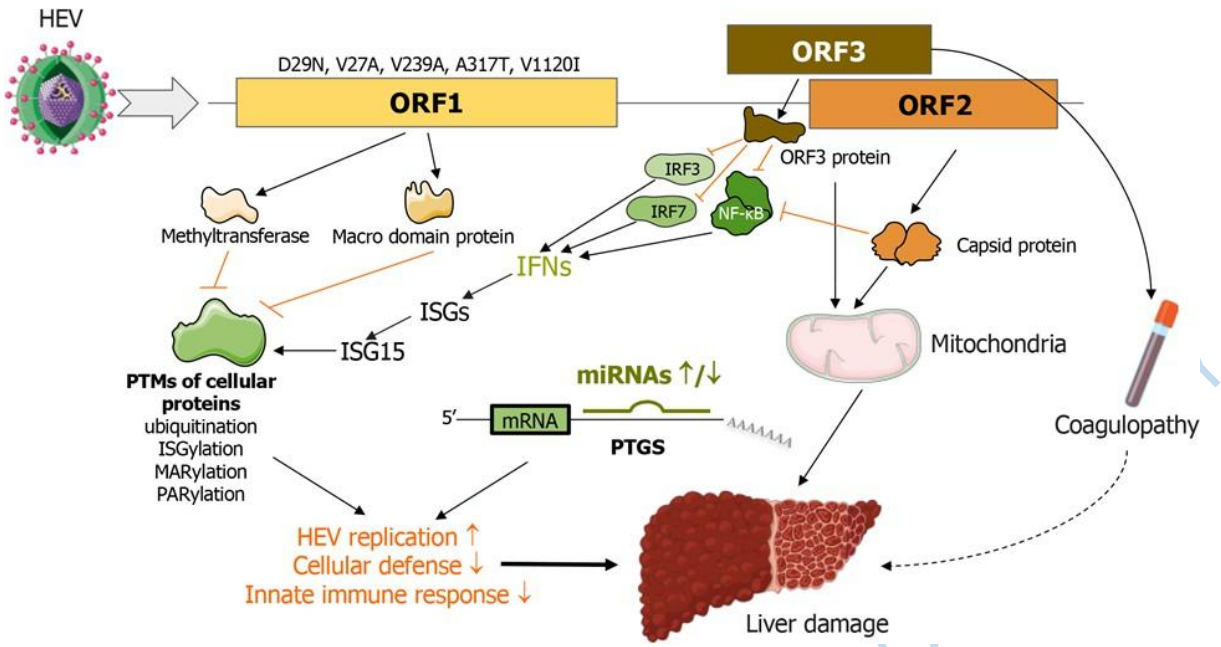

Figure 2.21: Several Viral Factors that Increase Hepatic Injury Induced By Hepatitis E Virus.

Source[123]

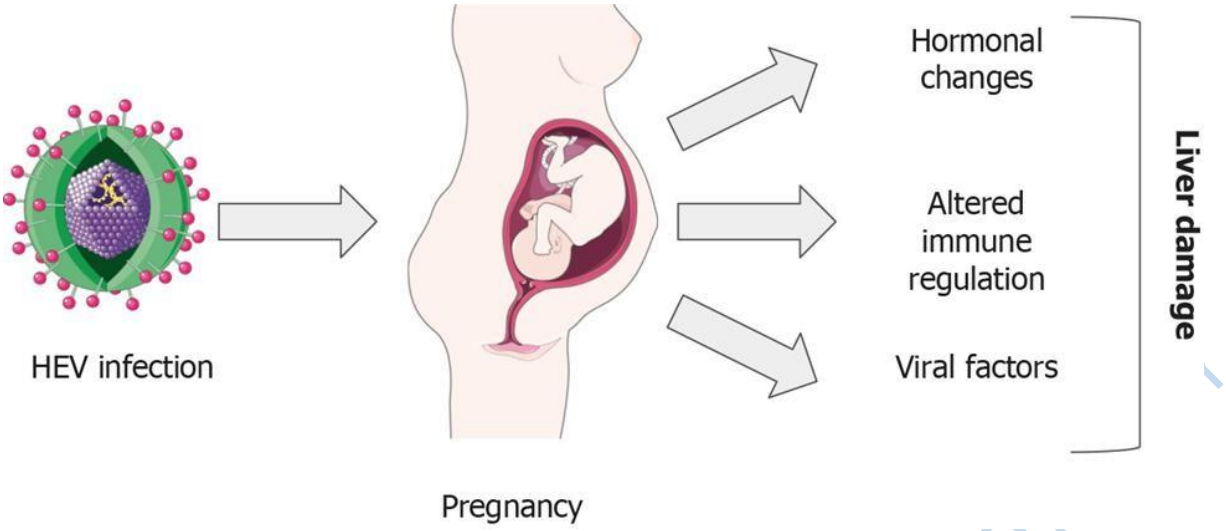

Figure 2.22: Summary of the Adverse Effects of Hepatitis E Infection During Pregnancy.

Source[129]

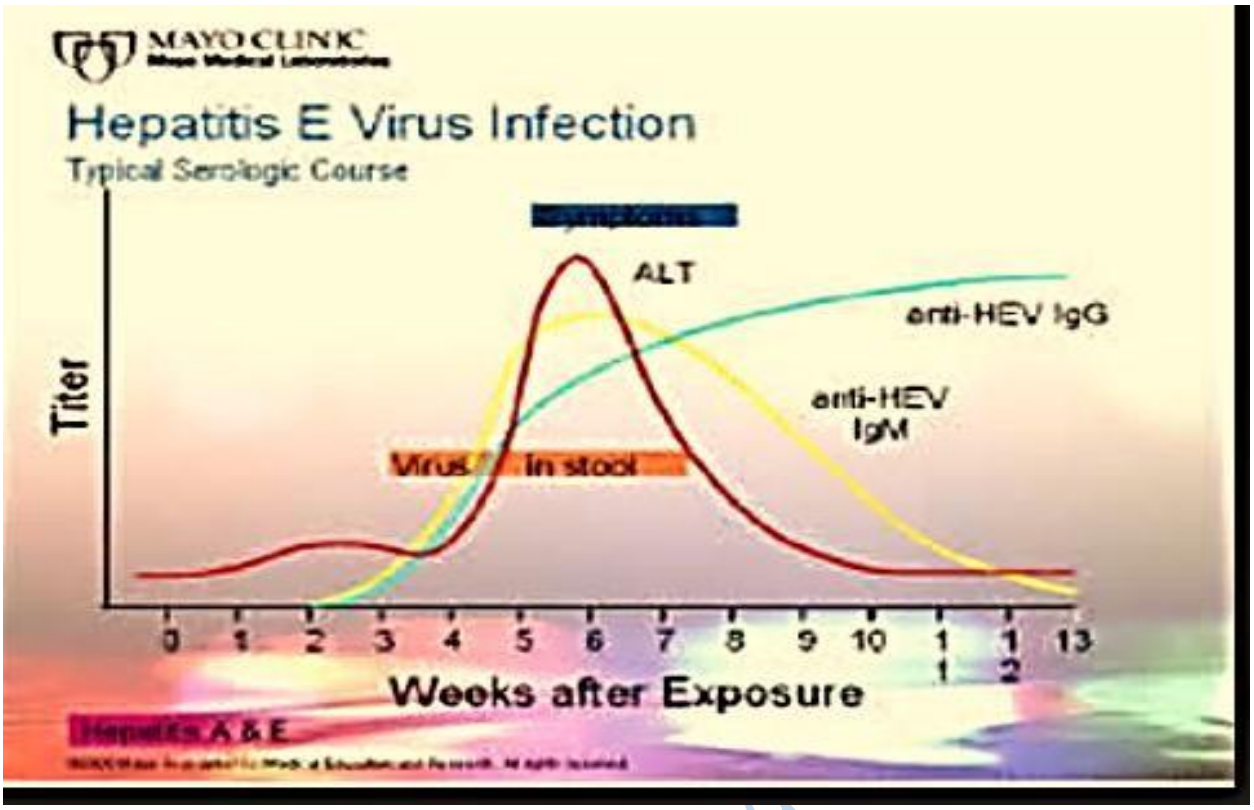

Figure 2.23: Antibody Level of Serum

Source[130]

2.24 Laboratory Diagnosis

Hepatitis E is an underdiagnosed disease, partly due to the use of serological tests with low sensitivity[134]. Diagnosis can be made indirectly by detecting antibodies against HEV in the serum, or directly by detecting the genome of the virus in blood or other body fluids[134]. There is no genotype-specific serological test[134]. One study that sought to determine the kinetics of anti-hepatitis E antibodies found that, at the symptom stage, anti-hepatitis E antibody levels peak, then remain at these levels for 8 weeks. After, the IgM levels fall rapidly, being below the detectable level in most patients after 32 weeks. IgG levels were found to be rising already when patients were symptomatic, reaching the peak at 4 weeks after onset of symptoms and remaining at high levels for more than 1 year. The exact duration of IgG response remains unknown[134].

These tests for anti-hepatitis E antibody screening are commercially available, but none of them has been approved by the Food and Drug Administration (FDA). Unfortunately, the sensitivity and specificity of these tests vary greatly and this could explain the discrepancies in rates of anti-hepatitis E antibodies published for the various populations studied. Until tests are approved by the FDA, physicians will rely on locally available tests. The tests for viral RNA in serum and feces are confirmatory, but still experimental[130]. One study compared six tests for anti-hepatitis E IgM antibodies in the serum of immunocompetent patients infected with the four types of hepatitis E, with sensitivity of tests between 72 and 98% and specificity between 78.2 and 95.6%[139]. Another study evaluated two anti-hepatitis E IgM antibody tests in immunocompetent and immunocompromised patients and showed that the sensitivity was 97.7% in immunocompetent patients and 85–87% in immunocompromised patients, with the two tests having high specificity (>99.5%)[140].

Trials evaluating anti-hepatitis E IgG antibodies have shown variable performance, with most available studies using serum from patients with recent infection, so that their ability to detect old/established infections remains unknown. The detection limits of these tests vary greatly and the IgG is sometimes undetectable after infection. These factors should be considered when interpreting seroprevalence data available in the literature[134]. Another important point is that the concentration of anti-hepatitis E IgG antibodies could be useful in determining which level of IgG would prevent infection after natural infection or administration of the vaccine. To this end, a vaccine study suggested that the antibody concentration of 2.5 IU/mL would be protective[134].

Regarding viremia, the peak occurs during the incubation period and the initial symptomatic phase[134]. Hepatitis E RNA in the blood becomes undetectable about 3 weeks after the onset of symptoms but can be detected in the stool for another 2 weeks. There is no correlation between levels of viremia and intensity of symptoms[134]. Thus, the initial examination for diagnosis of hepatitis E should be the anti-hepatitis E IgM antibody, leaving the HEV RNA detection by RT-PCR for suspected cases with anti-hepatitis E IgM negativity, especially in the immunocompromised[131,132,134].

Chronic hepatitis E is diagnosed by the detection of HEV RNA in feces or serum after a minimum of 3 to 6 months after the diagnosis of hepatitis E. Thus, IgM and IgG serological tests are not used to diagnose or exclude chronic disease[131]. Very recent data, in the context of transplanted patients, found that there is no spontaneous clearance of HEV between 3 and 6 months after acute infection and this suggests that chronic infection should be considered when replication lasts more than 3 months[134]. One study showed that at diagnosis, transaminases were lower in patients who progressed to chronic disease. The mean alanine aminotransferase was 300 IU/L in chronic disease

and 1000 IU/L in acute disease[131]. There was also no correlation found between viral serum concentration and risk of progression to fibrosis[131].

Hepatic biopsies from patients with acute hepatitis E show a typical pattern of portal and lobular inflammation associated with hepatocyte necrosis. Cholestasis and ductal proliferation may also be observed in varying degrees, and even cases of destructive lymphocytic cholangitis have been reported. Similar to hepatitis C, steatosis and plasma cells can also be found[131]. In general, no distinct histological feature has been identified that allows for differentiation between hepatitis B and C, supporting the hypothesis that the cellular immune response largely determines the severity of the disease. The inflammatory cell infiltrate in uncomplicated acute hepatitis E is predominantly neutrophils[131].

An important differential diagnosis is drug-induced liver injury, especially in the elderly, for whom polypharmacy is common. In a more recent study from the United States, 3% of patients with "drug-induced liver damage" were misdiagnosed as they had positive hepatitis E tests in subsequent research. Studies like this show the importance of excluding other causes of a hepatocellular lesion before making the diagnosis of drug-induced injury, especially in patients with elevated transaminases[134,137].

2.25 Management

2.25.1 Treatment

Several stages of the HEV cell cycle may be potential targets for development of antiviral drugs[133]. Acute infection usually does not require treatment, but chronic infection should be treated by reducing immunosuppression in transplanted patients or by using antiviral therapy[134]. Chronic

hepatitis E may lead to spontaneous resolution in some cases, but may also lead to rapid progression to cirrhosis and death[131]. Hence, it is important to consider the treatment.

A study demonstrated that reducing T cells to target immunosuppression helped in eradicating hepatitis E spontaneously in transplanted patients, in up to 1/3 of the cases evaluated[131,134]. They reported that in the remaining 2/3 of cases antiviral therapy would be indicated. All published data are based on small series and case reports, since no randomized study was performed[134]. Nevertheless, one risk of reducing immunosuppression is the increased risk of rejection[137,138].

A 3-month course of pegylated-interferon therapy at a dose of 135 g/week was conducted with 3 liver-transplanted patients and 1 hemodialysis patient who had received a kidney transplant. A sustained virological response was obtained in 3 of the 4 patients. A 12-month course of pegylated-interferon therapy was also effective in treating chronic hepatitis E after liver transplantation. However, interferon cannot be used after kidney, heart and lung transplantation due to the risk of acute rejection[134].

Ribavirin, a guanosine analog, inhibits the replication of various RNA and DNA viruses[141]. Studies have shown that ribavirin alone at a dose of 600–800 mg/day for 12 weeks has led to sustained virological response in at least 2/3 of chronic hepatitis E cases. In addition, success with ribavirin led to its use to treat severe acute hepatitis E, with promising results[130]. A study was performed in which 59 transplanted patients (kidney, liver, heart, kidney, pancreas, lung) were treated with ribavirin at an average dose of 600 mg/day for a median of 3 months[142]. Fifty-four patients were genotyped and all were found to have genotype 3 infections. The researchers found that 95% of

patients at the end of treatment had viral clearance, while 78% had sustained virological response. About 60% of patients had hepatitis E recurrence and 40% of these patients had sustained virologic response after prolonged treatment with ribavirin. This study demonstrated that ribavirin is a good initial treatment option for chronic hepatitis E. The main side effect of ribavirin was anemia, seen in 54% of patients, with 12% requiring blood transfusion[131].

A recent systematic review evaluated the efficacy and safety of ribavirin treatment in 105 patients and of pegylated-interferon treatment in 8 patients with chronic hepatitis E. Sixty-four percent of patients treated with ribavirin had an undetectable virus level within 6 months after stopping treatment, while only 2 of 8 (25%) of the patients treated with pegylated-interferon achieved a sustained virologic response. The main side effect of ribavirin in that study was again anemia, with 35% of patients requiring erythropoietin and 10% requiring blood transfusion. On the other hand, in the pegylated-interferon group, 2 of the 8 patients developed acute transplant rejection[143].

Therefore, ribavirin monotherapy has been applied, with promising results in both adults and children. The mechanism of action of ribavirin against HEV is still unknown. Studies with the use of sofosbuvir (SOF), a nucleotide drug against hepatitis C virus, were effective in inhibiting the replication of genotype 3 HEV *in vitro*, and this effect was greater when SOF was combined with ribavirin[144]. However, to date, hepatitis E treatment is experimental, there are no guidelines and neither ribavirin nor interferon have been approved for this use[130,138]. On the other hand, there was a suggestion that, as an initial approach, immunosuppression reduction and, in case of no adequate response, ribavirin at 600–800 mg/day for 3 months (with anemia monitoring) should be started[131].

2.25.2 Prevention

In the endemic areas, prevention strategies should include improving hygiene and sanitary practices. In non-endemic areas, an important measure is to avoid consumption of undercooked meat[131,136]. Two vaccines have been developed to prevent hepatitis E infection[145]. A phase 2 study was performed with a recombinant vaccine with 2000 healthy adults, and 95.5% efficacy was found after three doses[135]. However, the vaccine did not progress from phase 2[131]. A results was published from a phase 3, double-blind, randomized study with more than 50000 participants in each arm[146]. Three doses of hepatitis E vaccine were given at 0, 1 and 6 months to participants, and the vaccine showed 100% efficacy at 12 months after vaccination. In the extension of the follow-up period, for up to 4.5 years, the vaccine showed efficacy of 86.8%[131,147]. To date, this hepatitis E vaccine garnered approval in China but has not yet been approved in other countries[130,145].

Even if the vaccine becomes available, many new studies will still be needed to clarify several other questions, such as duration of protection, need for reinforcement, safety and immunogenicity in specific groups (pregnant women, patients with chronic liver disease, patients with immunogenicity), vaccine efficacy in endemic areas against genotypes 1 and 2, and vaccine efficacy in preventing and relieving symptoms after exposure to HEV. Another major obstacle will be cost. Probably, because of these and other difficulties, vaccination approval has been slow[145].

2.26 Co-infection of HBV and HEV in Pregnancy

Pregnancy is associated with high levels of steroid hormones. These steroid hormones may promote viral replication. It also has a direct inhibition on hepatic cells, which may predispose to hepatic dysfunction/failure when exposed to infectious pathogens. Steroid hormones are

immunosuppressive and mediate lymphocyte apoptosis through NF-κB. NF- κB is a eukaryotic dimeric transcription factor which has a multiple cellular effects, including liver development and regeneration and its implications on the immune response[148]. In areas where chronic hepatitis B is endemic (CHB), such as China, co-infections of HEV on the CHB background are not uncommon. In fact, it was reported that HBV-HEV co-infections represented 20%-40% of all symptomatic acute hepatitis E (AHE) infections[149]. A clear territory-wise distribution of HBV-HEV superinfection has been observed in Africa where a higher prevalence of 56.7% was reported in Egypt in the northern part of the continent, while insignificant rates of 1% and 0% were reported in Kenya, the Eastern part of Africa[150]. In Nigeria, only a few HEV seroprevalence studies have been performed until today which detected a seroprevalence (IgG and total antibodies) between 7.0%-66.7% in different populations[115]. In some studies, the prevalence of HBV/HEV co-infection was analysed among Nigerian healthcare workers and animal handlers, reporting a coinfection rate of 27.3% and 22% respectively[150,151]. HEV co-infection in patients with underlying CHB can result in an acute exacerbation of their liver disease[149]. In patients with cirrhosis, a co-infection can result in decompensated liver disease and increase mortality[149]. Immunosuppression such as pregnancy is a risk factor for development of chronic HEV disease[149].

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Chapter Three

Methodology

3.1 Study Area

The research was carried out in Adeoyo Maternity Teaching Hospital and Akinyele Primary Health Centre, Ibadan, Oyo State, South-Western Nigeria. The study areas were purposively selected because some of the potential risk factors to be investigated such as type of toilet facilities, inadequate personal hygiene and improper waste disposal are commonly observed in those locations[1]. Adeoyo Maternity Teaching Hospital is located in Yemetu area where most houses use a latrine or lack a toilet, practise improper waste disposal and are unhygienic. Akinyele area was selected based on HEV route of transmission[1]. HEV is transmitted faeco-orally or zoonotically via the ingestion of raw or under-cooked meat and close contact to infected animals[1]. Akinyele is an area in Ibadan dominated by butchers and animal handlers.

3.2 Study Design

This is a facility-based cross-sectional study designed to assess the seroprevalence, associated risk factors and likelihood of coinfection with HBV and HEV.

3.3 Ethical Consideration

Ethical approval was obtained from the Health Planning, Research and Statistics Department of Oyo State Ministry of Health with the approval number NHREC/OYOSHRIEC/10/11/22. Informed consent was obtained from each of the pregnant women before participating in this study (Appendix I).

3.4 Study Period

Sample collection for this study was carried out between August 2023 and January 2024, Laboratory testing was conducted between February 2024 and August 2024, while data entry and analysis were conducted between March 2024 and October 2024.

3.5 Study Population and Sampling

The study population consisted of pregnant women attending Adeoyo Maternity Teaching Hospital and Akinyele Primary Health Centre, Ibadan, Oyo State, South-Western Nigeria. These were selected based on the availability of antenatal screening services. A well-structured psychosocial questionnaire (Appendix II) based on direct and indirect questions to obtain demographic characteristics such as age, trimester, occupation and education level was used. Behavioral characteristics as well as possible associated risk factors such as previous history of hepatitis, vaccination, blood transfusion, surgery, source of drinking water, type of toilet, personal hygiene, waste disposal, and interaction with animals were also recorded. Informed consent (Appendix III) was obtained from patients prior to administering of questionnaire, then followed by sample collection. Those who can neither read nor write were assisted using the local lingua franca, mainly Yoruba and indigenous dialects.

3.5.1 Sample Size

The Fischer's formula was used for sample size calculation.

Sample size: $N= Z^2 P (1-P)/ d^2$ where

n = minimum sample size,

z = standard normal deviation set at 95% confidence level (1.96)

P = prevalence of infection

d= degree of freedom set at 0.05

For HBV-HEV = 223 ≈ 300. This was calculated based on HBV prevalence; 6.49% among pregnant women in Nigeria[2].

3.5.2 Inclusion Criteria

1. Pregnant women in Ibadan of any gestational age
2. Attending antenatal clinic at the selected hospitals
3. Willing to provide written informed consent for participation
4. Not currently on ART

3.5.3 Exclusion Criteria

1. Pregnant women who were positive for HIV or HCV
2. With a history of severe liver disease unrelated to HBV/HEV
3. Decline to participate

3.6 Sample Collection

Five millimeter (5ml) of venous blood was collected from each participant aseptically by venepuncture from the cubital fossa into EDTA bottles. The blood was centrifuged at 3000rpm for 5 minutes to separate the plasma. The plasma extracted were stored in plain bottle at -20°C until tested.

3.7 Serological Tests

The plasma samples were first screened for HIV using Rapid response test strip (BTNX inc., Canada) and HCV using Accu-Tell Rapid anti-HCV test strip (ACCUBIOTECH, Beijing, China) according to manufacturer's protocol to rule them out as part of the exclusion criteria. The samples negative for HIV and HCV were further processed for the measurement of serum alanine aminotransferase (ALT/SGPT) levels using the test kits ALTP2: ACN 20140 (Randox, uk) and then screened for the presence of HBV serological markers (HBsAg, HBsAb, HBeAg, HBeAb, HBcAb) using Bio-Inteco and Elescys® enzyme-linked immunosorbent assay (ELISA) (Inteco Diagnostics, UK and Roche Diagnostics, Germany respectively) and HEV antibodies (IgM and IgG) using AccuDiag^{TM} enzyme-linked immunosorbent assay (ELISA) (Diagnostic Automation, Inc, USA) according to the manufacturer's instructions.

3.7.1 Detection of HIV Infection

The Rapid Response^{TM} HIV Human Immunodeficiency Virus Test Strip is a rapid, qualitative test for the detection of antibodies to HIV in whole blood, serum and plasma, to aid in the diagnosis of HIV infection. Interpretation of test results are: positive (two lines), negative (one line), invalid (no lines or no Control line).

3.7.2 Detection of HCV Infection

The test strip, specimen and controls were allowed to equilibrate to room temperature (15-30^{0C}) prior to testing. The pouch was brought to room temperature before opening it. The test strip was removed from the sealed pouch and used as soon as possible. The strip was placed on a clean and level surface. The dropper was held vertically and 1 drop of plasma (50µL) was transferred to the

specimen area, then 2 drops of buffer (100µL) were added. The test result was read at 10 minutes when the color lines appeared.

3.7.3 Liver Functioning Tests

3.7.3.1 Principle of Serum Alanine Aminotransferase (ALT/SGPT)

α-oxoglutarate + L-alanine → L-glutamate + pyruvate

ALT catalyzes the reaction of alpha-ketoglutarate with L-alanine to form L-glutamate and pyruvate. Alanine Aminotransferase is measured by monitoring the concentration of pyruvate hydrazone formed with 2,4-dinitrophenyl-hydrazine.

3.7.3.2 Assay Procedure

Reagent blank and sample tubes were set up, 100ul of distilled water and plasma sample were pipetted into blank and sample test tubes respectively. Five hundred microlitre of buffer (R1) was added, mixed and incubated for 30min at 37°C. Five hundred microlitre of 2.4-DNP (R2) was added, mixed and incubated for 20min at 20 to 25°C. Five hundred microlitre of NAOH (R3) was then added and mixed. The absorbance of sample was read against blank after 5 minutes.

3.7.4 Bio-inteco HBsAg II Immunoassay

3.7.4.1 Principle of the Bio-inteco HBsAg II Immunoassay

HBsAg ELISA is a solid phase assay that employs highly purified, high affinity, agglutinating sera for HBsAg having reactivity for both ad and ay subtypes. The microwell strips are coated with agglutinating sera for HBsAg. Another agglutinating sera for HBsAg is conjugated to horseradish peroxidase (HRP). The sample and the conjugate are added to the coated wells and incubated

simultaneously. The wells were washed to remove unbound components. Bound enzyme was then detected by the addition of substrate. The reaction was stopped with acid, the absorbance was then determined for each well at 450nm with Labtech ELISA reader. The cut off value was calculated and absorbance of all the wells were then compared with the cut off value. Any sample having absorbance more than the cutoff (>10 UI/mls) value is considered reactive.

The kit components include:

1. Coated microwells: Microwells coated with Agglutinating sera for HBsAg. Ready to use and comes in 96 wells,192 wells and 480 wells.

2. Positive control: HBsAg positive serum diluted in stabilizer solution with preservatives.

3. Negative control: Inactivated and stabilized human serum non-reactive for HBsAg.

4. Conjugate agglutinating sera of HBsAg-HRP Conjugate.

5. Conjugate activator: Buffered solution containing activator and preservatives.

6. Substrate: Solution containing tetramethyl benzidine (TMB) and hydrogen peroxide ready to use.

7. Wash buffer: Buffer containing surfactants (20x) to be diluted 20 times with distilled water.

8. Stop solution: Diluted Sulphuric acid ready to use.

9. Microwell holder, instruction for use, protocol sheet and plate sealer.

3.7.4.2 Assay Procedure

All reagents and plasma were brought to room temperature before used, the required number of microplates needed were then removed, ELISA Protocol sheet indicating the location of controls and specimens was prepared, controls were used in duplicates for all batches, hundred micro litre of undiluted controls (positive and negative) and study samples were added into separate wells,

50µL of activated conjugate was then added to all wells, it was then evenly mixed by gentle tapping, plates sealer was applied and incubated for 120 mins at 37°c.

Each well was then washed by filling 350µL diluted wash buffer and aspirating/flicking off six times with 1 minute soak time between each wash and then blot dried, hundred micro litre of substrate was then added to all wells and incubated at 22-28°C away from sunlight for 30minutes, hundred micro litre of stop solution was then added in the same sequence as substrate addition to stop the reaction.

The absorbance of each well was then read at 450nm within 30minutes of stopping the reaction, the results were then interpreted as positive or negative. (Positive >10UI/L, Negative < 10UI/L). The results obtained for each test were interpreted according to the ranges specified by the controls.

3.7.5 Bio-inteco Anti-HBs Immunoassay

3.7.5.1 Principle of the Bio-inteco Anti-HBs Immunoassay

HBsAb ELISA is a solid phase assay that employs highly purified, high affinity, agglutinating sera for Anti-HBs having reactivity for both ad and ay subtypes. The microwell strips are coated with agglutinating sera for anti- HBs. Another Agglutinating sera for anti-HBs is conjugated to horseradish peroxidase (HRP). The sample and the conjugate are added in the coated wells and incubated simultaneously. The wells were washed to remove unbound components. Bound enzyme was then detected by the addition of substrate. The reaction was stopped with acid. The absorbance was then determined for each well at 450nm with Labtech ELISA reader. The cut off value was calculated by given formular and absorbance of all the wells were then compared with the cut off value. Any sample having absorbance more than the cutoff (>10 UI/mls) value is considered

reactive.

The test procedures are similar to what has been previously described in (section 3.7.4.2).

Quality control: The results obtained for each test were interpreted according to the ranges specified by the controls.

3.7.6 Elecsys® HBeAg Immunoassay

The hepatitis B e antigen (HBeAg) is a product of the pre-C/C gene and is expressed in hepatocytes during active replication of the hepatitis B virus (HBV). It serves as an important diagnostic marker for assessing the activity and infectiousness of ongoing HBV infections. The detection of HBeAg is generally associated with the presence of large quantities of virus as it is a surrogate of viral replication[10]. HBeAg can be detected in serum shortly after HBsAg during acute HBV infections and usually disappears before HBsAg, when alanine aminotransferase (ALT) levels peak, followed by the presence of the corresponding antibody (anti-HBe)[11]. HBeAg can usually be detected when viral replication is high, both in self-limited infections and in chronic hepatitis B; its presence for more than 10 weeks is indicative of a transition to persistent infection. HBeAg seroconversion to anti-HBe suggests the end of active viral replication and is therefore associated with clinical resolution (self-limited) or remission (chronic disease), marking a transition from the immune-active phase of the disease to the inactive carrier state. HBV infections can occur without detectable HBeAg due to infection with HBV variants containing precore stop codon mutants. However, while the virus can no longer produce HBeAg, disease activity is ongoing and anti-HBe may be present[11]. The HBeAg test is, therefore, meaningful in association with the anti-HBe test for monitoring the course of HBV infection and the effect of treatment for chronic hepatitis B. The Elecsys HBeAg assay uses monoclonal anti-HBe antibodies (mouse) for detection of HBeAg.

3.7.6.1 Principle of the Elecsys® HBeAg Immunoassay

First incubation: HBe antigen from 35 µL sample, a biotinylated monoclonal HBeAg-specific antibody, and a monoclonal HBeAg-specific antibody labeled with a ruthenium complex[a] form a sandwich complex**. 2nd incubation**: After addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture was aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. ▪ Results are determined automatically by the software by comparing the electrochemiluminescence signal obtained from the reaction product of the sample with the signal of the cutoff value previously obtained by calibration. (a) Tris(2,2'-bipyridyl) ruthenium (II)-complex (Ru(bpy)).

For quality control, PreciControl HBeAg was used. Controls for the various concentration ranges was ran individually at least once every 24 hours when the test was in use, once per reagent kit, and following each calibration. The control intervals and limits were adapted to the laboratory requirements. Values obtained fell within the defined limits.

3.7.7 Principle of the Elecsys® HBeAb Immunoassay

Competition principle. Total duration of assay: 180 minutes. ▪ 1st incubation: Anti-HBe in the sample (35 µL) binds to the added HBeAg. ▪ 2nd incubation: After addition of biotinylated antibodies and ruthenium complex[a] -labeled antibodies specific for HBeAg, together with streptavidin-coated microparticles, the still-free binding sites on the HBe-antigens become

occupied. The entire complex is then bound to the solid phase via interaction of biotin and streptavidin, The reaction mixture was then aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances were then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which was measured by a photomultiplier. Results are determined automatically by the software by comparing the electrochemiluminescence signal obtained from the reaction product of the sample with the signal of the cutoff value previously obtained by calibration. (a) Tris(2,2'-bipyridyl) ruthenium (II)-complex (Ru(bpy)).

For quality control, PreciControl Anti-HBe was used. Controls for the various concentration ranges should were ran individually at least once every 24 hours when the test is in use, once per reagent kit, and following each calibration. The control intervals and limits were adapted to laboratory requirements. Values obtained fell within the defined limits.

3.7.8 Elecsys® Anti-HBc Immunoassay

This test is used for the in-vitro qualitative determination of total IgM and IgG antibodies to HBcAg in human serum and plasma. The assay reagent pack came with four color-coded reagent bottles; a transparent-capped bottle (M) containing 6.5 mL of streptavidin-coated microparticles at a concentration of 0.72 mg/mL, a white-capped bottle (R0) containing 5 mL of DTT [1,4-dithiothreitol (110 mmol/L) in citrate buffer (50 mmol/L)], a gray-capped bottle containing 8 mL of HBcAg [HBcAg (E. coli, rDNA) (>25 ng/mL) in phosphate buffer (100mmol/L and a pH of 7.4)] and finally a black-capped bottle which contains 8 mL of AntiHBcAg-Ab~biotin; anti-HBcAg-Ab~Ru(bpy)2 3 + [biotinylated monoclonal anti-HBc antibody (mouse) (>800 ng/mL); monoclonal anti-HBc antibody (mouse) labelled with ruthenium complex (>130 ng/mL) all in

phosphate buffer (100 mmol/L with a pH of 7.4)]. The anti-HBc Immunoassay calibrators are Cal1; the negative calibrator containing 1.0 mL of human serum and Cal2; the positive calibrator which contains 1.0 mL of Anti-HBc (human) [>8 PEI (Paul-Ehrlich-Institute) U/mL] in human serum.

3.7.8.1 Principle of the Elecsys® Anti-HBc Immunoassay

The test is based on a competitive immunoassay principle with three incubation stages. The first incubation involves pre-treatment of 40 µL of sample with the DTT (a reducing agent). The HBcAg is then added to the sample and the reaction mixture incubated, allowing the formation of a complex between the HBcAg and the anti-HBc antibodies in the sample. In the third and final incubation step, biotinylated antibodies and ruthenium complex-labelled antibodies specific for HBcAg together with streptavidin-coated microparticles are added to the reaction mixture resulting in the previously free binding sites on the HBcAg becoming occupied.

The entire complex becomes bound to the solid phase due to interactions between the biotin and streptavidin. To induce and measure chemiluminescence, the reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode (unbound reactants are removed with ProCell) to which a voltage is applied. Chemiluminescent emission is then measured by a photomultiplier and results are determined by the ELISA machine. This is done by comparing the electrochemiluminescence signal obtained from the reaction product of the sample with the signal of the cut-off value obtained by anti-HBc calibration.

For quality control purposes, Positive and Negative Control Anti-HBc containing control serum based on human serum in the negative and positive concentration ranges was used to monitor the accuracy of the Anti-HBc Immunoassays. The controls are PC AHBC1 (negative control) containing 1.3 mL of control serum (Human serum) negative for anti-HBc with a target range for

the cut-off index falling between 1.05-3.0 and PC A-HBC2 (positive control) which contains 1.3 mL of control serum; Anti-HBc antibodies (human) (~1 PEI-U/mL) in human serum with a target range cut-off index between 0.14-0.87.

3.7.9 AccuDiag™ HEV IgM Antibody

3.7.9.1 Principle of AccuDiag™ HEV IgM Immunoassay

The HEV IgM ELISA is a solid phase, two-step incubation, antibody capture assay. Polystyrene microwell strips are pre-coated with antibodies directed to human immunoglobulin M protein (anti-u chain). During the first incubation stage, the patient's serum or plasma sample is added. At this point, any IgM- class antibodies will be captured in the wells. Next, all other components of the sample are washed out, especially any IgG- class antibodies. What becomes visible after adding recombinant HEV ORF2 antigens conjugated to horseradish peroxidase (HRP-conjugate) is the specific HEV IgM captured on the solid phase. In the second incubation stage, the HRP-conjugated antigens will individually react with HEV IgG antibodies. Chromogen solutions are added after the wells are washed to remove the unbound HRP-conjugate.

At this point, when (anti-u)-(anti-HEV-IgM)-(HEV Ag-HRP) immunocomplex is present, a blue-coloured product appears which is the result of colourless chromogens hydrolyzed by the bound HRP conjugate. After stopping the reaction with sulfuric acid, the colour turns yellow. The colour intensity can be gauged proportionally to the amount of antibody captured in the wells, and to the sample, respectively. Colourless wells appear when samples are negative for HEV-IgM.

3.7.9.2 Assay Procedure

One hunded micro litre of specimen diluent was added into each ELISA plate well including the blank. Ten micro litre of samples and ten micro litre positive and negative controls were added into their respective wells using separate disposal pipette tip for each specimen, negative and positive control to avoid cross-contamination. The plate was covered with plate cover and incubated for 30 minutes at 37ºC. After incubation, each well was washed 5 times with diluted washing buffer and the wells were turned down onto blotting paper and tapped to remove any remainder.

One hunded micro litre of HRP-Conjugate Reagent was added into each well except for the blank. The plate was covered with plate cover and incubated for 30 minutes at 37ºC. Each well was then washed 5times with diluted washing buffer and the wells were turned down onto blotting paper and tapped to remove any remainder. Fifty micro litre of Chromogen A and 50µl Chromogen B solution was added together into each well including the blank and the plate was incubated at 37ºC for 15 minutes, avoiding light. Fifty micro litre of stop solution was added into each well, and mixed gently. The absorbance was then measured using dual filter machine at 450nm and 630nm. The cut-off value was calculated and the result was evaluated.

3.7.10 AccuDiag™ HEV IgG Antibody

3.7.10.1 Principle of AccuDiag™ HEV IgG Immunoassay

The principle of the HEV IgG ELISA is a solid phase, indirect ELISA system for identification of IgG-class antibodies to HEV (anti-HEV). This is accomplished in a two-step incubation formula. Recombinant, highly immunoreactive antigens related to the structural regions of HEV (ORF-2) are precoated on the polystyrene microwell strips. Anti-HEV specific antibodies (if present) will be

bound to the solid phase pre-coated HEV antigens during the first incubation stage. Next, the wells are washed so that any unbound serum proteins can be removed, after which rabbit anti-human IgG antibodies (anti-IgG) conjugated to horseradish peroxidase (HRP-Conjugate) was added. These HRP-Conjugate antibodies will be bound to any antigen-antibody (IgG) complex previously formed. This occurs during the second incubation phase. Also at this time, the unbound HRP-Conjugate is removed by washing. Chromogen solutions containing Tetramethylbenzidine (TMB) and urea peroxide was added to the wells. This is done in the presence of the antigen-antibody-anti-IgG (HRP) the bound HRP conjugate. After stopping the reaction with sulfuric acid, the blue colour turns yellow. The colour intensity can be gauged proportionally to the amount of antibody captured in the wells, and to the sample, respectively. Colourless wells appear when samples are negative for HEV-IgG.

The test procedures are similar to what has been previously described in (section 3.7.9.2).

3.8 Hepatitis B Virus Genotyping

HBV DNA's presence in patient serum is a confirmation of an active HBV infection, while the concentration of HBV DNA recorded gives an indication of viral replication and infectivity[3]. As HBV DNA is never completely cleared even after resolution of an infection, the distinction between a current and past HBV infection after detection by serological profiles must be accompanied by HBV DNA analysis[3]. In this study, direct detection of HBV DNA was achieved through real time PCR assays. Hepatitis B virus DNA was determined in all samples with serological evidence of HBV infection. A quantitative real time PCR assay was employed for the quantitative detection of HBV DNA in the selected samples. Samples with high HBV DNA concentrations were also

subjected to a nested PCR assay to amplify HBV for genome sequencing by making use of specific primers using sanger protocol[3].

3.8.1 HBV DNA Extraction

In order to isolate viral DNA and make them available for detection by the PCR assays, an extraction procedure was carried out to extract HBV DNA from serum samples using the DaAn Gene RNA/DNA Purification Kit (Spin Column Cat. # DA0590-D, Daan Gene Co. China). The DaAn Gene RNA/DNA Purification kit comes complete with Spin Column, Collection Tube, Lysis Buffer, Inhibitor Remover (Concentrated), Deionized Solution (Concentrated). Eluent, Proteinase K, while anhydrous ethanol was prepared locally. Before starting the DNA extraction process working buffers were first prepared. Ten (10) mL anhydrous ethanol was added into Inhibitor Remover (Concentrated) and stored at room temperature (15ºC-25ºC), 44 mL anhydrous ethanol was added into Deionized Solution (Concentrated) and was stored at room temperature (15ºC-25ºC).

The first step of the extraction process was to lyse host cells and this was performed by adding 50µL of Proteinase K, 200µL of sample and 200µL of lysis buffer into 1.5 mL sterile centrifuge tube, vortex for 15seconds and centrifuged at high speed for 10seconds, incubated at 72ºC for 10mins. The second step was the binding phase, where 250µL of ethanol was added to the mixture, vortexed for 15seconds and the mixture was transferred into the spin column, centrifuged at 12000rpm for 1 minute. The third step was the washing phase, five hundred (500) µL inhibitor remover with ethanol was added and centrifuged for 1 minutes at 12000rpm, 500µL deionized solution was added to the mixture and centrifuged at 12000rpm for 1minute, same step was repeated at room temperature (15ºC-25ºC), the spin column was then fitted in a new collection tube, was

then placed at room temperature (15ºC-25ºC), centrifuged at14,000g for 3 minutes in order to remove residual ethanol, the lid was then opened and incubated for 2minutes at 72ºC. Fifty (50) µL eluent preheated at 72°C was then carefully added right above the membrane of spin column, the tube cap was then well tightened after standing for 1 minute at room temperature (15ºC-25ºC), it was centrifuged at14,000g for 1 minute, then the pure nucleic acid was gotten from the centrifuge tube. All extracts were then stored at -20°C.

3.8.2 HBV Real Time PCR Assay

Principle of Assay

The qPCR assay is a highly sensitive nucleic acid detection method which allows for the simultaneous amplification and quantification of as little as a single copy of DNA within a test sample. Detection of DNA is reported during the course of the amplification process and as such results are accessible in 'real time', eliminating the need for post-PCR processing and thus saving time and resources. 'Real time' reporting is achieved by the use of fluorescent reporter dyes and probes which are designed to fluoresce only after binding specifically to DNA[4]. Thus, emission of fluorescence confirms the presence of DNA while a measure of the fluorescence emitted during the binding process correlates with the amount of DNA present within the test sample. A wide range of these DNA binding molecules are available, including double-stranded DNA binding dyes (SYBR® Green 1 and EvaGreen®), fluorescent probes (hydrolysis probes, dual hybridization probes and scorpion probes) and molecular beacons[4]. For the purpose of this study, the TEL-HBV-Viral Load Test kit v5 was used. The TEL-HBV Master Mix contained all the reagents and enzyme for the specific amplification of a 110 bp sequence of the HBV genomes. The amplification was performed in a 20µl reaction mixture containing: 15µl of mixture of TEL-HBV Master Mix, TEL-

HBV primers /probe mix and 5µl of the template. The real time PCR cycling parameters consisted of activation at 95°C for 1min in 1 cycle followed by 45 cycles consisting of 95°C for 10 sec, 60°C for 30 sec.

Quantification of the HBV-DNA: Using a prepared standard curve from the Tel-HBV-Viral load Test kit (Cat No TEL-300M9), the concentrations of the samples were determined. The Amplification kit has a Detection limit of <10.

3.8.3 HBV Nested PCR Assay

The nested PCR assay is a conventional PCR method used for the amplification of DNA in a two-step reaction process, using two pairs of nested primers targeting the desired gene region. The PCR reaction is automated; performed on a thermal cycler which is programmed to apply different temperature ranges to a reaction mixture in order to amplify multiple copies of DNA from a single template, with the assistance of a thermostable polymerase enzyme. This nested PCR assay was used to amplify the HBV genome in order to generate DNA products for molecular characterization. Duplicate serial dilutions of an HBV infected human serum with known viral DNA concentration from qPCR were prepared and HBV DNA extracted from each dilution. Viral DNA was extracted from 200 µL of plasma using the QIAamp DNA Mini Kit (Qiagen), following the manufacturer's instructions with a minor modification: 1% sodium deoxycholate was added during the proteinase K digestion step to enhance cell lysis and viral release. The extracted DNA was eluted in 50 µL of nuclease-free water and quantified using a Sanger 2000 spectrophotometer. Only sample with A260/A280 absorbance ratios between 1.7 and 2.0 was selected for downstream analysis.

The extract was then subjected to a nested PCR assay using primers specific for the HBV genomes. In the first round of amplification, outer primers targeting conserved regions of the S gene were

used to produce a 1,200 bp fragment. This was followed by a second-round (nested) PCR using inner primers located within the first amplicon, yielding a 278 bp product. The outer forward primer sequence was 5′-GCCTGTATTTTCCTGCTGGTGGC-3′ and the outer reverse primer was 5′-CTCCTCTGCCGATCCATACTG-3′. The inner forward primer was 5′-TCCGGAACAGTGAACCCTG-3′, and the inner reverse primer was the same as the outer reverse primer, 5′-CTCCTCTGCCGATCCATACTG-3′ (Table 3.1).

The PCRs were performed using MyFi 2× premix (Bioline, United Kingdom) [5]. PCR reactions were performed in a total volume of 25 µL. Each reaction mixture contained 2.5 µL of 10× PCR buffer, 0.5 µL of 10 mM dNTP mix, 0.5 µL each of 10 µM forward and reverse primers, 0.2 µL of Taq DNA polymerase, and nuclease-free water to make up the final volume. For the first round of PCR, 1 µL of extracted DNA was used as the template. Thermal cycling conditions were as follows: initial denaturation at 95°C for 3 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute; followed by a final extension at 72°C for 5 minutes. For the second-round (nested) PCR, 2 µL of the first-round product was used as the template, and the same cycling conditions were applied, except the annealing temperature was lowered to 53°C. PCR products were resolved by electrophoresis on 1.5% agarose gels stained with ethidium bromide. Bands of the expected sizes were excised and purified using the QIAquick PCR Purification Kit (Qiagen). The purified products were sequenced bidirectionally using the Applied Biosystems 3130xl Genetic Analyzer with BigDye Terminator chemistry.

The resulting sequences were edited and aligned using BioEdit v7.2 and compared with reference sequences of HBV genotypes A through H retrieved from the NCBI database. Mutational analysis of the S gene was performed using MEGA v11 and Geno2pheno [HBV] tools. Phylogenetic

relationships were inferred using the maximum-likelihood method with 1,000 bootstrap replicates to evaluate genotype clustering confidence.

To support the primer design and amplification strategy, a plasmid map of construct PP790594 (4,168 bp) was generated using SnapGene. The map illustates the location and orientation of the outer and inner primers relative to the HBV S gene region and highlights key restriction sites used in the cloning and amplification process (Figure 3.1).

Negative controls were included in all PCR runs to monitor for potential contamination. All assays were performed in duplicate to ensure the reproducibility and reliability of the results.

Table 3.1: The primer sequences, melting temperatures, and expected amplicon sizes

Primer	Sequence (5′→3′)	Tm	Amplicon Size (bp)
Outer Forward	**GCCTGTATTTTC CTGCTGGTGGC**	**58 ^{0}C**	**1278**
Outer Reverse	**CTCCTCTGCCG ATCCATACTG**	**58 ^{0}C**	
Inner Forward	**TCCGGAACAGT GAACCCTG**	**59 ^{0}C**	**1200**
Inner Reverse	**CTCCTCTGCCG ATCCATACTG**	**59 ^{0}C**	

Source: Author's Field Work, 2024

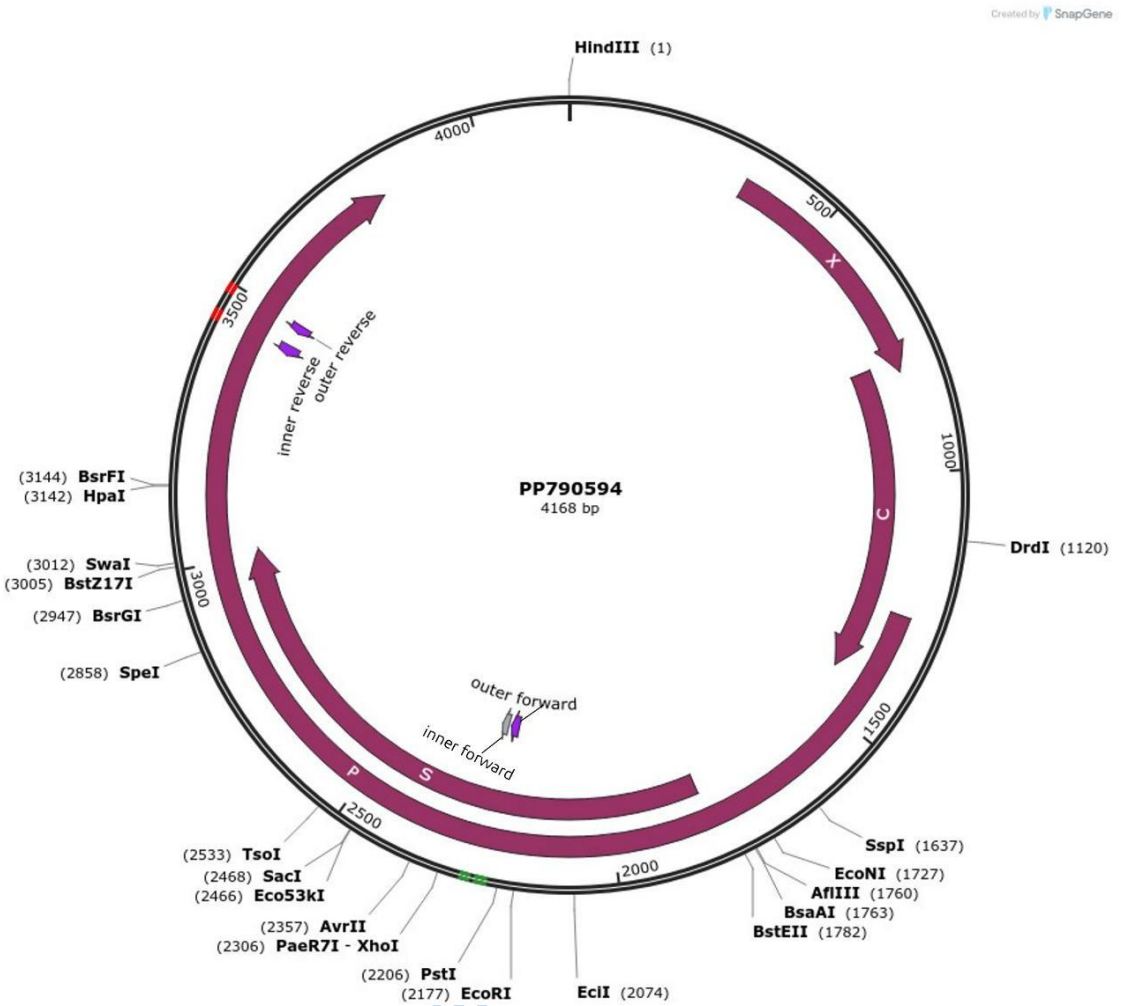

Figure 3.1: Map of construct PP790594 (4,168 bp) showing annotated restriction enzyme sites and locations of outer and inner primers used in the nested PCR protocol targeting the HBV S gene. Primer directions are indicated by purple arrows.

Source: Author's Field Work, 2024

3.8.4 Sequencing of HBV Genomes

Second round nested PCR products which were confirmed by agarose gel electrophoresis to be positive for the genome were sequenced using Sanger protocol.

Briefly, a dideoxy terminator sequencing reaction was performed on the second round nested PCR products, making use of the second-round primers for each respective gene and incorporating fluorescent chain termination dideoxynucleotides (ddNTPs). This was followed by exposure of the sequencing reaction products to a high resolution polyacrilamide electrophoresis reaction to separate short gene fragments (oligonuleotides). Using laser detection, the different wavelengths of the fluorescence emitted by each of the ddNTPs (ddATP, ddTTP, ddCTP, ddGTP) used were observed and then analyzed by a Spectrumedix computer software programme to generate sequenced data[6].

3.9 Reliability and Validity

To ensure validity of the study, internal controls (positive and negative controls) were included in all laboratory tests as appropriate. Where test kits were used, manufacturers‟ instructions were adhered to. The three medical research laboratories used namely (HBV Serology test with ELISA method was done at Bowen University Teaching Hospital Haematology Service Laboratory Ogbomoso, Oyo State, HBV DNA quantification with qPCR technique was done at Babcock University Teaching Hospital Molecular Laboratory, Ilisan Remo, Ogun State and genome sequencing with Nested PCR and sanger technique was carried out at Inqaba Biotech, Moniya, Ibadan) were all accredited and reference laboratory that routinely participates in external quality assurance programs for HBV and HEV testing.

3.9.1 Statistical Analysis

Data were analyzed using statistical packages within the Microsoft Excel, chi-square and SPSS version 16 (SPSS Inc., Chicago, Illinois, USA) software. The level of significance was set at $P<0.05$ at confidence interval of 95%. The information given by the pregnant women were used to investigate the associated risk factors of Hepatitis B and E viruses. The associated risk factors were determined based on the level of significance between pregnant women's life styles or histories filled in the questionnaire and the prevalence of Hepatitis B and E viruses.

3.9.2 Gene Sequence Processing and BLASTn Search

The raw **Sanger sequencing** data were processed using **BioEdit** to ensure sequence quality. The sequence was manually inspected for any ambiguities, and base calling errors were corrected. The processed sequence was then submitted to **BLASTn** for nucleotide sequence similarity search against the **NCBI GenBank database**. BLAST (Basic Local Alignment Search Tool) compares the query sequence to a comprehensive database of known HBV isolates and provides scores reflecting the degree of similarity. The analysis was specifically performed against HBV genotype E sequences to confirm the consistency of the study sample with this genotype. Query coverage and percent identity metrics were evaluated for each match to assess alignment quality.

3.9.3 Sequence Alignment Using MUSCLE

To assess the genetic diversity and conservation of the query sequence, a **MUSCLE (Multiple Sequence Comparison by Log-Expectation)** alignment was performed. The query sequence was aligned with several reference sequences of HBV g**enotype E** . The alignment revealed high conservation across critical regions, including the **PreS1**, **PreS2**, and **S-gene** regions.

3.9.4 Phylogenetic Analysis Using Phylogenetic Typing Tool

The **Hepatitis B Virus Phylogenetic Typing Tool** was used to assess the evolutionary relationship of the query sequence with known HBV isolates. This tool applies a phylogenetic tree-building algorithm combined with bootstrap analysis to evaluate the robustness of the genotype assignment. The query sequence was classified as **genotype E** with **100% bootstrap support**, confirming its assignment to this subtype..

3.9.5 Drug Resistance Mutation Analysis

Drug resistance analysis focused on identifying mutations in the SHB protein, particularly within the reverse transcriptase (RT) region, known for harboring mutations that confer resistance to antiviral treatments. Using the Geno2pheno [hbv] 2.0 tool, query sequence was screened for mutations associated with resistance to common HBV antivirals, such as lamivudine, adefovir, entecavir, tenofovir, and telbivudine. Geno2pheno compares the sequence data to a curated database of resistance-associated mutations, categorizing each detected mutation based on its known impact on drug efficacy.

For lamivudine, mutations at positions 204 and 180 were assessed, as changes at these loci are associated with significant reductions in drug binding and efficacy. The analysis included screening for the 204I, 204V, and 204S mutations, which are known indicators of lamivudine resistance. The presence of adefovir-associated mutations, particularly at positions 181 and 236, was also evaluated, as these mutations have been linked to reduced susceptibility to adefovir. Similarly, entecavir resistance mutations at positions 169, 250, and 204 were checked to determine the susceptibility profile. Finally, the analysis also examined resistance to tenofovir and telbivudine, focusing on mutations in the RT region that may compromise these drugs' efficacy. The presence

or absence of compensatory mutations, which may indirectly influence drug resistance by altering the structural conformation of the RT enzyme, was also documented.

3.9.6 Immune Escape Mutation Analysis

In addition to drug resistance, immune escape mutations within the small hepatitis B surface antigen (SHB) protein were examined using the Geno2pheno tool. These mutations are relevant because they can alter the recognition of hepatitis B surface antigen (HBsAg) by the host immune system, potentially impacting vaccine effectiveness and immune response. The query sequence was scanned for polymorphisms at specific amino acid positions known to be associated with immune escape, particularly at positions 100 and 109 within the SHB protein. These positions have been linked to altered antigenicity, potentially allowing the virus to evade immune surveillance. The polymorphic sites detected in the study sample were annotated to determine whether they were naturally occurring variations or escape-associated mutations.

3.9.7 Ethical Consideration

Ethical approval was obtained from the Health Planning, Research and Statistics Department of Oyo State Ministry of Health with the approval number NHREC/OYOSHRIEC/10/11/22. Informed consent was obtained from each of the pregnant women before participating in this study (Appendix I).

Endnotes

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Chapter Four

Results and Discussion of Findings

4.1 Results of Findings

Social Demographic Characteristics and Associated Risk Factors in Pregnant Women

A total of 297 pregnant women attending Adeoyo Maternity Teaching Hospital, Yemetu and Akinyele Primary Health Centre, Ibadan, Oyo State, Nigeria were recruited into this study. The highest number of pregnant women 92 (31.0%) were within the age range of 21-25years and the lowest 21 (7.1%) were within the age range of ≥36years. Majority of pregnant women 187 (63.0%) were secondary school holders and the lowest 27 (9.1%) were primary school holders. Majority of pregnant women 207 (69.7%) were self-employed and the lowest 34 (11.4%) were unemployed (Table 4.1). Majority of pregnant women 134 (45.1%) were in second trimester while the lowest 74 (24.9%) were in first trimester. One hundred and forty-one pregnant women (47.5%) took well water while 156 (52.5%) took tap water. Fifty-four (18.2%) used latrine while 243 (81.8%) used water closet. Seven (2.4%) had history of jaundice, 2.4% had been vaccinated with HBV, 7.1% had history of blood transfusion, 5.4% had surgical operation, 23.6% had pig contact (Table 4.2).

Prevalence of HEV Antibodies and HBV Serological Markers among Pregnant Women

HEV IgG seroprevalence among 297 women was 30 (10.1%) while none had detectable anti-HEV IgM antibody (Table 4.3). The seroprevalence of HBsAg, HBsAb, HBeAg, HBeAb, HBcAb among 297 pregnant women were 7.4%, 12.1%, 0.7%, 6.7% and 7.1% respectively (Table 4.4). The prevalence of HBsAg and HEV IgG simultaneously was 5 (1.7%) (Table 4.5).

Prevalence of HBV DNA and HEV RNA among Pregnant Women

The prevalence of HBV-DNA (≥10 - ≤999) among pregnant women with HBsAg seropositivity was 86.4%. Two (9.1%) of the pregnant women with HBsAg seropositivity had high viral load (Table 4.6).

Associations between ALT and HEV IgG and HBsAg

None of the pregnant women wilth high ALT level was positive for HEV IgG antibody (Table 4.7). Three (50.0%) of the pregnant women with high ALT level were positive for HBsAg. There was a significant association between ALT and HBsAg (Table 4.8).

Association between Social Demographic Characteristics of Pregnant Women and HEV IgG and HBsAg

HEV IgG antibody was highest (15.7%) among the age group 31-35years and lowest (0.0%) among the age group ≥36years. HEV IgG antibody was highest (11.2%) among the secondary school holders and lowest (7.2%) among tertiary school holders. HEV IgG antibody was highest (16.1%) among the employed and lowest (5.9%) among the unemployed. There was no significant association between social demographic characteristics of pregnant women and HEV IgG antibody (Table 4.9). HBsAg was highest (11.5%) among the age group 26-30years and lowest (4.3%) among the age group 31-35years. HBsAg was highest (19.3%) among tertiary school holders and lowest (0.0%) among primary school holders. HBsAg was highest (12.5%) among the employed and lowest (5.9%) among the unemployed. There was no significant association between social demographic characteristics of pregnant women and HBsAg (Table 4.10).

Associations between Risk Factors and HEV IgG and HBsAg

The HEV IgG antibody was highest (13.5)%), (7.8%), (11.5%), (10.3%), (14.3%), (14.3%), (12.5%), and (12.9%) among pregnant women in third trimester stage, women who took well or spring water, women who used water closet toilet type, women who did not have history of Jaundice, women who received HBV vaccine, women who were transfused with blood, women who had undergone surgical operation and women who had contact with animals respectively. There were no significant associations between Risk Factors and IgG Antibody (Table 4.11). HBsAg was highest (10.8%), (10.9%), (7.8%), (42.9%), (7.6%), (14.3%), (7.8%) and (10.0%) among pregnant women in first trimester stage, women who took tap water, women who used water closet toilet type, women who had history of Jaundice, women who did not receive HBV vaccine, women who were transfused with blood, women who did not undergo surgical operation and women who had contact with animals respectively. There were no significant associations between Risk Factors and HBsAg (Table 4.12).

Molecular Characterization of Samples with Serologic Evidence of HBV Infection

A qPCR assay was used in screening for the presence of HBV DNA in 22 samples of pregnant women positive for HBsAg in order to determine the presence of active HBV infection (figure 4.1). Out of the 22 samples, only 2 of the samples showed high viral load (> 1000 IU/mL).

The 2 samples with HBV DNA concentrations >1000 IU/mL were subjected to nested PCR to detect and amplify the HBV gene. Of these 2 samples for which the HBV gene was amplified, 1 was detectable as seen on the agarose gel documentation of HBV gene amplicons (Figure 4.2).

HBV gene amplicon from the detectable sample was then subjected to partial genome sequencing and nucleotide and amino acid sequences analyzed and the result of the genome sequenced was

documented (Figure 4.3). The sequence was submitted to the NCBI GenBank database with the accession number PV851423.

Sequence Similarity

The BLASTn search results revealed that the query sequence exhibited high similarity to **Hepatitis B virus Genotype E** (NC_003977.2) with up to 99.2% identity to the top hit. The matched several known **HBV** sequences, with the highest similarity consistently observed among **Genotype E** strains. These findings confirm the classification of the query sequence as genotype E (Table 4.13).

MUSCLE Alignment Consensus

This figure presents the consensus sequence obtained by aligning the query sequence with multiple HBV Genotype E strains. The alignment highlights conserved regions, demonstrating thequery's close similarity to other genotype E isolates. A few mutations were identified in the **PreS2** and **S-gene** regions; however, these variations are not predicted to significantly affect the overall structure or function of the encoded protein (figure 4.4).

Phylogenetic Tree of HBV Subtypes

The phylogenetic tree, generated using the **Genome Detective Platform**, shows that the query sequence clusters with other **HBV genotype E** sequences. The tree clearly positions the query within the **Genotype E** clade, indicating a close evolutionary relationship with other isolates of this genotype. The **100% bootstrap support** further strengthens the reliability of this phylogenetic classification. Overall, the analysis confirms the identification of the query sequence as **HBV Genotype E**, with a high degree of genetic similarity to other genotype E strains (figure 4.5).

Drug Resistance Analysis

The Geno2pheno analysis revealed that the HBV sample was susceptible to all major HBV antiviral drugs used in HBV treatment. No key resistance-associated mutations were detected, including 204I and 180M (linked to lamivudine resistance), 181T and 236T (associated with adefovir resistance), and 169T, 250V, and 204V (associated with entecavir resistance). The absence of these mutations suggests that the strain is likely responsive to the therapies and indicates that the women were not undergoing HBV treatment at the time of sampling. Additionally, no resistance-conferring mutations were detected in the reverse transcriptase (RT) region for tenofovir or telbivudine (Table 4.14).

Immune Escape Mutation Profile

The immune escape mutation analysis revealed low-frequency polymorphisms in the small hepatitis B surface (SHB) protein, specifically at positions 100 and 109. Mutations 100C and mutation 109R, both of which are associated with potential alterations in immune recognition, were identified. However, these mutations are not considered to have a significant impact on immune escape in HBV genotype E (Table 4.15).

Table 4.1: Social Demographic Characteristics of Pregnant Women

Variables	Category	Frequency	Percentage(%)
Age Group	≤20	27	9.1
	21-25	92	31.0
	26-30	87	29.3
	31-35	70	23.6
	≥36	21	7.1
Educational Status	Primary	27	9.1
	Secondary	187	63.0
	Tertiary	83	28.0
Occupation	Employed	56	18.9
	Self-employed	207	69.7
	Unemployed	34	11.4
Total		**297**	**100**

Source: Author's Field Work, 2024

Table 4.2: Prevalence of the Risk Factors Associated with HBV and HEV in Pregnant Women

Variables	Category	Frequency	Percentage(%)
Trimester	1st	74	24.9
	2nd	134	45.1
	3rd	89	30.0
Drinking Water Source	Well water	141	47.5
	Tap water	156	52.5
Toilet Type	Latrine	54	18.2
	Water closet	243	81.8
History of Jaundice	No	290	97.6
	Yes	7	2.4
HBV Vaccination	No	290	97.6
	Yes	7	2.4
Blood Transfusion	No	276	92.9
	Yes	21	7.1
Surgical Operation	No	21	94.6
	Yes	16	5.4
Pig Contact	No	227	76.4
	Yes	70	23.6
Total		**297**	**100**

Source: Author's Field Work, 2024

Table 4.3: Prevalence of HEV Antibodies among Pregnant Women

Variables		Frequency	Percentage(%)
HEV IgM	Neg	297	100.0
(Recent Infection)	Pos	0	0.0
HEV IgG	Neg	267	89.9
(Past Infection)	Pos	30	10.1
Total		**297**	**100**

Source: Author's Field Work, 2024

Table 4.4: Prevalence of HBV Serological Markers among Pregnant Women

Variables		Frequency	Percentage(%)
HBsAg	Neg	275	92.6
(Infection)	Pos	22	7.4
HBsAb	Neg	261	87.9
(Protected)	Pos	36	12.1
HBeAg	Neg	295	99.3
(Highly Infectious)	Pos	2	0.7
HBeAb	Neg	277	93.3
(Clearance)	Pos	20	6.7
HBcAb	Neg	276	92.9
(Past Exposure)	Pos	21	7.1
Total		297	100

Source: Author's Field Work, 2024

Table 4.5: Prevalence of HBsAg and HEV IgG Simultaneously in Pregnant Women

Variables		Frequency	Percentage(%)
HBsAg/HEV IgG	Pos	5	1.7
	Neg	292	98.3
Total		**297**	**100**

Source: Author's Field Work, 2024

Table 4.6: Prevalence of HBV DNA among Pregnant Women

Variables	Categories (IU/mL)	Frequency	Percentage(%)
HBV DNA	<10	1	5.0
(Active Infection)			
	(≥10 - ≤999)	19	86.4
	≥1000	2	9.1
Total		**22**	**100**

Limit of Detection for HBV DNA: <10

High Viral Load for HBV: ≥1000

Source: Author's Field Work, 2024

Table 4.7: Association between ALT and HEV IgG among Pregnant Women

Variables	Categories (U/L)	HEV IgG Pos (%)	Total	X^2	Df	P-value
ALT Level	Normal	27 (10.0)	270			
	Borderline	3 (14.3)	21	1.082	2	0.439
	High	0 (0.0)	6			

Source: Author's Field Work, 2024

Table 4.8: Association between ALT and HBsAg among Pregnant Women

Variables	Categories (U/L)	HbsAg Pos (%)	Total	X^2	Df	P-value
ALT Level	Normal	18 (6.7)	270			
	Borderline	1 (4.8)	21	16.300	2	0.01
	High	3 (50.0)	6			

Source: Author's Field Work, 2024

Table 4.9: Association between Social Demographic Characteristics and HEV IgG Antibody in Pregnant Women

Variables	Category	HEV IgG Pos (%)	Total	X^2	P-value
Age Group	≤20	2 (7.4)	27		
	21-25	10 (10.9)	92		
	26-30	7 (8.0)	87	3.433	0.488
	31-35	11 (15.7)	70		
	≥36	0 (0.0)	21		
Educational Status	Primary	3 (11.1)	27		
	Secondary	21 (11.2)	187	5.622	0.132
	Tertiary	6 (7.2)	83		
Occupation	Employed	9 (16.1)	56		
	Self employed	19 (9.2)	207	6.697	0.244
	Unemployed	2 (5.9)	34		

Source: Author's Field Work, 2024

Table 4.10: Association between Social Demographic Characteristics and HBsAg in Pregnant Women

Variables	Category	HBsAg Pos (%)	Total	X^2	P-value
Age Group	≤20	2 (7.4)	27		
	21-25	6 (6.5)	92		
	26-30	10 (11.5)	87	3.861	0.533
	31-35	3 (4.3)	70		
	≥36	1 (4.8)	21		
Educational Status	Primary	0 (0.0)	27		
	Secondary	16 (8.5)	187	3.129	0.567
	Tertiary	16 (19.3)	83		
Occupation	Employed	7 (12.5)	56		
	Self-employed	13 (6.3)	207	4.375	0.192
	Unemployed	2 (5.9)	34		

Source: Author's Field Work, 2024

Table 4.11: Associations between Risk Factors and HEV IgG Antibody in Pregnant Women

		HEV IgG			
Variables	**Category**	**Pos (%)**	**Total**	**X²**	**P-value**
Trimester	1st	4 (5.4)	74		
	2nd	14 (10.4)	134	1.023	0.600
	3rd	12 (13.5)	89		
Drinking Water Source	Well water	11 (7.8)	141	3.358	0.340
	Tap water	11 (7.1)	156		
Toilet Type	Latrine	2 (3.7)	54	2.975	0.566
	Water closet	28 (11.5)	243		
History of Jaundice	No	30 (10.3)	290	0.806	0.440
	Yes	0 (0.0)	7		
HBV Vaccination	No	29 (10.0)	290	0.574	0.449
	Yes	1 (14.3)	7		
Blood Transfusion	No	27 (9.8)	276	1.559	0.509
	Yes	3 (14.3)	21		
Surgical Operation	No	28 (10.0)	281	0.107	0.245
	Yes	2 (12.5)	16		
Pig Contact	No	21 (9.3)	227	1.301	0.347
	Yes	9 (12.9)	70		

Source: Author's Field Work, 2024

Table 4.12: Associations between Risk Factors and HBsAg in Pregnant Women

Variables	Category	HBsAg Pos (%)	Total	X^2	P-value
Trimester	1st	8 (10.8)	74		
	2nd	9 (6.7)	134	3.101	0.415
	3rd	5 (5.6)	89		
Drinking Water Source	Well water	5 (3.5)	141	4.613	0.113
	Tap water	17 (10.9)	156		
Toilet Type	Latrine	3 (5.6)	54	1.274	0.085
	Water closet	19 (7.8)	243		
History of Jaundice	No	21 (7.2)	290	0.539	0.369
	Yes	1 (14.3)	7		
HBV Vaccination	No	22 (7.6)	290	2.304	0.710
	Yes	0 (0.0)	7		
Blood Transfusion	No	19 (6.9)	276	0.961	0.212
	Yes	3 (14.3)	21		
Surgical Operation	No	22 (7.8)	281	3.422	0.743
	Yes	0 (0.0)	16		
Pig Contact	No	15 (6.6)	227	1.144	0.254
	Yes	7 (10.0)	70		

Source: Author's Field Work, 2024

Table 4.13: Top 10 BLASTn Results for Query Sequence

Rank	Sequence Description	Max Score	Total Score	Query Coverage	E-value	Percent Identity	Accession
1	Hepatitis B Virus clone A2	2032	2365	99%	0	99.20%	PP790594.1
2	Hepatitis B virus isolate	2032	2032	99%	0	99.20%	KX186584.1
3	Hepatitis B virus isolate A58	2028	2028	98%	0	99.20%	HM363609.1
4	Hepatitis B virus isolate SDAC_086	2023	2023	98%	0	99.11%	KF170786.1
5	Hepatitis B virus isolate 235-01	2023	2023	98%	0	99.11%	DQ060830.1
6	Hepatitis B virus isolate GU1405	2021	2021	98%	0	99.11%	GQ161821.1
7	Hepatitis B virus isolate GU1520	2021	2021	98%	0	99.11%	GQ161818.1
8	Hepatitis B virus isolate GU732	2021	2021	98%	0	99.11%	GQ161799.1
9	Hepatitis B virus isolate GU625	2021	2021	99%	0	99.02%	GQ161814.1
10	Hepatitis B virus isolate bne446	2021	2021	99%	0	99.02%	FN594763.1

Source: Author's Field Work, 2024

Table 4.14: Drug Resistance Profile Analysis

Drug Resistance Profile	Drug	Key Resistance Mutation	Result
Lamivudine	204I, 180M	Absent	Susceptible
Adefovir	181T, 236T	Absent	Susceptible
Entecavir	169T, 250V, 204V	Absent	Susceptible
Tenofovir	None	Absent	Susceptible
Telbivudine	80I, 80V	Absent	Susceptible

Source: Author's Field Work, 2024

Table 4.15: Immune Escape Mutation Profile

Escape Mutation Profile	Position	Mutation	Immune Escape Potential
SHB	100C	Present	Low
SHB	109R	Present	Low

Source: Author's Field Work, 2024

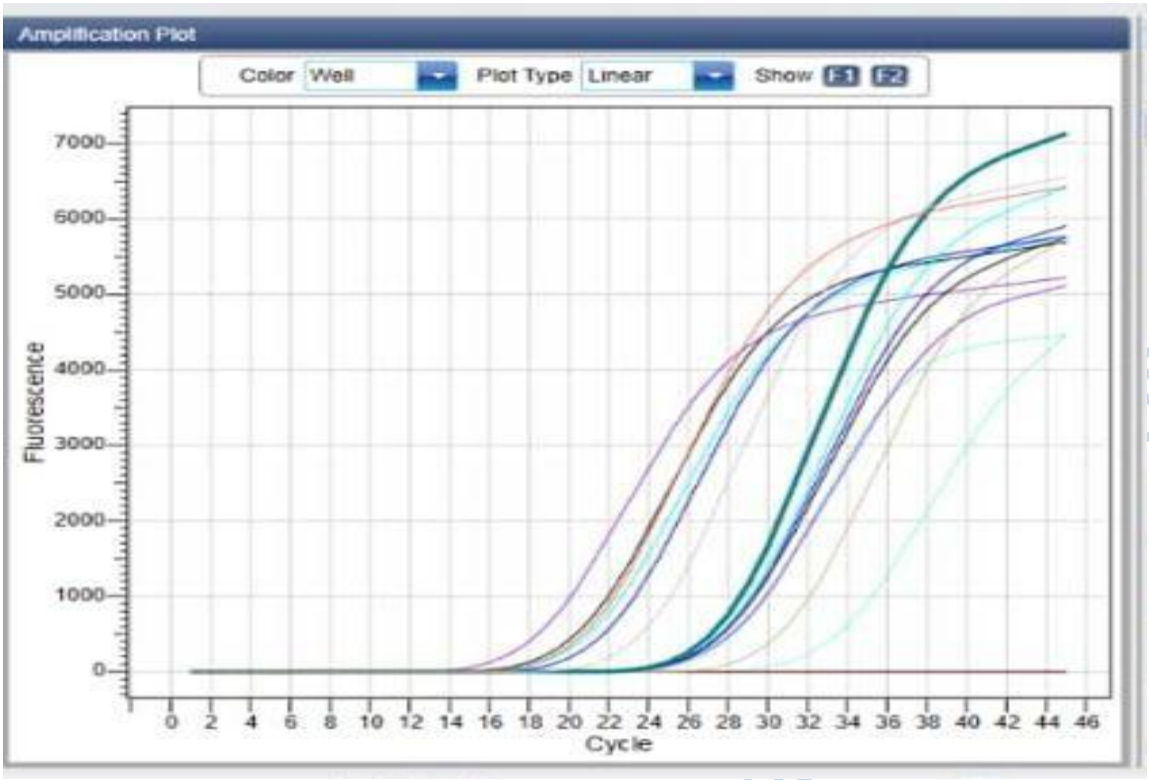

Figure 4.1: Graphical Representation of Amplification Curves During HBV qPCR Assay

Source: Author's Field Work, 2024.

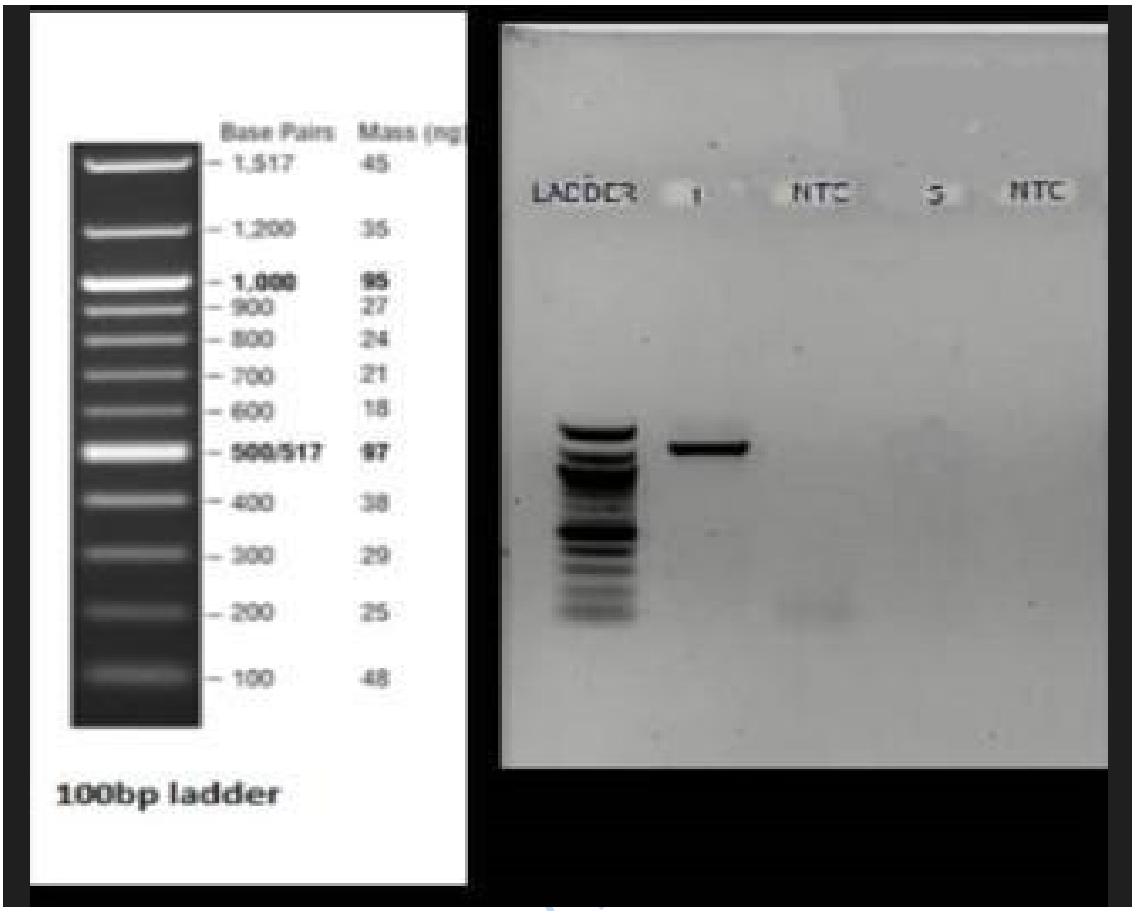

Figure 4.2: Agarose Gel Documentation of HBV Gene Amplicons [MW=100bp DNA Molecular Weight Marker, NTC=Negative Test Control, Study Samples Number 1 and 5]. The HBV Amplicon was Detectable in Sample 1 and undetectable in Sample 5.

Source: Author's Field Work, 2024.

TCATACTACCCCTTCTTTCATCTCGTCAATCTTCTCGAGGATTGGGGACCCTGCACCG
AACATGGAAAGCATCACATCCGGATTCCTAGGACCCCTGCTCGTGTTACAGGCGGG
GTTTTTCTTGTTGACAAAAATCCTCACAATACCGCAGAGTCTAGACTCGTGGTGGAC
TTCTCTCAATTTTCTAGGGGGAGCTCCCGTGTGTCTTGGCCAAAATTCGCAGTCCCCA
ATCTCCAATCACTCACCAACCTCTTGTCCTCCAATTTGTCCTGGCTATCGCTGGATGT
GTCTGCGGCGTTTTATCATCTTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTG
GTTCTTCTGGACTATCAAGGTATGTTGCCCGTTTGTCCTCTAATTCCAGGATCATCAA
CCACCAGTACGGGACCCTGCCGAACCTGCACGACTCTTGCTCAAGGAACCTCTATGT
TTCCCTCATGTTGCTGTTCAAAACCTTCGGACGGAAATTGCACTTGTATTCCCATCCC
ATCATCATGGGCTTTCGGAAAATTCCTATGGGAGTGGGCCTCAGCCCGTTTCTCCTG
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CTTTCAGTTATATGGATGATGTGGTATTGGGGGCCAAGTCTGTACAACATCTTGAGT
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CCCTGTTAACCGGCCTATTGATTGGAAAGTATGTCAAAGAATTGTGGGTCTTTTGGG
CTTTGCTGCCCCTTTTACACAATGTGGATATCCTGCTTTAATGCCTCTATATGCGTGT
ATTCAATCTAAGCAGGCTTTCACTTTCTCGCCAATTTACAAGGCCTTTCTGTGTAAAC
AATACCTGAACCTTTACCCCGTTACCCGGCAACGGCCAGGTCTGTGCCAAGTGTTTG
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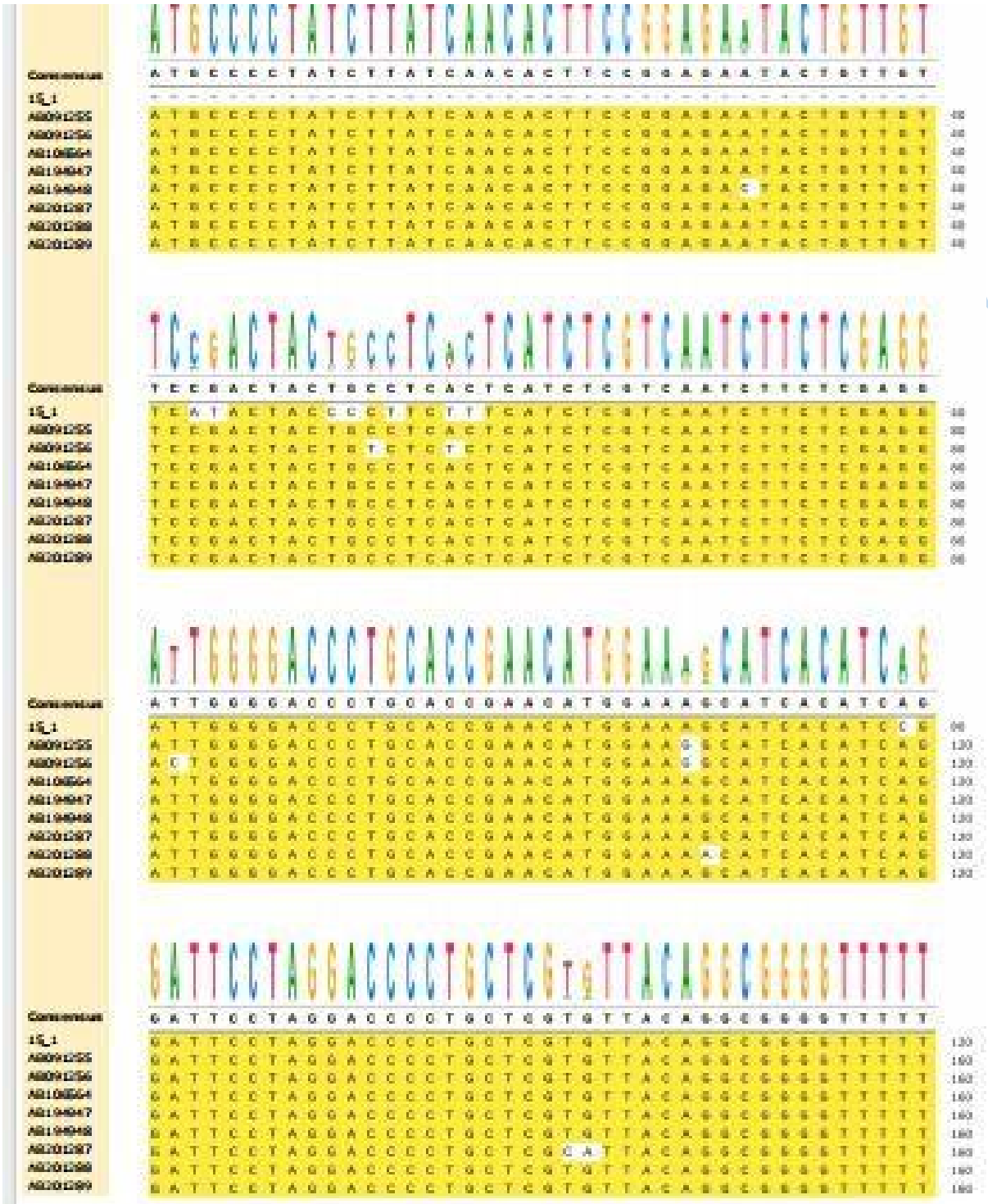
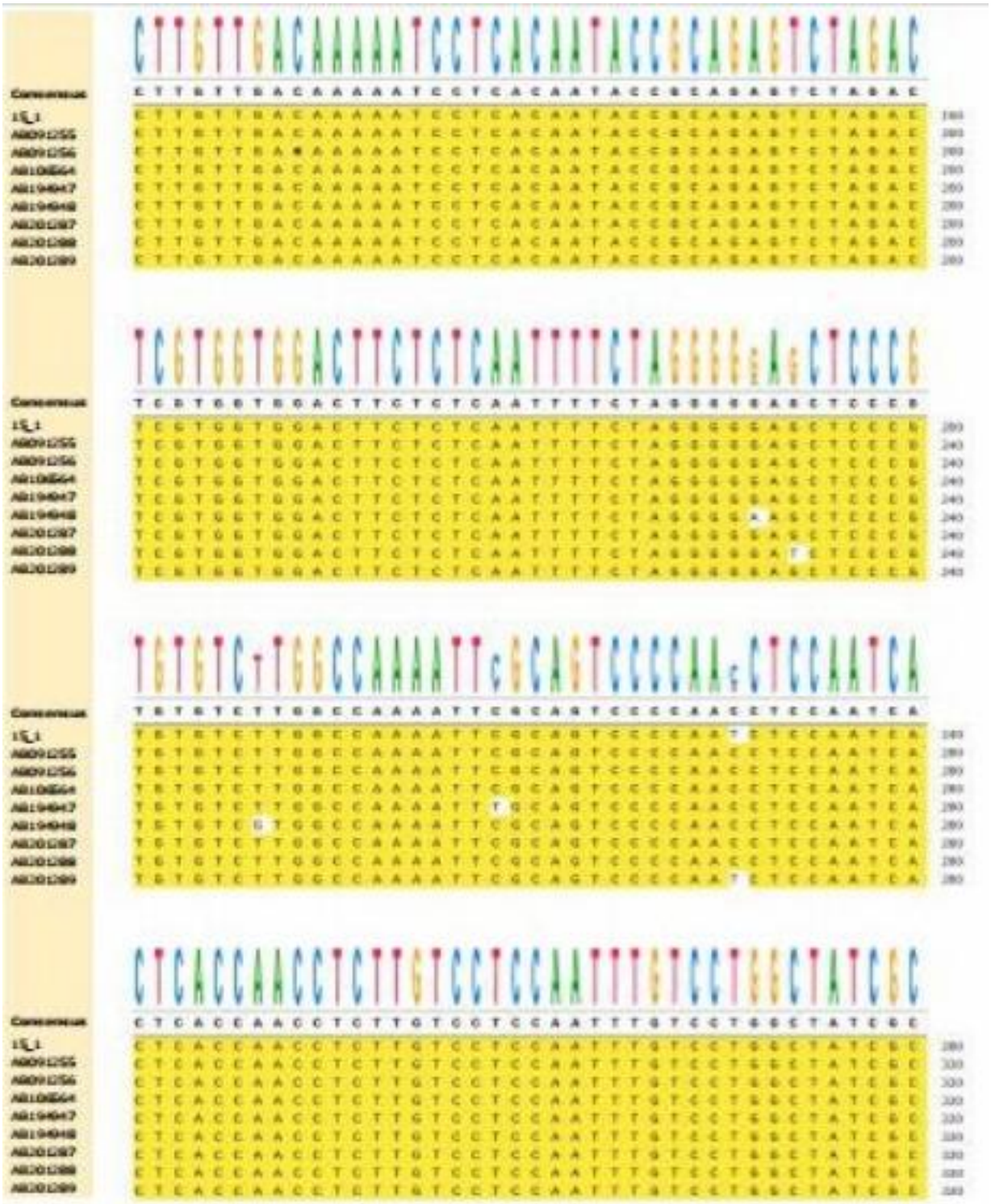
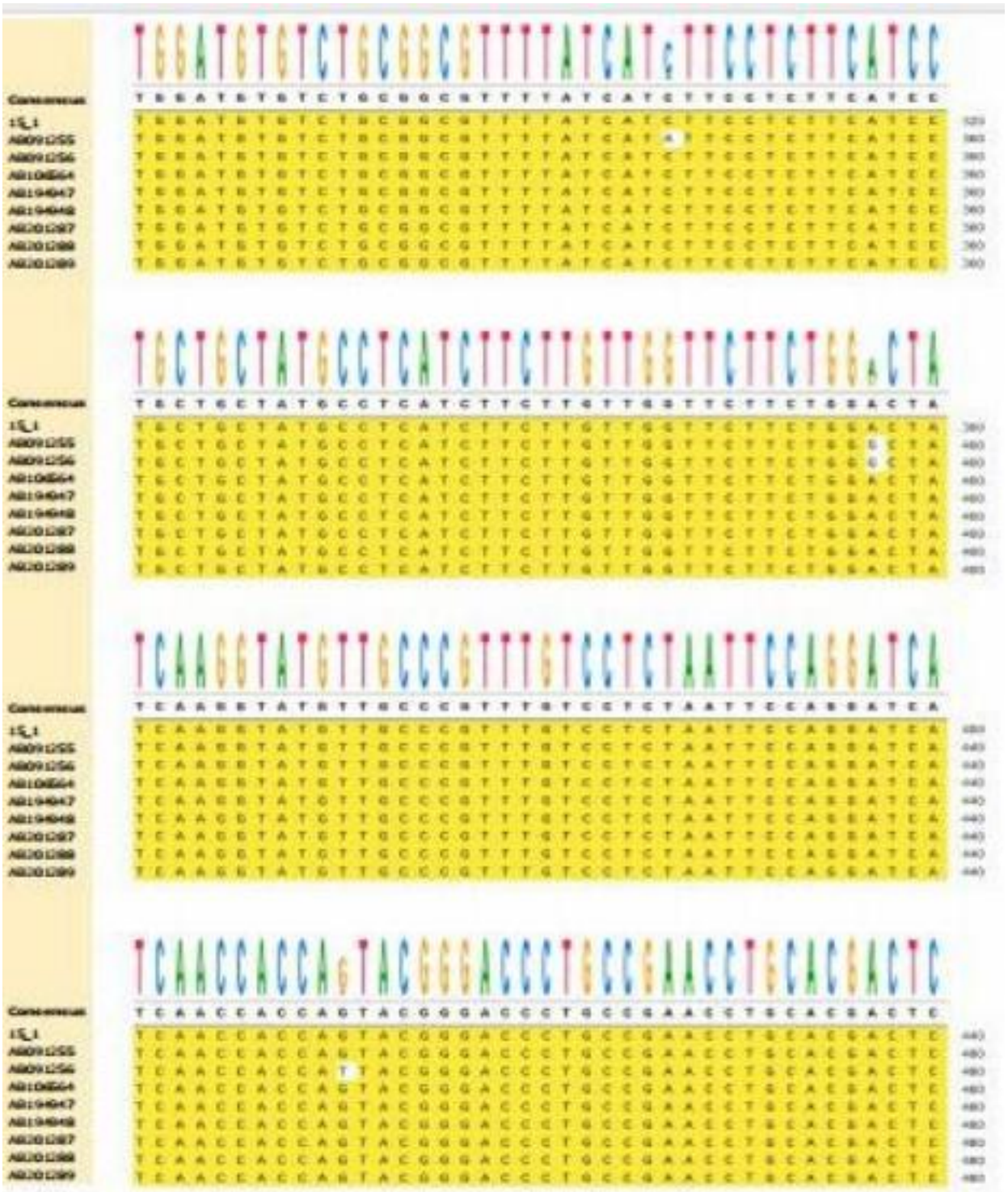
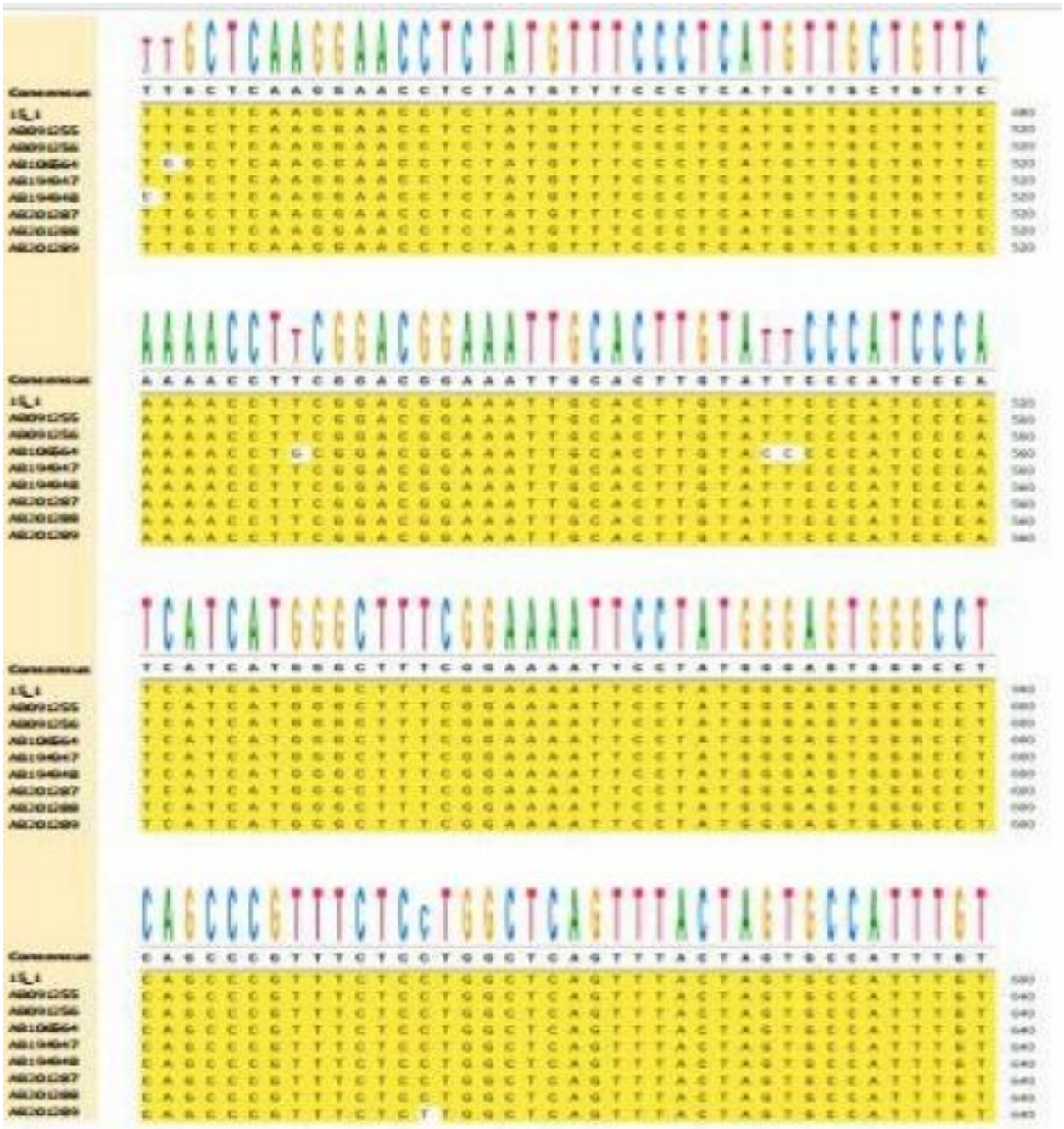
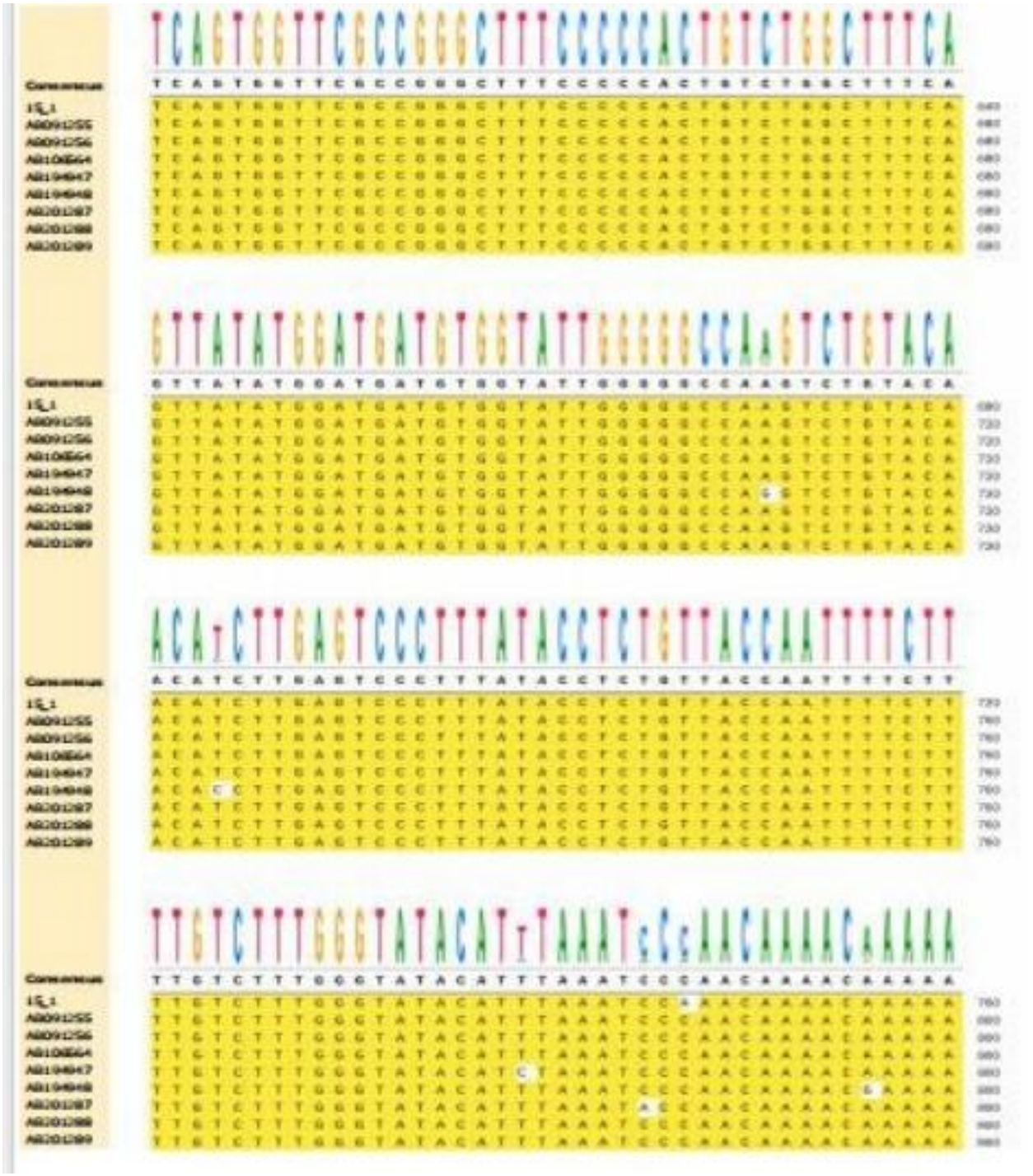
Figure 4.3: Partial Genome Sequence Result
Source: Author's Field Work, 2024.

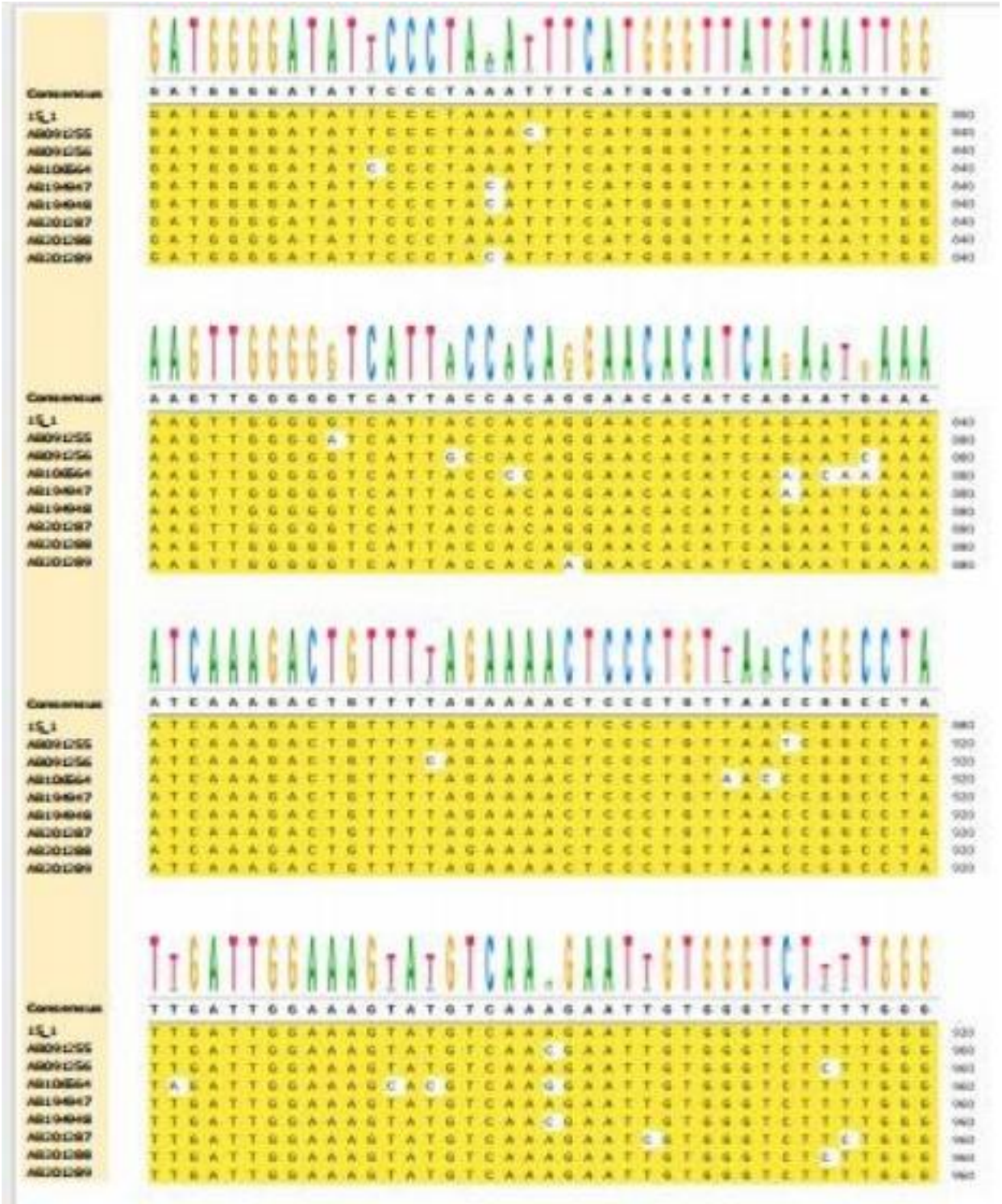


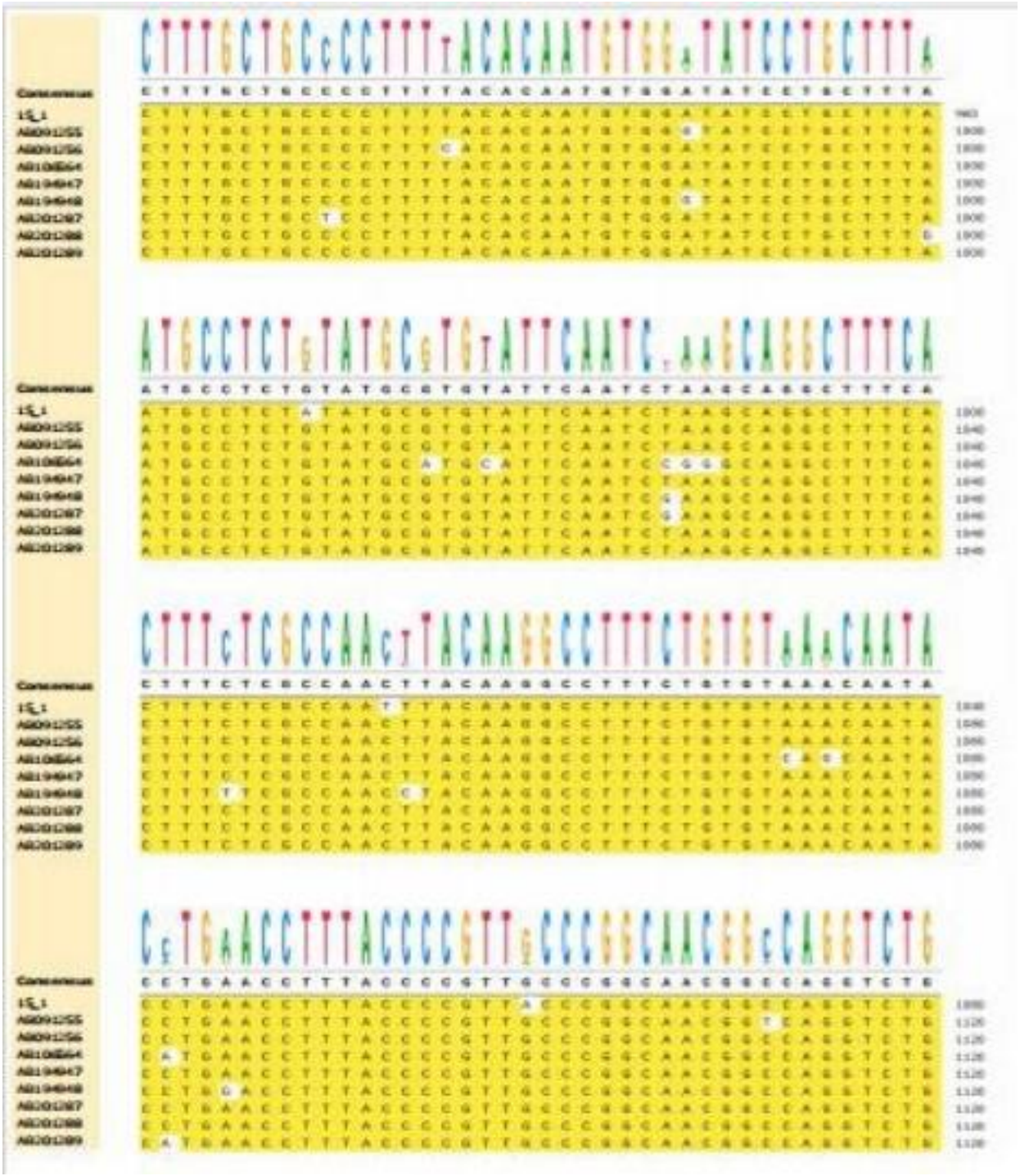












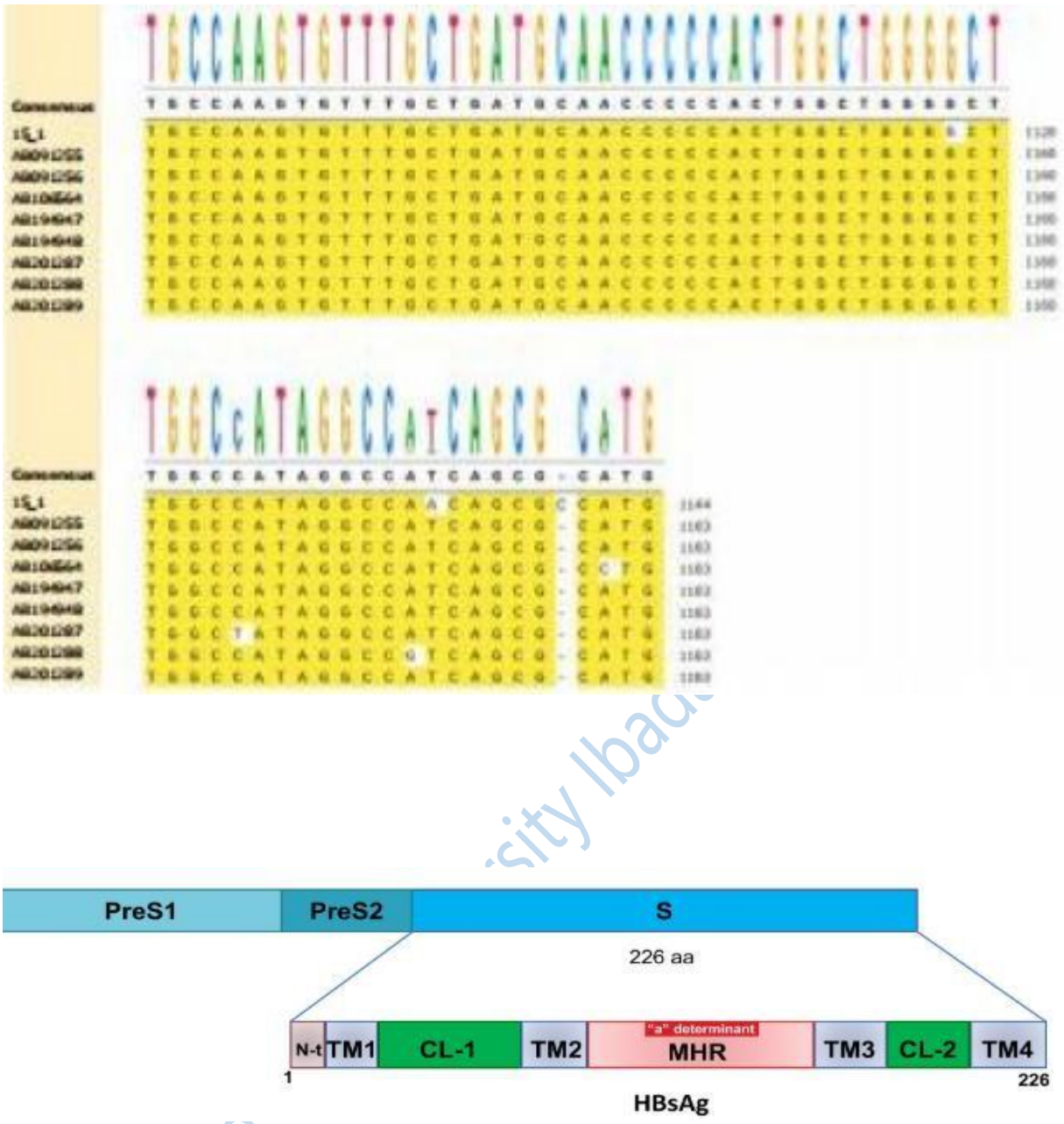


Figure 4.4: Nucleotide Sequence Alignment of Genotype E Isolates using MUSCLE

Source: Author's Field Work, 2024

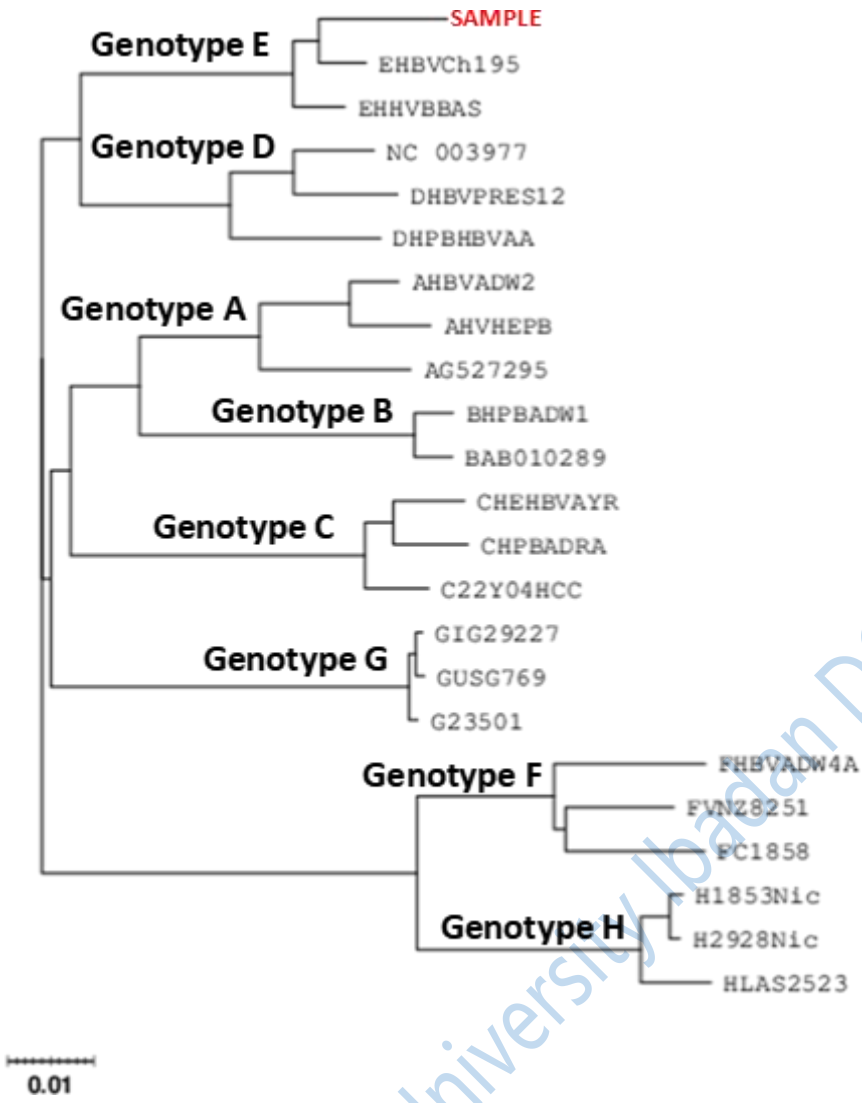


Figure 4.5: Phylogenetic Tree of HBV Genotypes. Study Sequence is Labelled Sample, while Reference Sequences from GenBank are Unlabelled. All Showing Genotype E.

Source: Author's Field Work, 2024

4.2 Discussion of Findings

Hepatitis B and E infections remain a serious public health issue globally. Although most acute HEV infections tend to be sub-clinical, self-limiting, and mild, the overlap of HBV and HEV infections can present additional risks, which frequently leads to severe complications and poor outcomes in pregnancy[1]. In this study, prevalence rate of HBV and HEV infections among pregnant women attending antenatal consultations were investigated in Ibadan, Oyo State, Nigeria.

The seroprevalence of HBsAg, HBsAb, HBeAg, HBeAb, HBcAb among the pregnant women attending Adeoyo Maternity Teaching Hospital and Akinyele Primary Health Centre, Ibadan, Oyo State, Nigeria were 7.4%, 12.1%, 0.7%, 6.7% and 7.1% respectively. The 7.4% overall prevalence for HBsAg in pregnant women attending antenatal clinics in Ibadan, Oyo State falls within figures reported for other African countries[1]. In related studies in different parts of Nigeria, higher prevalence rates of 11.6% were reported among pregnant women in Maiduguri, 10.5% in Lagos and 8.3% in Zaria[2,3]. Lower reports included 2.2% in Benin City, 4.3% in Port Harcourt and 5.7% in Ilorin[2]. Though within the same continent, the result of this study is higher than the 3% reported in pregnant women in Tanzania and 5.9% in Ethiopia[4,5]. Similar studies in other parts of the world reported 6.17% in Hong Kong, 10% in India, 12.9% in Papua New Guinea, 12% in Taiwan, 14.3% in Burkino Faso[6,7,8,9,10]. These agree with reports that the global prevalence of chronic HBV infection varies, highest in Africa, Asia and the Western pacific (>8%) to intermediate (2-7%) in Southern and Eastern Europe and lowest (< 2%) in Western Europe, North America, and Australia[11]. These variations may be due to differences in geographical area, age range of study participants, vaccination rate of HBV vaccine, income level and socio-cultural practices.

Anti-HBs are found following effective immunization against HBV either due to past infection or vaccination. This study shows HBsAb prevalence of 12.1% which is in line with the result (12.7%)

of similar study in Brazil[12]. Also, lower report of 3.2% was found among pregnant women in Kaduna, Nigeria[2]. Surprisingly, the result of this study disagreed with some pregnant women who claimed to have been vaccinated with HBV as none of this pregnant women had detectable antibody to HBV. This shows that 12.1% of the pregnant women in this study who had HBsAb had past infection with hepatitis B. This means they are no longer contagious and are protected from getting HBV in the future.

HBeAg correlates with the presence of high level of HBV replication and infectivity. Of all the pregnant women, 0.7% showed increased infectivity with HBV. This shows they are infectious and are able to spread the virus to their newborn and other people. A similar study in Central Nigeria showed higher HBeAg seropositivity of 10%[2]. HBeAg positive patients have high level of serum HBV-DNA which results in high levels of viral load and increases the risk of cirrhosis or chronic liver failure and hepatocellular disease in pregnant women[2]. The HBeAg positive pregnant women in this study had high viral load, which may result in vertical transmission. These most times lead into increased maternal and childbirth morbidity and mortality, as observed in Central Nigeria from various literatures[2].

HBeAb is an antibody to HBeAg which appears after the clearance of HBeAg. Twenty (6.7%) of the pregnant women had HBeAb and this showed decreased infectivity with HBV. A similar study in Bauchi, Nigeria showed higher HBeAb seropositivity of 17.2%[17].

HBcAb are antibodies to HBcAg indicating a resolved infection with HBV. Of all the pregnant women, 7.1% with detectable HBcAb showed resolved infection that is they had past infection with the virus. A lower HBcAb prevalence (4.2%) of similar study was reported in Brazil[12].

There was no significant relationship between HBV seropositivity in the pregnant women in terms of age, education, source of water supply, type of toilet, education, animal contact, previous history

of Jaundice, previous histories of blood transfusion and previous surgical operation ($p>0.05$).

In this study, the overall prevalence of HEV antibodies among pregnant women attending Adeoyo Maternity Teaching Hospital and Akinyele Primary Health Centre, Ibadan, Oyo State, Nigeria was 10.1%. HEV IgM antibody which indicates recent infection with HEV was not detected in this study and this shows the women were not currently infected with HEV as at the time of sampling but HEV IgG antibody detected in this study shows that the women had been exposed to HEV at one time or the other.

The seroprevalence of HEV IgG (10.1%) antibody among the pregnant women attending antenatal consultations in Ibadan, Oyo State is lower than the results of similar studies conducted in Plateau State, Nigeria (42.6%), Accra, Ghana (28.6%), Egypt (84.3%), Ethiopia (59.0%) and Sudan (31.1%) but higher than the seroprevalence in Mexico (5.7%) and Osun State, Nigeria (6.3%)[20,21,22,23,24,25]. The variations in prevalence are likely to be related to the rural urban differences in study areas. A likely explanation may be in the differences in socioeconomic as well as cultural and hygiene practices. Another reason for this disparity is the differences in the population and resources of each state or nation. People living in heavily populated countries with limited access to essential water, sanitation, hygiene, and health services may be greatly affected. Epidemic diseases related to water may arise in a more crowded and heavily populated nation due to the increase in the pressure on water and sanitary facilities than in a less crowded and low populated nation[20].

Earlier reports having the common assumption that HEV infects pregnant women in higher rates has been disproved by later studies that show that no distinction actually exists between pregnant and non-pregnant women in terms of prevalence of anti-HEV seropositivity[20,26]. HEV IgM which was not detected in this study shows that the participants in this study have not been recently

exposed to HEV infection and also reflects the low incidence of HEV infection in this study population. A similar study on pregnant women in Plateau State and Osun State, Nigeria reports a similar IgM antibody prevalence of 0.9% and 0.6% respectively[20,25]. There have been reports of HEV IgM prevalence in different populations in Nigeria such as 1.4% in HIV positive individuals in Ibadan and 5.45% in HIV positive individuals in Ogbomoso[27,28]. No significant relation was identified between HEV seropositivity in the pregnant women in terms of age, source of water supply, type of toilet, (P value >0.05).

There had been similar reports from studies in Turkey, however, it was reported that risk of positivity tends to reduce with level of education, also in this study the prevalence of HEV seropositivity was lower in pregnant women with tertiary education (7.2%) than in women with primary (11.1%) and secondary education (11.2%) only[26].

Pregnant women, particularly those in the second and third trimesters, are more affected during hepatitis E outbreaks, and have worse outcomes also, mortality rates among pregnant women, especially those infected in the third trimester, range from 15% to 25%[29]. Frequencies of abortions, stillbirths, and neonatal deaths are also increased among pregnant women with HEV infection[30]. In this study, the highest prevalence (13.5%) of HEV IgG antibodies was seen in pregnant women in the third trimester of pregnancy, which means that 86.5% are not yet protected and are at risk of HEV infection at this stage of pregnancy. Hence, there is a need for personal hygiene for this category of women.

Growing evidence suggests that individuals who work in contact with swine such as pig farmers, veterinarians, and slaughterhouse workers are at increased risk of acquiring HEV infection[31]. This evidence was also observed in this study as the prevalence of HEV antibody was higher among pregnant women who had contact with pig (12.9%) compared with those who did not have contact

with pig (9.3%). As observed from this study, many of the pregnant women live in the densely populated parts of Ibadan, where domestic animals share habitat with humans. This study is consistent with the results of similar studies in Sweden with the seroprevalence of 13% in exposed and 9.3% in people unexposed to swine[32].

Contaminated water or water supplies are important sources of HEV infection especially following outbreaks in humans[33]. Insufficient drinking water treatment and low standards of sanitation have been implicated in major outbreaks in developing countries where contamination of drinking water with animal or human faeces is common[33]. In this study, there was no significant difference between pregnant women who took well or spring water (7.8%) and those who took tap water (7.1%) ($p>0.05$). All sources of water could likely function as a vehicle for HEV transmission and that surface water only increased the risk[20]. Certain precaution should be taken, such as the consumption of boiled water, to curb the rate of infection through consumption of contaminated water.

HEV infection is emerging as a potential new threat to blood safety after several cases of transfusion-transmission were reported from non-epidemic countries[34]. Likewise, this study also shows higher prevalence of HEV antibody among the women who were transfused with blood (14.3%) than those who were not transfused with blood (9.8%). Evidence of HEV transmission through blood transfusion among hemodialysis patients was reported in Japan and UK[35,36]. HEV transmission was also reported among illegal blood donors in the US[37]. Credible reports of transmission of HEV by blood transfusion, therefore, warrant redefining of the donor screening policy by blood banks, especially in endemic areas.

HEV in patients with chronic HBV has been found associated with adverse clinical outcomes in several geographical settings[38]. It was reported that HBV and HEV represented 20%-40% of all symptomatic acute hepatitis E (AHE) infections[38]. The HBV and HEV IgG prevalence rate of 1.7%

observed in this study population shows that a few pregnant women with HBV infection simultaneously had previous exposure to HEV and HEV IgG has been shown to contribute to poor feto-maternal outcomes in a similar study[40]. However, the prevalence rate is lower than the 15.8% HBV and HEV IgG prevalence rate among animal handlers, 8.1% rate among blood donors in Tehran, 13.4% rate in Iran's previous studies found in the general population of the Fars province and 46.1% rate reported among adults in Khuzestan province[31]. During the long course of chronic hepatitis B infection, emphasis should be placed on reducing the chance that patients might be simultaneously exposed to HEV by taking appropriate precautions, such as consumption of boiled water and well-cooked food, in regions where it is endemic.

HBV infection is a serious public health problem, causing most of the acute and chronic liver disease[38]. In some studies, a higher HBV seroprevalence in individuals with liver disease in comparison to healthy individuals has been observed[38]. This is in line with this study as there is significantly high prevalence of HBV among pregnant women with elevated amino transferase (ALT) level compared to those with normal or at borderline ALT level indicating liver injury or damage in pregnant women. ALT is found in highest concentration in liver compared with other tissues of the body such as kidney, heart and muscle[39].

HBV DNA was screened and quantified in individuals with serological evidence of HBV infection. Molecular characterization of HBV genes was performed in HBV DNA positive samples to identify sequence variations, and the resulting sequences were compared with existing HBV data in Genbank. Partial genome sequence of HBV from this individual was successfully generated and analyzed as illustrated in (figure 4.3). Phylogenetic analyses confirmed that the HBV isolate from the study populations was genotype E, (Figure 4.5). To date, ten genotypes (A–J) have been identified, each with distinct geographical distributions, reflecting the virus's high genetic

heterogeneity. Notably, Nigeria is considered the most probable origin of genotype E[44].

HBV genotyping is important for several reasons. Firstly, it supplies information on the molecular epidemiology of the virus worldwide including phylogenetic and phylogeographic histories. Also, it provides a clue on the correlation between the course of disease and viral strain[45]. Lastly, it gives insight into the impact of human migration on viral evolution[46].

The genotype result from this study, is in alignment with HBV epidemiology studies which reported the most predominant HBV genotype in Nigeria to be the genotype E[44]. The study reported the circulation of genotype E (44.8%) within the study group, another study that reportedly identified five HBV genotypes within their study population also reported HBV genotype E as being the most predominant (97.1%)[47,48]. Individuals infected with genotype E have been reported to have high viral loads with high frequency of HBeAg-positivity and transmit HBV perinatally[49]. This report aligns with the present study result as the HBV DNA positive in this study has an extremely high viral load (1,650,000 IU/mL) with a HBeAg-positivity. This increases the risk of perinatal transmission of HBV from the HBV DNA-positive individual to her unborn child if no preventive measures are taken. This underscores the importance of maternal HBV screening, vaccination during pregnancy, and timely birth-dose vaccination for infants to prevent mother-to-child transmission.

The genome sequence of HBV sample was then subjected to drug resistance mutations and immune escape mutation or polymorphism check. Drug resistance mutations are a major public health concern because they can make infections difficult or impossible to treat which can lead to severe illness, disability and death. They also make it easier for infections to spread, which can increase the risk of serious infectious diseases. Drug resistance analysis focused on identifying mutations in the SHB protein, particularly within the reverse transcriptase (RT) region, known for harboring

mutations that confer resistance to antiviral treatments. The mutations reduce the binding affinity of a drug to its target or render the target constitutively active. The sample maintained susceptibility to current antiviral drugs, which means the hepatitis B infection can be treated with the major antiviral drugs to prevent the spread of infection. Immune escape is a phenomenon when the host immune system is incapable of responding against an infectious agent. Immune escape mutations and polymorphism within the small hepatitis B surface antigen (SHB) protein were examined. These mutations are relevant because they can alter the recognition of hepatitis B surface antigen (HBsAg) by the host immune system, potentially impacting vaccine effectiveness and immune response. The sample also showed low-frequency mutations and polymorphisms within SHB protein, particularly at amino acid positions 100 and 109 which were not considered to have a significant effect on immune escape for genotype E within the study population.

Overall, this study has been able to investigate the prevalence rate of HBV and HEV infections among pregnant women attending antenatal consultations in selected hospitals in Ibadan, Oyo State, Nigeria.

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Chapter Five

Conclusion

5.1 Summary of Findings

This study investigated prevalence of Hepatitis B and E viruses, the rate of HBV and HEV IgG co-positivity, associated risk factors for infection, the impact of these infections on liver function and partial genome sequencing of HBV in pregnant women attending antenatal clinics in Ibadan, Oyo State, Nigeria.

5.2 Conclusion

This study demonstrates high prevalence of HBV infection among the pregnant women which could be transmitted from mother to the offspring especially at birth. A significant number of pregnant women in the study population had been previously exposed to HEV; this demonstrates the endemicity of HEV infection in Ibadan, Oyo State and the need for personal hygiene. Also, a limited number of pregnant women with increased infectivity with HBV had high viral load, which may lead to increased maternal and childbirth morbidity and mortality.

A few pregnant women with HBV infection in this study simultaneously had previous exposure to HEV. There was significantly high prevalence of HBV among pregnant women with elevated amino transferase (ALT) level compared to those with normal or borderline ALT levels indicating liver injury or damage in pregnant women. The partial genome sequence of the HBV sample analyzed was confirmed as genotype E, exhibiting high genetic similarity to known genotype E isolates common to the HBV strain specific to Nigeria and West African countries and maintaining susceptibility to current antiviral drugs.

Low-frequency mutations and polymorphisms observed within SHB protein, particularly at amino acid positions 100 and 109 were not considered to have a significant effect on immune escape for genotype E within the study population.

5.3 Recommendations

There is need to increase access to antiviral therapy, especially for pregnant women infected with HBV to prevent mother-to-child transmission. Even though hepatitis B screening has been incorporated into the routine antenatal screening, those tested HBsAg positive should be tested for ALT to rule out liver injury or damage. Government and non-governmental organizations should intensify efforts to enlighten the general population on the public health importance of the diseases.

There is need to increase access to vaccination against the spread of HBV and intensify personal hygiene enlightenment campaigns on HEV among pregnant women. There is need for development of future HEV vaccination programs to precisely target the high risk populations who are prone to having worst outcome. Since HEV is self-limiting and short-lived, HEV IgM was not detected; therefore, further surveys with a larger sample size among pregnant women in Nigeria is necessary to determine the burden of HEV IgM and to validate the findings of this study. Continuous surveillance is necessary to monitor the potential HBV drug resistance emergence.

5.4 Contribution to Knowledge

This study has provided information on the endemicity of HBV and HEV among pregnant women in Ibadan, Oyo State, Nigeria. The study has provided valuable data for increasing the overall understanding of the devastating diseases caused by HBV infections.

This study showed that HBV infection can affect the liver functioning in pregnant women. Concerted efforts must be put in place if the global target of elimination on HBV by year 2030 will

be achieved in Nigeria. The findings revealed that HBV genotype E remains manageable with existing therapies to prevent vertical transmission.

5.5 Suggested Areas for Further Research

In terms of laboratory testing, screening for HBV DNA by real time PCR (qPCR) could not be performed in all HBsAg negative study samples due to financial constraints. Thus, the prevalence of occult HBV infection within the entire study population could not be established.

Furthermore, there was lack of follow-up for the women after delivery to establish the risk of mother-to-child transmission. It is therefore suggested that such be incorporated into future studies.

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Appendix I

TELEGRAMS..... TELEPHONE.....

MINISTRY OF HEALTH

DEPARTMENT OF PLANNING, RESEARCH & STATISTICS DIVISION

PRIVATE MAIL BAG NO. 5027, OYO STATE OF NIGERIA

Your Ref. No. .....

All communications should be addressed to the Honorable Commissioner quoting

Our Ref. No. AD 13/479/782

Date 29th FEBRUARY, 2024

NAME OF PRINCIPAL INVESTIGATOR: AKINDELE IBUKUN

TITLE OF STUDY: HBV-HEV CO-INFECTIONS AND ASSOCIATED RISK FACTORS AMONG PREGNANT WOMEN ATTENDING ANTENATAL CONSULTATIONS IN IBADAN, OYO STATE, NIGERIA.

RESEARCH INSTITUTION: LEAD CITY UNIVERSITY, IBADAN.

NREC ASSIGNED NUMBER: NHREC/OYOSHRIEC/10/11/22

DATE OF RECEIPT OF VALID APPLICATION: 21/02/2024

RE: NOTIFICATION OF EXECUTIVE APPROVAL OF PROTOCOL

This is to notify you that the Oyo State Ministry of Health Research Ethics Committee (HREC) has concluded to give executive approval to your research proposal after necessary reviews and corrections under the regulations guiding experiment in human subjects.

2. This approval is for a period of (1) one year from 29th February, 2024 to 29th January, 2025. If there is hindrance in starting this research, please inform the Oyo State HREC so that dates of approval can be adjusted accordingly. Note that no activity related to this research may be conducted outside these dates. No changes are permitted in the research without prior approval by Oyo State HREC.

3. All forms and questionnaires used in this study must carry the HREC assigned number and the duration of HREC approval. You are to note further that the National Code of Health Research Ethics requires you to comply with all Institutional guidelines, rules and regulation of the codes. Please ensure that any adverse effect from your study is quickly reported to the HREC Oyo State Ministry of Health, Ibadan.

4. You are expected to submit a **report** to this committee every three (3) months from the date of this approval. The Oyo State HREC reserves the right to conduct compliance visit on your research sites without previous notification.

5. I thank you.

RESEARCH ETHICAL REVI... COMM... Signature & Date ... MINISTRY OF HEALTH, OYO

Dr. Abbas Gbolahan
Director, Planning, Research & Statistics
Secretary, Oyo State Research Ethics Review Committee

Appendix II

Questionnaire on Psychosocial Factors Associated with the Seroprevalence of HBV and HEV

SECTION A: RESPONDENT'S INFORMATION

Instruction: Tick (√) as relevant and answer appropriately.

1. Serial Number: _____

2. Age:

   - ≤ 20 years [ ]
   - 21-25 years [ ]
   - 26-30 years [ ]
   - 31-35 years [ ]
   - ≥ 36 years [ ]

3. Occupation: _____

4. Level of Education:

   - No Formal Education [ ]
   - Primary [ ]
   - Secondary [ ]
   - Tertiary [ ]

5. Trimester:

   - 1st [ ]
   - 2nd [ ]
   - 3rd [ ]

6. Drinking Water Source:

- Spring water [ ]
- Well water [ ]
- Tap water [ ]
- Other (specify): _____

7. Type of Toilet:

- Latrine [ ]
- Water Closet [ ]
- Other (specify): _____

SECTION B: KNOWLEDGE AND HISTORY

Instruction: Tick (√) Yes or No

1. Have you heard of Hepatitis before? Yes [ ] No [ ]
2. Do you have any history of Hepatitis in the past? Yes [ ] No [ ]
3. Have you received Hepatitis vaccine before? Yes [ ] No [ ]
4. Have you received blood transfusion before? Yes [ ] No [ ]
5. Have you undergone surgery before? Yes [ ] No [ ]
6. Do you rear pigs or come into contact with pigs regularly? Yes [ ] No [ ]
7. Have you experienced jaundice (yellowing of eyes and skin)? Yes [ ] No [ ]

Appendix III

Informed Consent

My Name is AKINDELE Ibukun Akinwumi, I am a student of the Department of Biological Science, Lead City University, Ibadan. I am conducting a Study titled: "Hepatitis B and E viruses co-infection and associated risk factors among pregnant women attending antenatal consultations in Ibadan, Oyo State, Nigeria" as part of requirements for award of Doctor of Philosophy (Ph.D) degree in Medical Virology at Lead City University, Ibadan. This study will involve collection of human blood samples from each pregnant woman attending antenatal consultations in selected hospitals in Ibadan and the co-infection rate will be determined.

Hepatitis B and E viruses cause a very devastating disease in immunosuppressed individual such as pregnant women. In patients with chronic HBV infection, co-infection with HEV is a common cause of liver failure. HBV and HEV are transmitted vertically from infected mother to infant thereby resulting in both maternal and fetal complications. The risk for a pregnant woman who is positive for hepatitis B virus gotten through perinatal, sexual or blood contact, to be infected with hepatitis E virus is high. This can be as a result of consumption of contaminated water or lack of proper hygiene leading to the co-infection of the two viruses in a pregnant woman which is more overwhelming to the immune system. Hence, the need to test for HBV and HEV co-infection in pregnant women.

Please note that your answers to the questionnaire will be made confidential. In filling this questionnaire, your name will not be needed, you will only be given a number. Your genuine information along with other people's information given will be used to determine the associated risk factors of Hepatitis B and E viruses and the result of this study will sensitize the health care system on the burden or spread of HBV-HEV co-infection among pregnant women attending antenatal consultations in Ibadan, Oyo State, Nigeria, for early diagnosis and proper management to prevent both maternal and fetal death.

About 5ml of blood will be taken from you for the aforementioned purpose. The process of taking the sample will not cause you any harm or injury but slight pain. You are free to refuse to take part in this study, you also have a right to withdraw at any given time if you choose to during the course of this study without any consequences whatsoever. I, therefore, humbly request you to ask any question that you may have concerning the study.

Thank you.

Akindele Ibukun,

Department of Biological Sciences,

Lead City University, Ibadan.

08032182307.

Consent: Now that the study has been well explained to me and I fully understand the content of the process, I will be willing to take part in the program.

…..……..

First and second name of Participant

…… ……

Signature/Thumbprint of Participant **Date**

Bio-data

A. Personal Data

Name:	Ibukun Akinwumi AKINDELE
Matric Number:	LCU/PG/001705
Nationality:	Nigerian
State of Origin:	Oyo State
Local Government Area:	Ibadan North-East Local Government
Date of Birth:	25th June, 1982
Religion:	Christianity
Email Address:	ibkakindele02@gmail.com
Telephone Number:	08032182307
Next of Kin/ Phone no:	Akindele Funmike Adesola/08038304423
Address:	88, Adeyemo Road, Okebadan, Ibadan, Oyo State, Nigeria.

B. Educational Background

Master degree in Medical Microbiology and Parasitology (2014-2018)
Ladoke Akintola University of Technology, Ogbomoso, Oyo State.

Bachelor of Medical Laboratory Science degree (BMLS) (2004-2010)
Ladoke Akintola University of Technology, Ogbomoso, Oyo State.

Loyola College, Ibadan, Oyo State. (1994-2000)

C. Work Experience

- Lecturer II, Medical Lab Science Department,
  Ajayi Crowther University, Oyo (June 2021 – Present)
- Medical Lab. Scientist: Kindness Medical Diagnostic Centre,
  Ibadan, Oyo State (Nov.2019 – April 2021)
- Assistant Lecturer, Medical Lab Department,
  Lead City University, Ibadan (Nov.2017 – Oct.2019)
- Adjunct Lecturer, Medical Lab Department,
  Lead City University, Ibadan (April 2015– Nov.2017)
- Technologist II, Dept. of Microbiology,
  Lead City University, Ibadan (Jan.2014 – April.2015)
- Medical Lab. Scientist: Molete Diagnostic Centre,
  Ibadan, Oyo State (Dec.2012 – Jan.2014)
- Medical Lab. Scientist: Emma Medical Laboratory ,
  Oyo, Oyo State (June 2012 – Dec.2012)
- National Youth Service Corps: State Hospital,
  Asubiaro, Osogbo, Osun State (July 2011 – June 2012)
- Internship: Oyo State Hospitals Management Board,
  Adeoyo, Ring Road, Ibadan (June2010 -May2011)

D. Professional Qualifications with Dates

- Associate in Medical Laboratory Science (AIMLS) (2013)

E. Publications

- Adefioye, O.J., Olaleye, E.D., Feruke-Bello, Y.M., Fasogbon, R.O. **Akinwumi, I.A**., Olulowo, O.R. Genotyping of ESBL-Producing E. coli from Food-producing Animals, Animal Food Products and Humans in South-West, Nigeria. Ajayi Crowther J. Pure Appl. Sci. 2024, 2(3), pp. 1-12. | doi: https://doi.org/10.56534/acjpas.2024.03.01.01

- Akinjinmi A. A. Amballi A.A. Abdulrahman Abdulbasit Opeyemi Aderinto Nicholas Adeniyi Akinbobola Ayokunle, **Akindele I. A.** AbdulBasit Opeyemi Muili. Relationship between Serum Leptin, Lipid Metabolism, HbA1c, and Renal Function in Individuals with Type 2 Diabetes Mellitus and Obesity and in Individuals with Type 2 Diabetes Mellitus without Obesity.medRxiv (2023). Preprint doi: https://doi.org/10.1101/2023.02.20.23286131

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- Oloyede S. Bolaji, Olutoyin C. Adekunle, Akindele A. Ajayi, Abolaji T. Adeyemo , Abiodun R. Ojewuyi, Abdulwahab A. Ibrahim, Adeyemi T. Adeyemo, Adeola O. Ajayi, **Akinwumi I. Akindele**, Oluwaseyi A. Adeyeba. Prevalence of Pathogenic Intestinal Parasites and Enteropathogenic Bacteria in Faecal Samples Obtained from Abattoirs in Ogbomoso, Oyo State, Nigeria. AJVS. (2023) Vol. 77(2): 17-28.

- Oloyede S. Bolaji, Olutoyin C. Adekunle, Akindele A. Ajayi, Abolaji T. Adeyemo, Margaret A. Adekanle, Oluwatoyin J. Idris, Nimatullah A. Adeoye, **Akinwumi I. Akindele**. Prevalence of Gastrointestinal Helminths In Cattle Reared In Owo, Ondo State, Nigeria. (2023) AJVS. Vol. 78(1): 28-35.

- Bolaji O.S, Ajayi A, Adekunle O.C, Adebayo T.O, Adeyemo A.T, Adekanle M, Idris O, Adeyemo A.T, Ajayi A.O, Adejumobi O.A, **Akindele I A**, Arogunyo M.K. Prevalence of Trichomoniasis, Syphilis and Hepatitis B Virus among Pregnant Women in Ejigbo, Osun State. FJLS. (2023) Vol. 3(1).

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F. Major Conferences/Workshops Attended

- Second International Conference of the Faculty of Natural Sciences, Ajayi Crowther University, Oyo, Oyo State (2022). Theme: "Achieving Sustainable Development Through Scientific Intervention"

- Advanced Digital Appreciation Programme for Tertiary Institutions, Statistical Package for Social Sciences at Ajayi Crowther University, Oyo, Oyo State (2022)

- Association of Medical Laboratory Science Council of Nigeria Conferences Faculty of Basic Natural Sciences international conference, Lead City University Ethical conduct among higher institutions Lead City University workshop

G. Referees

- Prof. M. O. **Akiibinu**
  Dept. of Medical Laboratory Science, College of Health Sciences
  Ajayi Crowther University, Oyo
  08038613172

- Prof. Rosemary Audu
  Dept. of Microbiology,
  Nigeria Institute of Medical Research, Lagos
  08035017790

- Prof. O.O. **Opaleye**
  Dept. of Microbiology, College of Health Sciences
  Lautech, Ogbomoso
  08160471660

Signature **Date**

The University Compliance Certification

This is to certify that the thesis written by **Ibukun Akinwumi AKINDELE** with Matric no **LCU/PG/001705** in the department of **Medical Virology**, Faculty of Natural and Applied Sciences, Lead City University, is in full compliance with the approved university format and style.

Signature **Date**

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