CHAPTER ONE

INTRODUCTION

1.1 Background

Viral hepatitis is a systemic disease primarily involving the liver. It is caused by five distinct hepatitis viruses (A, B, C, D, and E) and is a highly widespread public health problem in the African Region (WHO, 2016). All the five hepatitis viruses can cause severe disease, but the highest numbers of deaths result from liver cancer and cirrhosis – a condition in which there is irreversible scarring of the liver following chronic hepatitis B and C infection (WHO, 2016). Most cases of acute viral hepatitis in children and adults are caused by one of the following agents: hepatitis A virus (HAV), the etiologic agent of viral hepatitis type A (infectious hepatitis); hepatitis B virus (HBV), which is associated with viral hepatitis B (serum hepatitis), hepatitis C virus (HCV), the agent of hepatitis C (common cause of post transfusion hepatitis); hepatitis D (Delta Virus) and hepatitis E virus (HEV), the agent of enterically transmitted hepatitis.

Hepatitis B virus (HBV) and hepatitis C virus (HCV) have several important similarities including worldwide distribution, hepato-tropism, similar modes of transmission and the ability to induce chronic infection that may lead to liver cirrhosis and hepatocellular carcinoma (Desikan and Khan, 2017; D'Almeida *et al*., 2017). Hepatitis B and C virus have similar routes of transmission which are through blood transfusion, sharing of needles to inject drugs and sexual activity, co-infection with these viruses is a common (Chung, 2006). Since both viruses are individually known to cause the pathologies mentioned above, co-infection with both HBV and HCV would be expected to be linked with higher morbidity as well as mortality and impact health-care resource utilization (Desikan and Khan, 2017; Marcelin *et al*., 2017). HBV and HCV

co-infections in HIV positive individuals are of utmost importance due to the underlying consequences such as the hepatological problems associated with these viruses, which have been shown to decrease the life expectancy in the HIV-infected patients (Chung, 2006). The burden of these co-infections in sub-Saharan Africa is still unclear (Umutesi *et al*., 2017).

Human Immunodeficiency Virus (HIV), Hepatitis B and C viruses (HBV and HCV) are the three most common chronic viral infections documented worldwide (Soriano *et al*., 2006). Viral hepatitis is a recognized major public health problem worldwide, but more prevalent in developing countries. Hepatitis B virus (HBV) is a DNA virus of the family *Hepadnaviridae* and the causative agent of hepatitis B infection (Pungpapong *et al*., 2007). It is 50-100 times more infectious than HIV and 10 times more infectious than hepatitis C virus (HCV). Many carriers do not realize that they are infected with the virus, thus it is referred to as a "silent killer" (Samuel *et al*., 2004). The minimum infectious dose is so low that such practices like sharing a tooth brush or a razor blade can transmit infection (Chang, 2008). HBV and HCV also shares similar routes of transmission with HIV (Willey *et al*., 2008). Approximately 350 million people are infected with HBV worldwide (Eke *et al*., 2011; Liu and Hou, 2006) with Nigeria classified among the group of countries endemic for HBV infection. Hepatitis C virus is an RNA virus of the *Flaviviridae* family and appears to have humans and chimpanzees as the only species susceptible to its infection (Polyak, 2006). About 170 million people are infected with HCV worldwide (Liu and Hou, 2006). Apart from being detected in blood, it has also been detected in semen (Cavallero *et al*., 2008) and saliva (Chen *et al*., 2009). HBV and HCV account for a substantial portion of liver diseases worldwide and infected individuals can remain asymptomatic for decades. However, more than 80% of them become chronic carriers which result in an increased risk of liver cirrhosis, liver cancer and liver failure 20 - 30 years later (Volf

et al., 2008). The global distribution of hepatitis B infection varies greatly. WHO, 2016 estimates that there are 350 million people with chronic HBV infection and 170 million people reside in Africa with chronic HCV infection. HBV is estimated to result in 563,000 deaths and HCV result in 366,000 deaths annually (Abel and Solomon, 2012). The prevalence of HBV carriers varies substantially between regions, from 7% to 35% (Emechebe *et al*., 2009). The wide range is largely related to differences in age at the time of infection, a factor that is inversely related to the risk of chronic infection. In areas where the prevalence is high, such as Africa, Southeast Asia and China more than half the population is infected at some time in their lives, and more than eight percent are chronic carriers of HBV, the result of either neonatal transmission (vertical) or transmission from one child to another (horizontal) (UNAIDS 2012). Areas with low levels of endemicity include North America, Western Europe, and Australia, where only a minority of people comes into contact with the virus, as a result of horizontal transmission among young adults (UNAIDS 2012).

The prevalence of serological markers (HBsAg) of exposure to HBV in sub-Saharan Africa is high, up to 90% in many areas (WHO, 2016). Unfortunately, most people with chronic viral hepatitis are not aware of their status and do not receive appropriate treatment. Viral hepatitis B and C are blood-borne infections, with significant transmission occurring in early life and through unsafe injections and medical procedures, and less commonly through sexual contact. Mother- to-child transmission of hepatitis B virus is a major mode of transmission in high prevalence settings. Hepatitis B affects an estimated 100 million people in the African Region (mainly in West and Central Africa) and an estimated 19 million adults in the African Region are chronically infected with hepatitis C (WHO, 2016).

Globally, of the 370 million chronic infections, 2–4 million have HIV, which modifies the natural history of HBV infection (Puoti *et al*., 2002). The prevalence of HIV/HBV, HIV/HCV and HIV/HBV/HCV in Nigeria as observed in a North Central population study was 20.6%, 11.1% and 7.2%, respectively (Forbi *et al*., 2007). However, more studies are needed to give a better picture of these co-infected patients in the country. Epidemiological survey showed that about 5% of the world populations are asymptomatic carriers (Volf *et al*., 2008). Chronic HBV or HCV infection is the major cause of mortality among children and adults who might be HIV seronegative. Nigeria is an endemic area of viral hepatitis, and studies carried out by various researchers had shown that HBV and HCV infections are highly prevalent among Nigerians. Studies across Nigeria have shown varying prevalence of HBV/HIV co-infection from 9.2 % to as high as 70.5 % while that of HIV/HCV co-infection ranged between 0.5 % and 14.7 % (Balogun *et al*., 2012; Denue *et al*., 2012; Lesi *et al*., 2007 and Nwokedi *et al*., 2006). Alao *et al*. (2009) analyzed the results of HBsAg screening among blood donors in General Hospital, Otukpo an urban area of Benue State, Nigeria, over a three-year period (2006 – 2008), with a view to establishing the prevalence rate in this region of the Middle Belt of Nigeria. Two thousand and five hundred (2,500) samples were screened for HBsAg over the three-year period. The seropositivity rate among donors tested was found to be 20%. According to a recent study by Okonko *et al*. (2010), HBV prevalence of 67% was found among hepatocellular carcinoma patients in North Eastern Nigeria. The prevalence of HCV was reported by Muktar *et al*. (2005) as 5.2% in Zaria, North-Western Nigeria. In a research conducted by Okonko *et al*. (2010) it showed that the prevalence of hepatitis B virus (HBV) infection among patients of Abeokuta, South-Western Nigeria was 4.0%, while Alikor and Erhabor (2007) reported 12.4% in children attending tertiary health institution in Niger Delta, South-South of Nigeria. Pennap *et al*. (2010)

also reported that the prevalence of HbsAg and Anti- HCV in Keffi, Nassarawa State of North Central, Nigeria were 13.3% and 13.3% respectively. In view of the advantage of early detection and therapy, this study is designed to determine the seroprevalence of HBV and HCV and apparently their co-infections among HIV patients.

1.2 Statement of the Problem

Many people having the HBV and HCV are asymptomatic, and therefore not identified. These people serve as the source of the transmission of the virus and might develop chronic hepatic inflammation, which can slowly progress to severe liver diseases such as cirrhosis and hepatocellular carcinomas. HBV and HCV chronically infected HIV patients are at increased lifetime risk for cirrhosis and hepatocellular carcinoma.

Despite the negative impact of HBV and HCV co-infections on mortality and morbidity rates among HIV-infected individuals, detailed information on the prevalence of HCV/HIV and HBV/HIV co-infection in Nigeria is scanty. Although vaccination against HBV is becoming available, integration of treatment and diagnosis in HIV programs has not been achieved. This lack of prevention strategies is partially due to high costs associated with these interventions. Information generated by this study will help inform policy makers of a baseline for HIV patients that are co-infected with HBV or HCV. With appropriate consideration, this could be used to increase attention for integration of HBV and HCV interventions into HIV prevention and control programs. In Nigeria, centres where one can assess his or her hepatitis status are many, but for quantitative determinations, they are rare and expensive. Samples have previously been taken outside the country to foreign laboratories. This costly enterprise keeps the test out of reach of the average citizen. However, the HBV DNA and HCV RNA test is essential to hepatitis

B and C virus management. Therefore this study was designed to determine the prevalence of HBV and HCV in HIV patients on HAART.

1.3 Justification for the Study

Infections due to Hepatitis B and C virus are of public health concern around the world. Viral hepatitis, especially hepatitis B and C, form a considerable percentage of liver diseases worldwide and infectious individuals remain asymptomatic for several years (Pennap *et al*., 2010). However, more than 80% of them become chronic carriers, which results, in an increased risk of liver cirrhosis, liver cancer and liver failure 20- 30 year later (Pennap *et al*., 2010; Volf *et al*., 2008). In view of the advantage of early detection and therapy, it is essential to determine the prevalence of HBV and HCV among individuals with a view to providing baseline data for further research, public health policy formulation and awareness campaign for the need to know one's hepatitis status especially in hepatitis B and C viruses' endemic areas. This study would be useful to health managers and planners to develop appropriate preventive services, allocate resources, decide on priorities, and target certain populations and assessment of related risk factors.

1.4 Aim of the Study

The aim of this study was to determine the epidemiology of Hepatitis B and C virus infections amongst HIV patients on Highly Active Antiretroviral Therapy in Southeastern Nigeria.

1.5 Objectives are:

1 To determine prevalence of Hepatitis B and C co-infections amongst HIV patients on Highly Active Antiretroviral Therapy (HAART).

2 To determine seromarkers of Hepatitis B, C and D virus in HIV patients on Highly Active Antiretroviral Therapy (HAART).

3 To determine the effects of Co- Infection of HIV and Hepatotropic Viruses on Selected Biochemical, immunological and Hematological Profiles of Patients.

4 To measure Hepatitis B and C viral loads in co-infections.

5 To determine Hepatitis B and C genotypes prevalent in positive patients.

6 To determine genetic relatedness of Hepatitis B and C genotypes (homology with consensus sequence).

CHAPTER TWO

LITERATURE REVIEW

2.1 Virology/Structure of Hepatitis B Virus

The HBV virion (virus particle) is ~42 nm in diameter and is one of the smallest enveloped viruses infecting animals. The icosahederal nucleocapsid, consisting of HBcAg, contains both the viral DNA and DNA polymerase. This capsid is enclosed in an outer lipid membrane (envelope), which is derived from the host hepatocyte and into which all three surface antigens (small, medium and large) are embedded, to form the infectious, or Dane, particle, in addition to this infectious virion, non-filamentous and spherical sub-viral particles (22 nm in diameter) lacking a protein core and viral nucleic acid, also occur.

Hepatitis B virus is a double-stranded DNA virus and is a member of the *Hepadnaviridae* family. Electron microscope of HBV positive serum reveals three morphologically distinct forms of the particle. The small, 22 nm spherical particles and the tubular forms of roughly the same diameter are composed of the virus-surface protein embedded in lipid and are synthesized in vast excess over the 42 nm, double-shelled virions (Zuckerman *et al*., 2009). The virus consists of a core capsid, which contains viral DNA and this is surrounded by an envelope containing surface antigen (HBsAg). Both whole, intact virions and incomplete virus particles, consisting entirely of HBsAg, are produced during replication of HBV. The HBsAg particles vary greatly in morphology and are found in high concentrations in early acute infection and continue to be produced in chronic disease. Similarly, Kudesia and Wrighitt (2009), described Hepatitis B virus (HBV) as a member of the *Hepadnaviridae* family of viruses, and has a double-stranded circular DNA and a DNA polymerase enzyme. It has two major proteins: hepatitis B surface antigen (HBsAg), which is an outer protein expressed in excess when the virus replicates in the liver; and hepatitis B core antigen, an inner protein, which is expressed only within hepatocytes in the liver.

A third protein, hepatitis B e antigen (HBeAg), is also shed in the blood when the virus replicates, and its presence is associated with high infectivity (Kudesia and Wrighitt, 2009).

2.1.1 Genome of Hepatitis B Virus

The HBV genome is composed of circular, partially double-stranded DNA that is enclosed within a nucleocapsid (or core antigen). This genome is surrounded by a spherical envelope or surface antigen. The genome encodes both core and surface proteins and a DNA polymerase, which also acts as a reverse transcriptase. The variable expression of core and surface antigens and antibodies to these proteins, serve as useful markers of past, current or chronic infection. However, the genomes of a variety of isolates of HBV have been cloned, and the complete nucleotide sequences determined (Zuckerman *et al*., 2009). There is some variation in sequence (up to 12% of nucleotides) between these isolates, and up to eight genotypes (A to H) have been described on the basis of >8% nucleotide sequence divergence, and recombinant forms have also been recognized. However, the genetic organization and other essential features are conserved. The genome is around 3200 bp in length and analysis of the protein-coding potential reveals four conserved open reading frames (ORFs). These four ORFs are located on the same DNA strand, and the strands of the genome have accordingly been called ‘plus’ (incomplete strand) and ‘minus’ (complete strand), Other features include a motif of 11 bp, which is directly repeated near to the 5’end of each strand of genomic DNA (DR1 and DR2) and plays an essential part in the replication strategy, two transcriptional enhancer (Zuckerman *et al*., 2009).

2.1.2 Life Cycle and Replication of Hepatitis B Virus

HBV replication is a complex process as shown in figure 2.1, which involves several stages: binding of the virion to the host hepatocyte, transport of the virus within the cell, conversion of relaxed circular DNA (rcDNA) to covalently closed circular DNA (cccDNA),

transcription, expression of core and polymerase proteins, encapsidation, reverse transcription, synthesis of positive strand DNA, circularization, progeny capsid trafficking and formation of a closed circular DNA pool (Jilbert *et al*., 2002). The specific cellular receptor or receptors, which the virus uses to gain entry to the hepatocyte, have not yet been identified. Evidence suggests that the HBV envelope attaches irreversibly to a receptor, or receptors, and is taken into the cell via endocytosis (Urban *et al*., 2010; Jilbert *et al*., 2002).

After entering the cell, the envelope is removed and the viral core is transported along micro-tubules, passes through the nucleopore, and enters the nucleus, where the rcDNA is converted to cccDNA by host enzymes (Urban *et al*., 2010; Jilbert *et al*., 2002). During this process, the positive viral DNA strand is completed and the 5' covalently-linked viral polymerase is removed. The negative strand of the cccDNA in the nucleus serves as the template for the transcription of viral RNA by host RNA polymerase II. The mRNA synthesized for the LHBs, MHBs, SHBs, and X proteins is subgenomic in length, whereas that synthesized for the HBeAg, core and polymerase proteins, are longer than the genome (Jilbert *et al*., 2002). Both the 5' and 3' ends of the pregenomic RNA contain an encapsidation signal (Ɛ), which takes the form of a stem-loop structure with a bulge. The binding of the polymerase protein to the 5' copy of this bulge initiates encapsidation of the RNA pregenome and reverse transcription (Urban *et al*., 2010). Negative strand DNA synthesis, by reverse transcription is then initiated by the polymerase protein.

Nucleocapsids containing double-stranded DNA and the polymerase protein either return to the nucleus or are secreted from the cell as infectious virions (Urban *et al*., 2010; Jilbert *et al*., 2002). Those which are secreted from the cell first move into the lumen of the endoplasmic

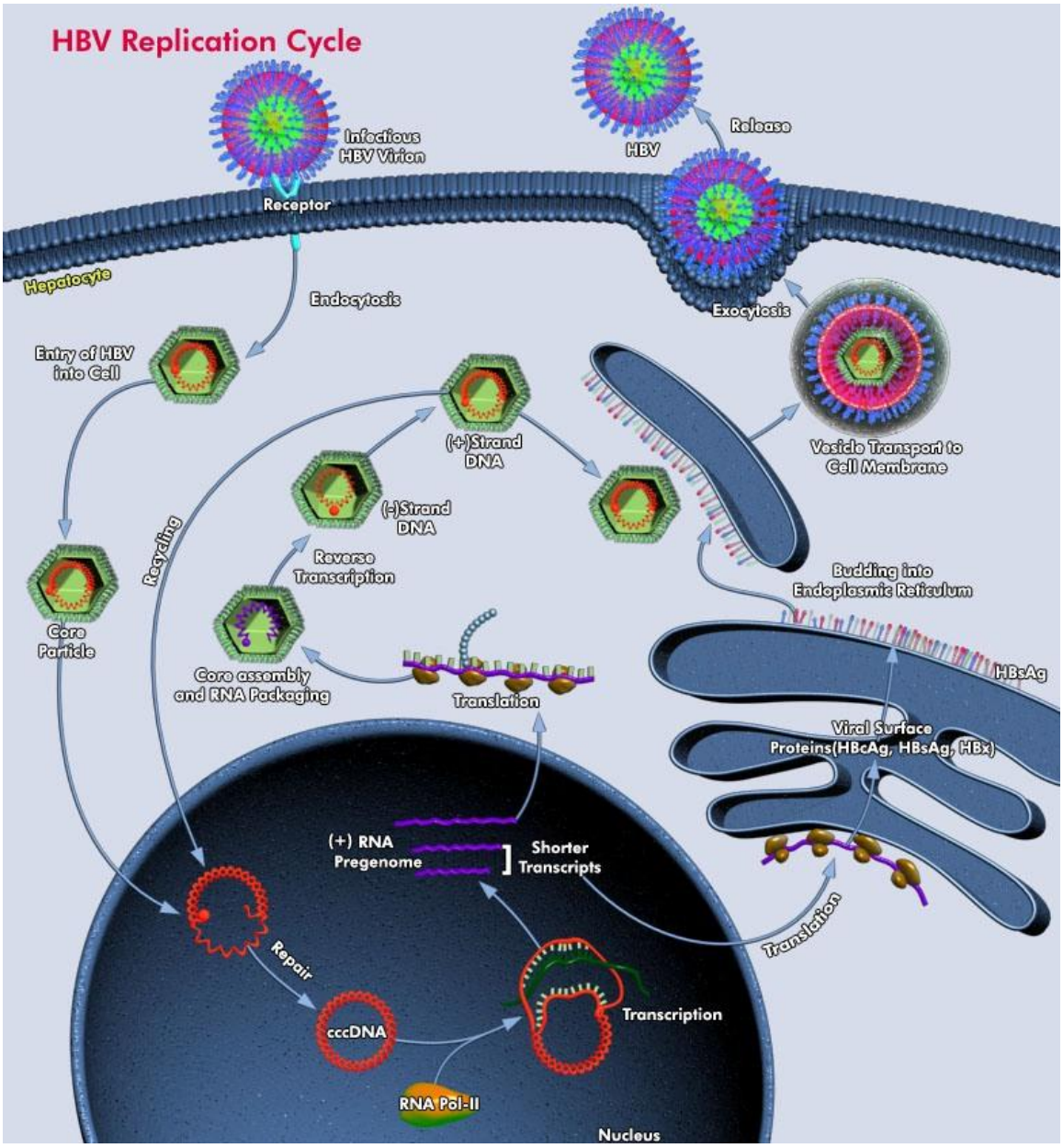

Figure 2.1: Replication of HBV

Source: (Urban *et al*., 2010)

reticulum, where they are enveloped and then move through the Golgi body into a secretory vesicle, which is released from the hepatocyte (Ganem and Prince, 2004). Both infectious virions and non-infectious sub-viral particles may be released by the hepatocyte (Urban *et al*., 2010).

2.1.3 Epidemiology of HBV Infection

Hepatitis B is the leading cause of chronic liver disease worldwide including hepatitis, cirrhosis and hepatocellular carcinoma (Okechukwu *et al*., 2014). After acute infection acquired in adulthood, 90- 95% of the infected individuals develop a broad, multi-specific cellular immune response that eliminates the virus and lead to the development of protective antibodies to HBsAg (Hoffman and Thio, 2007). Approximately, 400million persons worldwide are reported to have chronic hepatitis B which is defined as the presence of HBsAg for a minimum of 6 months, and between 500,000 and 1 million persons die annually of HBV related disease (Hoffman and Thio, 2007); translating to approximately 2 people each minute (Hepatitis B Foundation, 2014). In the United State of America, 12 million Americans have been infected (1 out of 20 people) with more than one million people chronically infected. Estimates of 40,000 new people are to become infected each year while 5,000 will die each year from hepatitis B and its complications with 1 health care worker each day (HBF, 2014).

Sub-saharan Africa, the Pacific, the Amazon and southern part of Eastern Europe are areas of high endemicity with the prevalence rate of above 7% (Emechebe *et al*., 2009). Low prevalence areas (0.2 - 0.5%) are the Northern, Western and Central Europe as well as Northern America and Australia, while middle endemicity areas such as the eastern Europe, the Mediterranean, Russia, South West Asia, Central and South America have a prevalence of between 2% and 7% (WHO, 2002). Screening for HBV in Nigeria is neither a standard practice in government or private medical health institutions.

However, the overall prevalence ranges from 1.3% (Okonko *et al*., 2012) among blood donors in Ibadan; South West Nigeria to 44.7% (Bukbuk *et al*., 2005) recorded among primary School Pupil in Hawal valley, Borno State, in north eastern Nigeria. Highest HBV rates appears to be more distributed in the North Eastern states, followed by the South Western States but lowest rate was concentrated in the South Eastern States of Nigeria. When these literatures were considered according to the population groups encountered, the least prevalence was found in studies carried out among pregnant women, followed by researches in blood donors while very high HBV rates were surprisingly recorded in community based studies, (Gambo *et al*., 2012; Bukbuk *et al*., 2005). But high HBV prevalence were encountered in literatures among high risk groups like prison inmates (Adoga *et al*., 2009), Hospital patients and Health care workers (Amuta *et al*., 2012).

2.1.4 Mode of Transmission of Hepatitis B Virus

The predominant routes of transmission vary according to the endemicity of the HBV infection. In areas of high endemicity, perinatal transmission is the main route of transmission, whereas in areas of low endemicity, sexual contact among high-risk adults is predominant (Rosini *et al*., 2003). Hepatitis B is transmitted more efficiently than HIV through sexual, vertical and percutaneous routes (Thio, 2004). HBV is reported to be 100 times more infectious than HIV. A quantity of blood as small as 0.05 ml or of plasma 0.0001 ml can transmit HBV infection (Banker, 2003). Transmission of HBV and HIV has been observed in numerous forms of human contact: perinatal (Mother to Child), sexual, needle sharing, and occupational/health care related (Shepard *et al*., 2006). HBV seroprevalence have marked geographic variation, and the degree of HBV endemicity often correlates with the predominant mode of transmission. In

highly endemic settings, perinatal and horizontal (exposure to chronically infected household members) route are reported to be responsible for disease transmission (Shepard *et al*., 2006).

Hence, transmission of HBV and HIV in a highly endemic country is multifactorial ranging from unavoidable to avoidable ones like blood transfusion. Screening blood for transfusion in developed societies and even some less developed could involve detection of HBV markers (HBsAg, HBcAb, HBV-DNA), HIV and HCV (Al-Waleedi and Khader, 2012; Rosini *et al*., 2003); however, most intending blood donors in Nigeria are only screened of HBsAg and HIV (Ado *et al*., 2010: Olukayode *et al*., 2009) and rarely HBsAg, HIV and HCV (Egah *et al*., 2005). There is paucity of information on screening of intending blood donors for HBcAb and HBV-DNA. This could constitute yet another factor in the transmission of HBV through occult hepatitis B infected individuals in Nigeria. Occult hepatitis B is defined as the presence of HBV viraemia without HBsAg in the presence or absence of anti HBs antibodies and can be identified by a molecular assay to quantify HBV-DNA (Thabit *et al*., 2012). All forms of occult HBV could be infectious in immune-compromised individuals, but detection of HBVDNA in individuals with only HBcAb in their blood is higher and has been associated with infectivity in both immune-compromised and immune-competent individuals, especially in blood transfusions (Thabit *et al*., 2012). Nebbia *et al*. (2007), corroborates the fact that occult HBV infection is common in HBsAg negative, anti HBc positive patients and poses a diagnostic challenge.

There are conflicting reports on the association of blood transfusions in Nigeria with HBV transmission. Some studies could not associate prevalence of HBV with history of previous blood transfusions (Eke *et al*., 2011; Mbaawuaga *et al*., 2008a; Mbaawuaga *et al*., 2008b). However, other studies reported strong association between prevalence of HBV and previous blood transfusions (Yakasai *et al*., 2013; Sule *et al*., 2011; Okonko *et al*., 2010). Adesunkanmi

et al. (2003) also report that cutaneous, percutaneous and mucous membrane exposure to patient's blood and body fluids are common events during general surgical operations.

2.1.5 Serological Markers for Hepatitis B Virus

The hepatitis B surface antigen (HbsAg) is the first marker that appears in the blood following infection with hepatitis B virus (HBV) some days or weeks before clinical symptoms manifest. It is a lipoprotein polypeptide, which constitutes the external envelope of the HB virus. The detection of HbsAg in human serum or plasma indicates an ongoing HBV infection. Testing of additional HBV markers is needed to define the specific disease state. Therefore, presence of Anti- HBcIgM indicates the infection is acute, while presence of HbsAg and absence of Anti-HBc IgM signify the infection is chronic (Kudesia and Wreghitt, 2009). Anti–HBcIgM is the only marker that can distinguish between acute and chronic infections. In addition, the presence of HbeAg and absence of Anti- Hbe clearly spell out that the patients have higher infectivity and also having higher risk developing liver diseases. Conversely, presence of Anti-HBe and absence of HbeAg indicate that the patients have low infectivity and fewer tendencies to develop liver diseases.

2.1.6 Pathogenesis of Hepatitis B Virus

HBV is a non-cytopathic virus that causes a spectrum of liver diseases ranging from acute hepatitis (including fulminant hepatic failure) to chronic hepatitis, cirrhosis and hepatocellular carcinoma (Fields *et al*., 2001). The majority of patients recover from their infection. Those who become persistently infected, many have mild liver disease with little or no long-term morbidity or mortality (Fields *et al*., 2001). The eventual outcome, extent and severity of HBV infection depend on a variety of viral and host factors.

Although host responses play a major role in disease pathogenesis and viral clearance, the

natural history of HBV infection is often predicated on a variety of viral adaptive mechanisms. Frequently, viral mutations in critical regions of the viral genome are the result of these adaptive mechanisms (Zuckerman *et al*., 2009). Although the early events of primary HBV infection remain largely unknown, hepatocellular injury most likely begins with antigen recognition by HBV-specific cytotoxic T-lymphocytes (CTLs) with the induction of apoptosis in HBV-positive hepatocytes. Recruitment by the CTLs of host-derived, antigen-nonspecific inflammatory cells including macrophages, neutrophils, and other lymphocytes—leads to formation of necro-inflammatory foci. Infiltrates of these inflammatory cells are primarily responsible for hepatocellular death.

At this stage, clinical evidence of hepatitis becomes apparent with elevation of aminotransferases. During acute self-limited infection, the pathology is mild to moderate, and viral replication is effectively controlled by host immune responses with resolution of hepatitis. In some cases, these virus-nonspecific inflammatory responses are markedly augmented by yet-unknown host determinants or viral factors, resulting in massive hepatocellular injury and Fulminant hepatitis. On the other hand, chronic hepatitis results from continuous hepatic injury as a consequence of ineffective viral clearance. In asymptomatic carriers, tolerance to HBV antigens confers little or no immune-mediated injury in the infected liver (Zuckerman *et al*., 2009).

2.1.7 Natural History of HBV Infection

In adult and childhood acute infection can either resolve or progress to chronic infection. Chronic infection can either develop into an inactive carrier state, or progress to chronic hepatitis and liver cirrhosis and hepatocellular carcinoma (liver cancer) (deFranchis *et al.*, 2003). Various stages of chronic HBV infection are recognized, as detailed in Table 2.1**.** Two variants of chronic

hepatitis are possible: HBeAg-positive and HBeAg negative chronic hepatitis. Serological patterns of acute and chronic infections are shown in Table 2.2.

2.1.7.1 Acute Infection

Infections, which develop within 6 months of HBV exposure, and then resolve within 6 months, are considered acute (deFranchis *et al.*, 2003). These are self-limiting and are indicated by elevated alanine transaminase (ALT) levels and the presence of anti-HBc in the serum (deFranchis *et al.*, 2003; Goodman, 2002). Liver injury or disease is a result of the host immune system initiating apoptosis (programmed cell death) and/or necrosis of infected hepatocytes (Goodman, 2002). Fulminant hepatitis is a severe manifestation of an acute infection.

2.1.7.2 Chronic Infection

If the host immune system is unable to clear the virus from the hepatocytes, the infection will become chronic, which is formally defined as the persistence of HBsAg in the serum for longerthan six months (Farrell and Denstag, 2002). The extent of liver damage, which results from the continuing inflammatory response, varies between individuals (Goodman, 2002). Several factors may increase the chances that an infection will develop to chronicity, including the age and immune status of the individual, as well as the persistence of HBeAg. Individuals infected at birth have a 90% chance of developing chronic infection and this decrease to less than 5% for those infected during adulthood (Farrell and Denstag, 2002). Acute HBV infection in individuals with compromised immunity, such as those infected with HIV, is more likely to develop into chronic HBV (Farrell and Denstag, 2002).

Persistence of HBeAg for more than three months is an indication that the infection is likely to become chronic (Farrell and Denstag, 2002). Seroconversion of HBeAg to anti-HBe is associated with a decrease in both viral replication and liver damage (Farrell and Denstag, 2002).

However, the loss of HBeAg may also be associated with the development of mutant HBV strains.

Four phases of the natural history of chronic HBV are recognized, although all infected individuals do not necessarily experience each phase: (1) immune tolerance, (2) immune clearance (HBeAg-positive) or immune active phase, (3) inactive carrier (HBeAg-negative) and (4) HBsAg clearance and/or reactivation (McMahon, 2009; Yim and Lok, 2006). The immune tolerance phase, which lasts from 1 to 4 decades, is characterized by elevated levels of HBV DNA in the serum, HBeAg positivity, normal ALT levels and reduced immunological response to HBV infection (McMahon, 2009; Yim and Lok, 2006).

The liver may, however, show some symptoms of infection (Kao and Chen, 2002). During the second phase, HBV DNA levels and ALT levels may fluctuate; the liver may show active inflammation or fibrosis and HBeAg seroconversion may occur (McMahon, 2009; Yim and Lok, 2006). In the third phase, ALT levels return to normal, anti-HBe is present, liver inflammation and fibrosis decrease and HBV DNA serum levels become undetectable (McMahon, 2009; Yim and Lok, 2006).

This phase may persist indefinitely in some individuals, resulting in a positive prognosis (Yim and Lok, 2006). However, in some individuals, a fourth phase occurs, characterized by the reactivation of HBV replication, elevated HBV DNA serum levels elevated ALT levels and further liver damage (Yim and Lok, 2006).

Table 2.1: Stages of chronic HBV infection

Characteristic	Immunotolerant	Immunoactive	Inactive Carrier
HBsAg	Yes		
Anti-HBs	No	No	Yes
HBeAg	Yes	Possible	No
Anti-HBe	No	No	Yes
HBV DNA	High	Decrease	Very low
Aminotransferases	Normal/Slightly elevated	Increase	Normal
Symptoms	None	Possible	
HBV Replication	'Yes	Yes	Very low

Source : (deFranchis *et al.*, 2003).

2.1.7.3 Cirrhosis

Cirrhosis is defined as "a diffuse process characterized by fibrosis and the conversion of normal liver architecture into structurally abnormal nodules" (Yim and Lok, 2006). It is considered to be an advanced stage of liver fibrosis in which the hepatic vasculature and architecture are distorted such that blood flow in the liver is compromised (Schuppan and Afdhal, 2008).

2.1.7.4 HCC

Hepatocellular carcinoma (HCC, liver cancer) is one of the leading causes of death worldwide, particularly in southern China and sub-Saharan Africa, where it is responsible for 10% of all deaths (Bunz, 2008). Important risk factors for HCC include infection with HBV, infection with hepatitis C virus, exposure to aflatoxin and alcohol-induced liver disease (Bunz, 2008). Fibrosis disrupts interactions between hepatocytes, which are normally quiescent, resulting in uncontrolled growth. Hepatocytes, which are not eliminated via apoptosis or the immune response, can therefore become fully transformed (Bunz, 2008).

2.1.8 Occult HBV Infection

Presence of HBV in a sample is routinely determined by an enzyme -linked immunosorbent assay (ELISA) test for HBsAg. This test is relatively inexpensive, particularly when samples are processed in bulk, and is easy to administer, a positive result for HBsAg indicates the presence of HBV.

However, a variant form of HBV infection, in which HBsAg test results are negative, has been described (Raimondo *et al.*, 2013 and 2007; Hu, 2002). Such infections are termed "occult HBV infections" (OBI). The clinical and virological relevance of OBI are considered particularly challenging to understand, with the issues being debated for the last three decades (Raimondo *et al.*, 2008).

Table 2.2: Serological patterns in HBV infection

Serology	**Incubation**	**Acute Infection**	**Recovery**	**Chronic >6months**	**Chronic <6months**	**Immune Carrier**	**Inactive**
HBsAg	+	+	-	+	+	-	+
Anti-HBs	-	-	+	-	-	+	-
Anti-HBcIgG	-	+/-	+	+	+	-	+
Anti-HBcIgM	-	+	+/-	+/-	+/-	-	*
HBeAg	+	+	-	+	-	-	-
Anti-HBe	-	-	+	-	+	-	+
HBV DNA	+	+	-	+	+	-	+

Source: (Torbenson and Thomas, 2002)

Key: * means not applicable

OBI has been referred to as "inapparent HBV infection", "serologically silent hepatitis B", "silent hepatitis B", "surface antigen negative carriers" and "unrecognized hepatitis B infection" (Torbenson and Thomas, 2002).

OBI is defined by the *Taormina* expert panel as "the presence of HBV DNA in liver (with detectable or undetectable HBV DNA in the serum) of individuals testing HBsAg-negative by currently available assays. When detectable, the amount of HBV DNA in the serum is usually very low (<200 IU/ml) (Raimondo *et al.*, 2008). OBI is usually detected by the analysis of serum samples (Raimondo *et al.*, 2008). Samples, which are HBsAg-negative, with serum HBV DNA levels <200 lU/ml, are considered "true" OBI. Samples in which serum HBV DNA levels are similar to HBsAg-positive ("overt") infection, but are nevertheless HBsAg-negative, have been termed "false" OBI by the *Taormina* panel (Urbani *et al*., 2010). The clinical implications of OBI in immunocompro-mised individuals, such as those infected remains unclear (Raimondo *et al.*, 2007; Mphahlele *et al.*, 2006). Furthermore, HIV has been identified as a risk factor for the development of OBI (Mphahlele *et al.*, 2006).

2.1.9 HBV/HIV Co-Infection

The natural history of Hepatitis B virus is known to be complicated by HIV co-infection (Rockstroh, 2006).Hence HIV and HBV infections are both endemic in Sub-Saharan Africa (Hoffman *et al*., 2012; Firnhaber *et al*., 2010; Mphahlele, 2008). Because these two viruses share major risk factors, a number of HIV infected individual reportedly have past exposures to, or become chronic carriers of HBV. In Sub-Saharan Africa, HBV is predominantly transmitted in childhood and the majority of inhabitants are already exposed to, or are chronic carriers of HBV by the time they become exposed to HIV for the first time (Mphahlele, 2008). Hence, there is frequent detection of HBV in HIV infected individuals (and vice versa) in the region because

both viruses are highly endemic. Several studies in Nigeria have reported HBV-HIV co-infections. North Eastern Nigeria, Taura *et al*., (2008) and Mustapha and Jibrin (2004), reported the prevalence of HBV in patients with HIV in Gombe (26.5%) and Aminu Kano Teaching Hospital Kano (6%) respectively. Denue *et al*., (2012) determine the prevalence of HBV and HCV among HIV patients in University of Maiduguri Teaching Hospital and found 12.3% of the HIV patients co-infected with HBV. The study also observed that elevation of alanine aminotransferase (ALT) in serum of the patients was higher in HIV-HBV co-infected individuals than in those with HIV mono-infection. In North Central Nigeria, Forbi *et al*., (2007) studied the roles of triple infection with HBV, HCV and HIV on CD4 lymphocytes levels among HIV infected population and found HIV-HBV co-infection of 27.8%. Co-infections between HIV and HBV are common because both viruses share a number of characteristics (Mphahlele, 2008). These include transmission routes (vertical, parental and sexual) (Cooley and Sasadeusz, 2003). Both viruses are known to replicate using reverse transcriptase enzyme, which acts as an RNA-dependent DNA polymerase and also have a propensity to establish chronic infections (Mphahlele, 2008), which are difficult to treat with antiviral agents (Karayiannis, 2003). Their genomes also have a great capacity to mutate which leads to rapid emergence of mutant strains, some of which are resistant to widely applied antiviral agents (Cooley and Sasadeusz, 2003; Kamiya, 2003). It has also been reported that both HIV and HBV reverse transcriptase enzyme lack 3', 5'-exonuclease proof-reading activity and hence have a higher rate of misincorporation of bases during replication (Bartholomeusz *et al*., 2004). The genome of both HIV and HBV are known to integrate within the host genome, a process obligatory for the life cycle of HIV but rarely HBV (Barthlomeusz, 2004).

2.1.10 Diagnosis of HBV

The diagnosis of HBV infection is typically based on the evaluation of serological and virological markers of HBV in serum as well as the evaluation of biochemical and histological markers of the liver (Keeffe *et al*., 2006). The most useful detection methods are ELISA for detecting HBV antigens or antibodies (Jawetz *et al*., 2007). The detection of serological markers is based on Antigen-Antibody reaction, therefore divided into two classes, detection of Antigens or detection of Antibodies. The biochemical assessment of liver function include: total and direct bilirubin (TSB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein, albumin, globulin and coagulation profile.

2.1.10.1 Laboratory Diagnosis

HBsAg is the most important serological marker for identifying infection (Haaheim *et al*., 2002). Current immunoassays for HbsAg detect 100–200 HBsAg/ml of serum, corresponding to roughly 3×10^7 particles/ml. Most HBeAg-positive carriers have more than 10^5 genomes/ml of serum. Detection of HBsAg is the standard assay for infection and is used widely for screening, for example in transfusion centres. HBeAg is a marker of viraemia, but anti-HBe does not necessarily indicate clearance of virus replication. Immunity after infection with HBV is characterized by the presence of anti-HBs together with anti-HBc in serum. Immunity after vaccination is characterized by the presence of anti-HBs alone. Anti-HBc of the IgM class is a valuable marker of acute infection. It is present early in acute infection, disappears with resolution of infection and persists in chronic infection. IgM anti-HBc is essential for the diagnosis of acute infection, but is also seen occasionally in very active chronic hepatitis. Anti-HBc antibodies develop and persist after all HBV infections (Zuckerman *et al*., 2009). The loss of HBsAg and development of anti-HBc signals resolution of acute infection. Anti-HBs also

occurs post vaccination, but anti-HBc will not be present in such cases. Chronic infection is manifested by persistent HBsAg. Markers of viral replication such as HBeAg and HBV-DNA (non-PCR method) are detectable during the early high replication phase, but are not detectable during the later quiescent low replication phase. HBeAg is not a reliable marker of HBV replication when a precore variant is responsible for the infection. Such cases will be HBeAg negative, anti-HBe positive, but HBV-DNA (by a non-PCR method) positive (Haaheim *et al*., 2002).

2.1.10.2 Detection of HBV Antigens

1. Detection of hepatitis B surface antigen

Hepatitis B surface antigen is the first serological marker to appear after infection. Its persistence for more than 6 months indicates CHB infection (Keeffe *et al*., 2006). HBsAg appears at average of 6-8 weeks after exposure, 1-3 weeks before ALT becomes abnormal and 3-5 weeks before onset of symptoms or jaundice (Alter, 2003). This Antigen can be detected by many techniques; the commonly used are radioimmunoassay (RIA) and enzyme immunoassay. Since many immunoassays use monoclonal antibodies directed against the "a" determinant, amino acid substitution in this region may account for false-negative results in immunoassay (Levicnic-Stezinar, 2004). Thus, diagnosticians and health care industry need to increase their awareness of HBsAg mutation and how these mutants may alter current diagnostic and treatment algorithms (Coleman, 2006).

2.1.10.3 Detection of HBV Antibodies

1. Anti-HBs antibodies

Anti-HBs replace HBsAg as AHB infection is resolving. It generally persists for life time in over 80 % of patients and indicates immunity (Jawetz *et al*., 2007). Occasionally, anti-HBs

and HBsAg are both detectable in patients with CHB infection, a finding of no known significance (Keeffe *et al*., 2006). Anti-HBs may not be detectable until after a window period of several weeks to months (Berenguer and Wright, 2002). This marker is acquired through natural HBV infection, vaccination, or passive antibody immunization (Lin and Kirchner, 2004).

2. Anti-HBc Antibodies

It is the first antibody to appear. Demonstration of anti-HBc in serum indicates HBV infection, current or past. Anti-HBc IgM is present in high titer during acute infection and usually disappears within 6 months, and although it can persist in some cases of chronic infection, this test may therefore reliably diagnose AHB infection (Jawetz *et al*., 2007). High levels of IgM-specific anti-HBc are frequently detected at the onset of illness because this antibody is directed against the 27 nm internal core component of HBV and it is appearance in the serum indicate viral replication (Jawetz *et al*., 2007). Anti-HBc IgG predominates after 6 months and generally persists indefinitely in patients who have recovered from HBV infection. Anti-HBc IgG present in virtually at all patients who ever been exposed to HBV (Lin and Kirchner, 2004). Anti-HBc total is used with anti-HBs and HBsAg for screening populations at risk (Schiff, 2004).

2.1.10.2 Molecular Diagnosis

The amount of HBV-DNA in serum is a measure of the level of viral replication. Previously, serum HBV-DNA testing was performed using non-amplified hybridization. These assays (Dot blot hybridization, Liquid hybridization, North blot and branched DNA assays) have a limit of quantification of 10^5-10^6copies/ml and should no longer be used for routine management of patients with CHB infection (Keeffe *et al*., 2006). HBV-DNA assays provide very useful adjunct information concerning viral replication, especially in situations when

patients' serological profiles fall outside of classical pattern. The molecular testing of HBV consists of two categories (i) HBV-DNA quantification assays that measure the amount of HBV-DNA in peripheral blood, which reflects the level of HBV replication (viral load) in the liver (ii) assays that identify sequences or motifs of clinical or pathophysiological importance in the HBV genome (Pallier *et al*., 2006).

1. Polymerase Chain Reaction (PCR) Technique

Hepatitis B virus DNA detection based on a nested PCR approach detects as few as 10^2 - 10^3genome copies. In contrast to PCR assay that measure HBV-DNA titers only after the PCR cycle (end point measurement), Real-time PCR technology, based on continuous quantitative monitoring during the exponential phase of the PCR reaction, it is able to measure viral loads over a large dynamic rang (Bowden, 2002). New developments (Taq Man technology, molecular beacons) that decrease the number of handling steps reduce contamination, and increase throughput and the accuracy of quantification will further enhance the utility of these assays (Sablon and Shapiro, 2005).

2.1.11 Prevention and Control

Patients with chronic HBV should be screened for hepatitis C virus (HCV) infection and should be vaccinated against hepatitis A virus (HAV) if they are not immune. HIV/HBV-coinfected patients should be counseled to avoid or limit intake of hepatotoxins, including alcohol and high dosages of acetaminophen (Leutkemeyer, 2010).

2.1.11.1 Prevention of HBV Transmission

All HBsAg-positive patients should be counseled about reducing the risk of HBV transmission to close contacts. It is important to inform HIV/HBV-coinfected patients that HBV can be more

infectious than HIV and can be transmitted to household contacts via dried blood, open cuts, and shared toothbrushes or razors. Sex partners, household members, children with close physical contact, and those who share injection drug equipment with the patient should be screened for HBV and vaccinated if they are not actively infected. As with HIV prevention, condom use with sex and avoidance of shared needles and other equipment for injection drug use are recommended measures for reducing the risk of HBV transmission (Leutkemeyer, 2010).

2.1.11.2 HBV Therapy

The goal of HBV treatment for persons with HIV coinfection is to suppress HBV viral replication and minimize ongoing hepatic damage. Loss of HBsAg and seroconversion to HBsAb indicating resolution of active HBV disease are uncommon in HIV/HBV coinfection; therefore, indefinite treatment of HBV for coinfected patients often is required (Jawetz *et al*., 2007)

2.1.11.3 Indications for Treatment of HBV in HIV Infection

HBV therapy is recommended for all HIV/HBV-coinfected patients with abnormal alanine aminotransferase (ALT) values or HBV DNA levels of >2,000 IU/mL. Some guidelines cite >20,000 IU/mL as the threshold for treatment of HBeAg-positive patients (Fonseca *et al*., 2005); however, many experts recommend treatment of HBV for all HIV-coinfected patients in whom any HBV replication is present. If HBV viremia is low level (<2,000 IU/mL) and the ALT value is normal, a liver biopsy may be considered for patients who are not on HIV therapy, as those with no or limited fibrosis may not require HBV treatment. It is important to note that significant liver fibrosis may be present in persons with normal transaminases. If HBV treatment is deferred, transaminases and HBV DNA values should be monitored closely (Jawetz *et al*., 2007).

2.1.11.4 Treatment of HBV in the Setting of ART

As most HIV treatment contains one or more HBV-active agents (eg, lamivudine [3TC], emtricitabine [FTC], tenofovir [TDF]), patients with an indication for HBV treatment should be started on fully active ART that contains HBV-active nucleoside/nucleotide analogues, regardless of the CD4 cell count, to ensure that HIV is not partially treated(CDC, 2009) Similarly, any HIV/HBV-coinfected patient with an indication for ART should be started on HIV treatment that includes effective anti-HBV treatment. The combination of TDF with 3TC or FTC is recommended as a highly effective first-line treatment for HBV (CDC, 2009). Individuals who cannot take TDF because of renal insufficiency or other intolerance may consider entecavir treatment (renally dosed if necessary) for HBV in lieu of TDF. Treatment with 3TC or FTC as the only HBV-active agent in ART (ie, HBV monotherapy) is not recommended owing to a high risk of developing HBV drug resistance over time (CDC, 2009)

2.1.11.5 Treatment of HBV in the absence of ART

The current U.S. Department of Health and Human Services guidelines recommend fully active ART for HIV/HBV-coinfected patients who require HBV treatment, even if they do not have a current indication for HIV treatment (US Department of Human Health Services, 2009). However, in some circumstances, an individual may not tolerate HIV therapy or may wish to defer treatment for HIV. Treatment with HIV/HBV-active agents that are insufficient to fully suppress HIV should be avoided. Entecavir and telbivudine have been shown to have anti-HIV activity and to contribute to HIV drug resistance (Low *et al*., 2009; McMahon *et al*., 2007) as have traditional HIV-active agents used for HBV treatment (eg, 3TC, FTC, TDF). Whereas adefovir may be an option for treatment of HBV only, there is a concern that even low-dose

adefovir used for HBV could have anti-HIV activity. Pegylated interferon (IFN) is an option in this scenario, but there are limited efficacy and safety data in HIV coinfection, and long-term treatment with IFN is not feasible because of toxicity and poor tolerability.

2.1.11.6 Monitoring on Therapy

The goal of therapy is suppression of HBV below the assay level of detection. For patients on treatment, HBV DNA should be monitored at 3-6 month intervals, along with transaminases and HBeAg. Virologic response is defined as a ≥2 log10 copies/mL decrease from baseline HBV DNA after 6 months of therapy. TDF-based HBV treatment will fully suppress HBV replication for the majority of patients, but that could take several years to accomplish. Development of TDF resistance despite ongoing viremia has not been documented; it is unclear whether there is a role for additional anti-HBV agents such as entecavir for patients in whom HBV replication has not been suppressed after a year or more of therapy. It is important to note that HBsAg and HBeAg may remain detectable for years despite HBV DNA suppression, and in some cases indefinitely. HBV treatment in HIV-infected individuals can be associated with hepatic decompensation and transaminase flares caused by immune responses to HBV infection; however, other causes of decompensation or increased transaminases must be considered, including drug toxicity, viral hepatitis coinfection, medication non-adherence, and development of viral resistance to current HBV treatment. Coinfected patients should be cautioned against interruption of HIV and HBV therapy, as treatment interruption can be associated with HBV viral rebound and hepatic decompensation. As a sustained loss of HBsAg is uncommon among HIV-coinfected patients, most of these individuals require indefinite HBV treatment with nucleosides or nucleotides, which are given as a component of HIV treatment, if possible.

2.1.11.7 HCC SCREENING

HBV infection is associated with development of HCC even in the absence of cirrhosis. The 2010 American Association for the Study of Liver Disease guidelines recommend screening for HCC every 6-12 months with alpha-fetoprotein (AFP) and with ultrasound in groups at risk of HCC, including patients with chronic HBV who are >40 years of age and those of African American ethnicity (Bruix and Sherman, 2010). The guidelines also recommend ongoing HCC screening for patients with evidence of cirrhosis, regardless of age or other risk factors. The benefit of surveillance in patients with chronic HBV who are <40 years of age is not well established. However, given that liver disease progresses more rapidly in HIV/HBV-coinfected patients and that HCC may be more aggressive in HIV coinfection (Puoti *et al*., 2004) many experts recommend HCC surveillance for all HIV-infected patients with chronic HBsAg-positive HBV disease.

2.1.11.8 IMMUNIZATION AGAINST HBV IN HIV INFECTION

HBV vaccine has been suggested for all HIV-infected patients who lack HBsAb and do not have HBsAg positivity or occult HBV (Leutkemeyer, 2010). Although transient HIV viremia has been reported after vaccination in some studies, it does not appear to be clinically relevant. Patients with HIV infection are reportedly less likely to develop protective HBsAb after HBV vaccination, with a response rate of 18-71%, compared with >90% for HIV-uninfected adults (Kim *et al*., 2009; Shire *et al*., 2006). Lower seroconversion rates are associated with lower CD4 cell counts, detectable HIV RNA, and HCV coinfection (Fonseca *et al*., 2005). HIV infection also is associated with lower mean HBV HBsAb titers and faster decline of HBsAb levels over time (Kim *et al*., 2009). Administration of a higher dose of HBV vaccine ("double" or "renally"

dosed at 40 mcg) is reported to increase effectiveness compared with the standard 20 mcg dose (47% vs 34%), an effect that was strongest in patients with CD4 counts of >350 cells/μL (Fonseca *et al*., 2005). Those data have prompted some clinics to adopt 40 mcg dosing of HBV vaccine for all HIV-infected individuals, although data to support that practice are limited. Although low CD4 cell counts are associated with an impaired response to vaccination, HBV vaccination should not be deferred for patients with advanced HIV, as some individuals do develop protective antibody titers despite low CD4 cell counts. Postvaccination HBsAb levels should be checked 1-2 months after the vaccination series is completed to ensure titers of ≥10 IU/L (Mast *et al*., 2006). Revaccination should be considered for patients who have not attained protective titers, and use of the higher dose (40 mcg) vaccine for repeat vaccination should be considered (CDC, 2009). Several studies have reported that a second vaccination series, with 2 studies reporting a 40 mcg dosing for HBV vaccination, resulted in seroconversion rates of 50-76% (Bloom *et al*., 2009; Cruciani *et al*., 2009; deVries *et al*., 2008;). Adjuvants such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and CpG 7909 (Vaximmune) have shown promise in boosting responses to the HBV vaccine, but are in use only for research purposes at the present time (Kim *et al*., 2009; Cooper *et al*., 2008). For persons who develop HBsAb in response to vaccination, some experts recommend annual testing to ensure HBsAb titers remain adequate, extrapolating from recommendations for dialysis patients, in whom loss of protective antibody is associated with reduced protection against HBV (CDC, 2009).

2.2 Virology and Molecular Characteristics Hepatitis C Virus

Hepatits C virus is a positive stranded RNA virus, classified in the family, Flaviviridae, and genus Hepacivirus. The virus is 45-60nm in diameter with a 9.5kb genome encased by capsid proteins (Fields *et al*., 2001). The virus is enveloped and contains glycoprotein projections

on the surface. The viral genome encodes structural proteins namely nucleocapsid (p22, core proteins), the two envelope glycoproteins (gp35 and gp70); and the non-structural proteins, transmembrane protein (p23), metalloprotease, serine protease, RNA helicases (p8, p27), cofactors (p56, p158), IFN-resistance protein, RNA polymerase (p68) and p7 with unknown function, but speculated to be involved in viral assembly and release. This protein is technically, not a known structural element of HCV virions, but generally grouped with the core, E1, and E2 structural proteins. HCV core protein has been shown to directly trigger apoptosis by upregulation of the tumor suppressor, p53, and down regulation of cell cycle regulators p21 and p38 (Schuppan, 2003).

The RNA genome of hepatitis C virus carries a long open reading frame (ORF) encoding a polyprotein precursor of 3010 amino acids. Translation of HCV ORF is directed via an approximately 340 nucleotide long 5′ non-truncated region (NTR) functioning as an internal ribosome entry site which permits the direct binding of ribosomes in close proximity to the start codon of the ORF. The HCV polyprotein is cleaved by cellular and viral proteases to yield ten different products with the structural proteins, core (C), E1 and E2 (envelope glycoproteins), isolated from the N-terminal (N3) and non-structural (NS2-5) replicative proteins from the remainder. HCV has a positive sense, single stranded RNA, thus, on entry; the viral RNA gets modified to function as mRNA (Fields *et al*., 2001). Most of the reports regarding the processing of HCV proteins and genome function are based on *in vitro* studies using mammalian, insect cell, or bacterial expression systems. Kolykhalov and colleagues demonstrated an essential requirement for the enzymatic activities of the NS2/3 protease, the NS3-4A serine protease, the NS3 nucleoside triphosphatase (NTPase) and helicase, and the NS5B polymerase for chimpanzee infection (Fields *et al*., 2001). In addition, a requirement for elements contained within the 3'-

UTR of the HCV genome for successful replication of the virus has also been shown using the chimpanzee (Fields *et al*., 2001). These observations suggest that the *in vitro* data provide a good representation of events occurring during productive viral infection (Fields *et al*., 2001).

2.2.1 HCV Replication

Infection with a positive-strand RNA virus leads to rearrangements of intracellular membranes, a prerequisite to the formation of a replication complex that associates viral proteins, cellular components and nascent RNA strands. The HCV NS4B protein seems to be sufficient to induce the formation of a membranous web or membrane-associated foci (*Gretton et al*., 2005; Egger *et al*., 2002). It is not known whether NS4B recruits cellular proteins responsible for vesicle formation or induces vesicle formation by itself. The membranous web is derived from ER membranes (Bartenschlager *et al*., 2004). It is rich in cholesterol and fatty acids, the degree of saturation of which (that influences membrane fluidity) modulates HCV replication (Kapadia and Chisari, 2005). HCV replication was shown to occur in detergent-resistant membranes that co-localize with caveolin-2, an essential component of lipid raft domains (Shi *et al*., 2003). Indeed, lipid rafts are involved in the formation of the replication complex, through protein-protein interactions between hVAP-33 and both NS5A and NS5B HCV proteins (Gao *et al*., 2004; Shi *et al*., 2003). Overall, the membranous web consists of small vesicles embedded in a membranous matrix, forming a membrane-associated multiprotein complex that contains all of the nonstructural HCV proteins (Egger *et al*., 2002).

The precise mechanisms of HCV replication are still poorly understood. By analogy with other positive-strand RNA viruses, HCV replication is thought to be semi-conservative and asymmetric with two steps, both of which are catalyzed by the NS5B RdRp. The positive-strand

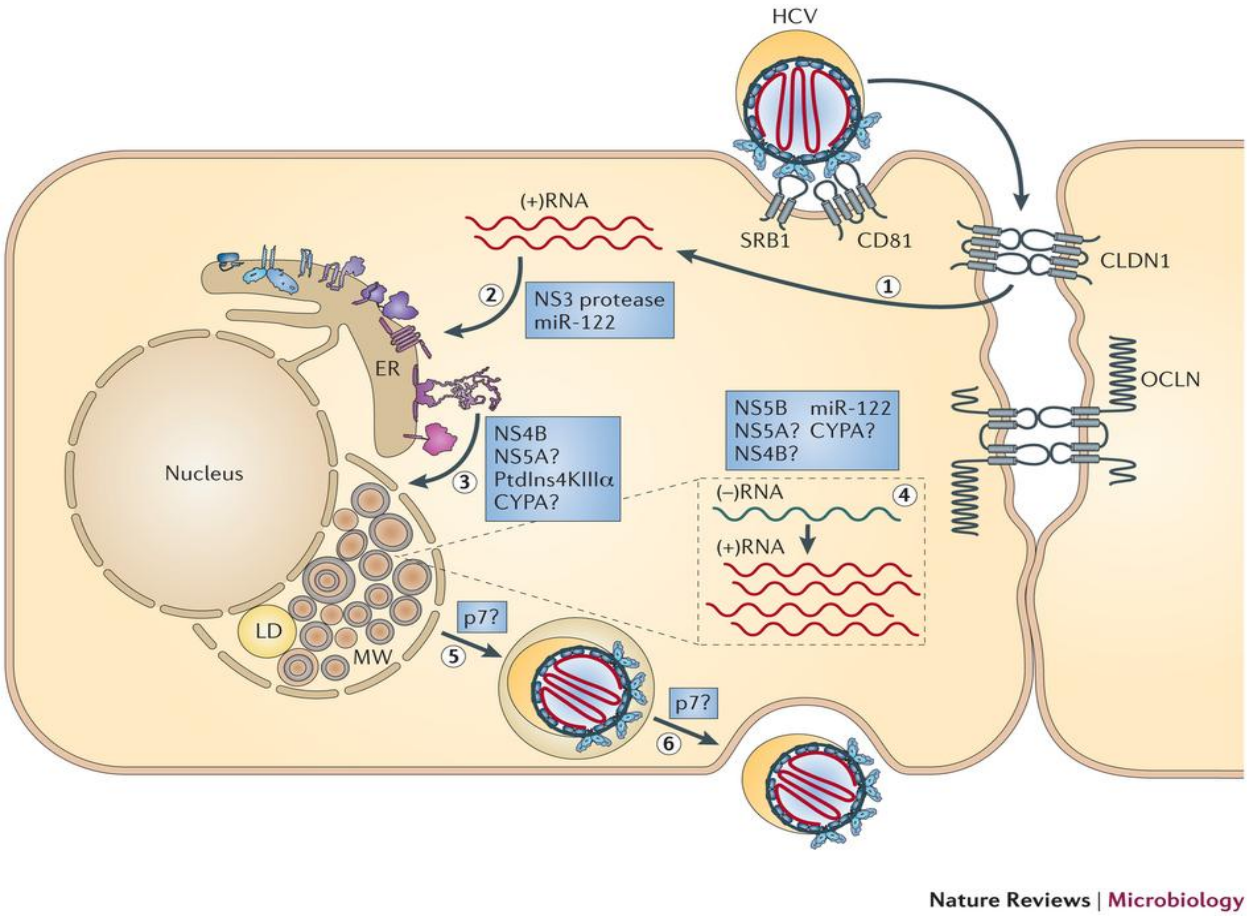

Figure 2.2: HCV Replication

Source: (Egger *et al.*, 2002)

genome RNA serves as a template for the synthesis of a negative-strand intermediate of replication during the first step. In the second step, negative-strand RNA serves as a template to produce numerous strands of positive polarity that will subsequently be used for polyprotein translation, synthesis of new intermediates of replication or packaging into new virus particles (Bartenschlager *et al*., 2004).

The positive-strand RNA progeny is transcribed in a five to ten fold in excess compared to negative-strand RNA. NS5B RpRd was initially thought to catalyze primer-dependent initiation of RNA synthesis, either through elongation of a primer hybridized to the RNA template or through a copy-back mechanism ((Bartenschlager *et al*., 2004). More recently, the HCV RdRp was shown to be capable of initiating *de novo* RNA synthesis under certain experimental conditions ((Bartenschlager *et al*., 2004).

Initiation of RNA strand synthesis at the 3′-end of the plus and minus strands involves domain I of the 5′ UTR, which can form a G/C-rich stem-loop, the 3′ UTR and a *cis*-acting replication element (5BSL3.2) consisting of 50 bases located in a large predicted cruciform structure at the 3′ end of the HCV NS5B-coding region (You *et al*., 2004). Initiation of RNA replication is triggered by an interaction between proteins of the replication complex, the 3′ X region of the 3′ UTR, and 5BSL3.2 that forms a pseudoknot structure with a stem-loop in the 3′ UTR (Astier-Gin *et al*., 2005; Friebe *et al*., 2005; You *et al*., 2004). A phosphorylated form of PTB was found in the replication complex and PTB was shown to interact with two conserved stem-loop structures of the 3′ UTR, an interaction thought to modulate RNA replication (Chang and Luo, 2005; Luo, 2004). Importantly, inhibition of PTB expression by means of small interfering RNAs reduced the amount of HCV proteins and RNA in HCV replicon-harboring Huh7 cells (Chang and Luo, 2005).

2.2.1.1 Virus Assembly and Release

Little is known about HCV assembly and release due to the lack of appropriate study models. Different variants of the HCV core protein, which can exist as dimeric, and probably multimeric forms as well, have been shown to be capable of self-assembly in yeast in the absence of viral RNA, generating virus-like particles with an average diameter of 35 nm (Acosta-Rivero *et al*., 2004a; Acosta-Rivero *et al*., 2004b). Recent reports suggested that the N-terminal portion of the core protein is sufficient for capsid assembly, in particular the two clusters of basic residues (Klein *et al*., 2005; Majeau *et al*., 2004). In bacterial systems, HCV core proteins efficiently self-assembled to yield nucleocapsid-like particles with a spherical morphology and a diameter of 60 nm, but the presence of a nucleic acid was required (Chang and Luo, 2005). Overall, particle formation is probably initiated by the interaction of the core protein with genomic RNA; HCV core can indeed bind positive-strand RNA *in vitro* through stem-loop domains I and III and nt 23-41 (Klein *et al*., 2005). It is tempting to speculate that the core-RNA interaction may play a role in the switch from replication to packaging.

Virus-like particles were produced in mammalian cells by using a chimeric virus replicon allowing high-level expression of HCV structural proteins in BHK-21 cell lines (Blanchard *et al*., 2002). Budding of virus-like particles of 50 nm in diameter in the dilated ER lumen was observed (Blanchard *et al*., 2003). Transfection of full-length HCV RNA in HeLa G and HepG2 cell lines led to the formation of virus-like particles with a diameter of 45 to 60 nm, which were synthesized and assembled in the cytoplasm and budded into the ER cisternae to form coated particles (Klein *et al*., 2005). Indeed, the HCV envelope glycoproteins E1 and E2 associate with ER membranes through their transmembrane domains (Blanchard *et al*., 2003), suggesting that virus assembly occurs in the ER. Structural proteins have been detected both in the ER and the

Golgi apparatus, suggesting that both compartments are involved in later maturation steps (Serafino *et al*., 2003). Moreover, the presence of N-glycan residues at the surface of HCV particles is also in keeping with a transit *via* the Golgi apparatus. The mechanisms underlying exportation of mature virions in the pericellular space have yet to be understood. Newly produced virus particles may leave the host cell by the constitutive secretory pathway.

2.2.2 Transmission

2.2.2.1 Blood Transfusion

HCV is spread primarily by contact with blood and blood products. Blood transfusions and the use of shared, unsterilized, or poorly sterilized needles and syringes have been the main causes of the spread of HCV.

2.2.2.2 Maternal-Infant Transmission

Maternal-infant transmission is not common. In most studies, only 5 percent of infants born to infected women become infected. The disease in newborns is usually mild and free of symptoms. The risk of maternal-infant spread rises with the amount of virus in the mother's blood and with complications of delivery such as early rupture of membranes and fetal monitoring. Breast-feeding has not been linked to spread of HCV (Lauer and Walker, 2002).

2.2.2.3 Sexual Transmission

Sexual transmission of hepatitis C between monogamous partners appears to be uncommon. Survey of spouses and monogamous sexual partners of patients with hepatitis C infection, shows that less than 5 percent are infected with HCV, and many of these have other risk factors to this infection. Spread of hepatitis C to a spouse or partner in stable, monogamous relationships occurs in less than 1 percent of partners per year. For these reasons, changes in sexual practices are not recommended for monogamous patients.

2.2.2.4 Sporadic Transmission

Sporadic transmission, when the source of infection is unknown, occurs in about 10 percent of acute hepatitis C cases and in 30 percent of chronic hepatitis C cases. These cases are usually referred to as sporadic or community-acquired infections. These infections may have come from exposure to the virus from cuts, wounds, or medical injections or procedures (Lauer and Walker, 2002).

2.2.2.5 Unsafe Injection Practice

In many areas of the world, unsafe injection practices are an important and common cause of hepatitis C. Use of inadequately sterilized equipment, lack of disposable needles and syringes, and inadvertent contamination of medical infusions are unfortunately well documented causes of transmission of hepatitis C. Careful attention to universal precautions and injection techniques should prevent this type of spread (Lauer and Walker, 2002).

2.2.2.6 Iatrogenic Medical or Dental Exposure

People can be exposed to HCV via inadequately or improperly sterilized medical or dental equipment. Equipment that may harbor contaminated blood if improperly sterilized includes needles or syringes, hemodialysis equipment, oral hygiene instruments, and jet air guns, etc. Scrupulous use of appropriate sterilization techniques and proper disposal of used equipment can reduce the risk of iatrogenic exposure to HCV to virtually zero (Lauer and Walker, 2002).

2.2.2.7 Drug use by Nasal Inhalation (Drugs which are "Snorted")

Researchers have suggested that the transmission of HCV may be possible through the nasal inhalation (insuffulation) of illegal drugs such as cocaine and crystal methamphetamine when straws (containing even trace amounts of mucus and blood) are shared among users (Lauer and Walker, 2002).

2.2.2.8 Occupational Exposure to Blood

Medical and dental personnel, first responders (e.g., firefighters, paramedics, emergency medical technicians, law enforcement officers), and military combat personnel can be exposed to HCV through accidental exposure to blood through accidental needle sticks or blood spatter to the eyes or open wounds (Lauer and Walker, 2002).

2.2.2.9 Body Piercing and Tattoos

Tattooing dyes, ink pots, stylets and piercing implements can transmit HCV-infected blood from one person to another if proper sterilization techniques are not followed (Lauer and Walker, 2002).

2.2.3 Clinical Features of Hepatitis C virus

2.2.3.1 Acute Hepatitis C

Early identification of acute hepatitis C is important, but may be difficult as the disease may be relatively silent in the acute phase; 75% of patients are not jaundiced and have nonspecific symptoms. Management of acute sporadic hepatitis C includes conventional supportive treatment and specific antiviral therapy. Therapeutic trials of IFN-α have been undertaken. Recent studies have indicated that treatment benefits those patients who have been treated early, but it may be reasonable to allow one to three months to determine which patients might convalesce spontaneously (Zuckermann *et al*., 2009). In those who do not appear to be convalescing two to four months after onset of the disease, antiviral treatment should be considered, as a high percentage of patients (>80%) may respond. The optimal form of treatment for acute hepatitis C is not yet determined but weekly pegylated IFN-α and ribavirin can be considered. Studies are in progress to determine whether a wait-and-see strategy is detrimental compared to immediate treatment (Zuckermann *et al*., 2009).

2.2.3.2 Chronic Hepatitis C

There is evidence that alcohol and hepatitis C may synergistically aggravate hepatic injury and the drinking of excess alcohol is discouraged because of this (Zuckermann *et al*., 2009). The patient should be advised not to donate blood. Patients can be told that the parenteral route is the most important route of transmission and that the virus is not easily transmitted except by this route. Treatment of hepatitis C has improved considerably. The aim of therapy is to achieve an undetectable HCV RNA six months following therapy (sustained virological response, SVR) (Zuckermann *et al*., 2009). A sustained response is associated with reduction in inflammation and severity of fibrosis. A substantial proportion of patients with chronic hepatitis C can be cured, although current treatments have limitations. HCV RNA should be measured in all patients to confirm viraemia. If the test is reproducibly positive, serum aminotransferases, bilirubin, alkaline phosphatases and prothrombin time should be measured. In patients whose lifestyle or geographic origin suggest they are at risk of other forms of viral hepatitis, HBsAg and HIV infection must also be considered.

Because autoimmune hepatitis is treated differently, it is particularly advisable to exclude this diagnosis by measuring titres of anti-smooth muscle and anti-liver-kidney microsomal antibodies, even in patients with a positive anti-HCV test. A liver biopsy is helpful in grading the degree of inflammation and staging the degree of fibrosis. Earlier guidelines recommended antiviral therapy for those patients with chronic hepatitis C who were deemed to be at highest risk of developing cirrhosis; that is, patients with chronic hepatitis C who had persistently increased serum ALT levels, detectable levels of HCV RNA and histological evidence of portal or bridging fibrosis or inflammation and necrosis. However, recent guidelines have been modified in the light of improved treatment responses, so that all patients with hepatitis C are

potential candidates for treatment; a liver biopsy can be informative and provides unique clinical information, but may not be mandatory for all patients (Zuckermann *et al*., 2009).

2.2.4 Epidemiology of Hepatitis C Virus

Hepatitis is one of the leading causes of mortality in the world (WHO, 2002). In Nigeria, infective hepatitis has been listed as one of the major causes of morbidity from notifiable diseases from 1996-2000 (Herfon, 2006). But from 1996-2004, there has been 142,714 cases of the disease and 641 reported deaths (Herfon, 2006). Infection with hepatitis C virus is a common problem worldwide, affecting millions of people across all populations. Most acutely infected patients develop chronic hepatitis and become a potential source of virus transmission and as many as one-fifth would develop cirrhosis and its complications (Knobler and Schattner, 2005).

Hepatitis C virus is a serious public health concern in developing countries like Nigeria, even in the world at large affecting over 300 million people. HCV prevalence in various African populations ranges from 0.2-40%, even though information on the epidemiology of HCV in developing countries is incomplete (Cheesebourgh, 2002). The prevalence of hepatitis C virus infection in sub-Saharan African populations is an estimated 3% with modest regional variations (Biggar *et al*., 2006). In Nigeria ranges from 5.8-12.3% have been reported, while in Eygpt and Cameroon there are seropositivity rates of 13.8- 45% (Laurent *et al.*, 2007; Fontanet and Rekacewicz, 2002).

The HCV prevalence rate in Cameroon is 13.8% and Egypt is the country with the highest HCV prevalence in the world, reaching 45% among adults older than 40 years of age in rural areas (Laurent *et al*., 2007; Cheesebourgh, 2002). The number of people infected with HCV in Egypt was estimated at around 8 million in 1999 by the Egyptian Ministry of Health and Population (Fontanet and Rekacewicz, 2002). The origin of this epidemic has been attributed to

mass treatment campaigns of intravenous injections for bilharziasis from 1960 -1982 (Fontanet and Rekacewicz, 2002).

Among a total of 1007 subjects, this included 681 males and 326 females, who were tested for HCV using ELISA technique, 62 subjects (6.2%) were positive (Nwokedi *et al*., 2006). The researchers concluded that even though Nigeria does not lie within HCV endemic region, the prevalence rate of 6.2% is quite significant. Thus, they suggested that HCV antibody screening should be included in the screening test carried out in blood banks and for patients with sexually transmitted disease (Nwokedi *et al*., 2006). HCV-related liver diseases are one of the most important liver injuries worldwide and most patients with the diseases die of hepatocellular carcinoma (Kyuichi, 2004). At present, it is estimated that there are over 100 million hepatitis C virus carriers in the world. It is particularly prevalent in Japan where most carriers (70%) are patients with chronic liver disease (Kyuichi, 2004). Approximately 2.7million persons in the United States have chronic hepatitis C infection and there are 175,000 new cases of hepatitis C reported in US per year (CDC, 2006). In the USA, non-A, non-B hepatitis (NANBH) infection is more common than HBV infection among intravenous drug abusers and those given blood transfusions (CDC, 2006). Chronic HCV infection may lead to cirrhosis, hepatocellular carcinoma and is a leading cause of liver transplantation in the United States of America (CDC, 2006).

Chronic HCV infection is considered to be a causative factor in hepatocellular carcinoma and several recent studies have established a strong association between HCV and hepatocellular carcinoma (CDC, 2006). In the absence of HBV markers, antibodies to HCV were detected in 44.5% of hepatocellular carcinoma patients in Spain, 43% in Japan, 16% in Italy, 7% in South Africa (CDC, 2006). Since HBV carriers often have chronic HCV infection, it is conceivable that

the 2 viruses may act together to cause hepatocellular carcinoma (HCC). It has been recently suggested that the risk of developing HCC is approximately 5% in cirrhotic patients with chronic hepatitis C and this is greater than the risk from hepatitis B (CDC, 2006). Among the six major HCV genotypes (types 1 to 6), genotypes 3 and 4 are foundfrequently in the Middle East and Africa, genotype 5 is found mainly in southern Africa, genotype 6 is found mainly in South East Asia, genotype 3 is found in Asia, genotypes 1b, 2a, 2b are found in Japan and Taiwan, genotype 4 is the predominant genotype in Egypt and Cameroon. Genotype 1 and 4 are the major HCV genotypes in Nigeria with novel sequences which have been designated provisionally as genotypes 1d and 4i (Faeji and Omilabu, 2015). Hepatitis C virus continues to be a disease burden in Nigeria and the world at large, and man is known to be its natural host (Oni *et al.,* 2007).

Association of HCV with HIV comes from infection among the same risk group namely intravenous drug abusers. Thus 240,000 people are concomitantly infected by HCV and HIV in the USA (Laurent *et al*., 2007). Although the prevalence of HCV infection among intravenous drug abusers is much higher than that of HIV infection; an alarming increases in the number of individuals infected with HCV and HIV are continually being reported. Multiple studies performed before antiretroviral therapy came into use have shown that while the course of HIV infection in people with HIV/HCV infection is not changed, and the course of HCV infection in people with HIV is more rapid (Laurent *et al*., 2007).

Alcoholism worsens the amount of liver damage from HCV infection. It is recommended that people with HCV infection should abstain from consumption alcohol (Laurent *et al*., 2007). Also, people with HCV infection should receive vaccination for hepatitis A or B, if they do not

already have immunity to these infections. This is because an acute case of hepatitis A or B can increase the severity of HCV infection and even be fatal (Laurent *et al*., 2007).

2.2.5 Diagnosis of Hepatitis C Virus

The common tests used for diagnosis of HCV infection were designed primarily for screening of blood donors. These assays are based on detection of serum antibody to various HCV antigens because these antibodies are nearly universally present in patients who are chronically infected with HCV. Acute HCV infections are relatively rare among blood donors, but the antibody tests often fail to detect these patients in the window period between the time of infection and the time of appearance of antibody detectable by the assay. Therefore, the antibody assays also have limited utility for diagnosis of patients with acute HCV infections. Tests for HCV RNA genome detection based on the PCR or other highly sensitive RNA detection systems have been used for the diagnosis of acute hepatitis. Although these tests are available for clinical testing and are being used in blood banking, they have not yet achieved final approval for these uses by the U.S. Food and Drug Administration (Fields *et al*., 2001).

2.2.5.1 Serologic (Antibody) Assays

Anti-HCV is the most important serological marker for identifying infection. It may not be detectable early in acute infection, but will develop in later serum samples. Anti-HCV is almost always present during chronic infection. In the presence of elevated liver tests, anti-HCV is highly specific and need not be confirmed by immunoblotting or HCV-RNA testing. However, in patients with normal liver enzymes, anti-HCV should be confirmed by one of these techniques. HCV-RNA determinations are usually not helpful for diagnosis other than in carriers with normal liver tests. They may be used, though, in following the response to treatment and modifying therapeutic regimens. Viral genotyping is currently of limited value in the clinic. It is

possible that future treatment will be modified according to viral genotype, HCV-RNA levels and the degree of hepatic injury (Haaheim *et al*., 2002).

2.2.6 Prevention of Hepatitis C Virus

Prevention of hepatitis C at present is based on prevention of exposure to contaminated blood by screening of blood and plasma donors, identification of carriers by testing high-risk individuals, and public health measures designed to prevent spread. No effective vaccine has been developed to prevent HCV infection. In developed countries, screening of blood donors has virtually eliminated transmission of HCV by transfusion. Even before the specific screening tests were available, HCV transmission by blood had been significantly reduced by elimination of paid donors, ridged donor screening implemented to reduce HIV transmission, and possibly the use of surrogate tests for hepatitis C infection (Zuckermann *et al*., 2009).

Source plasma for manufacture into various plasma products, such as clotting factors, is now all screened by the specific assays. Because these products are all made from pools containing plasma from thousands of donors, most of these pools continue to have low levels of HCV. Presently, these plasma-derived products all must undergo some specific process designed to eliminate or inactivate viruses, especially lipid-enveloped viruses that include HCV, HBV, and HIV. At the present time, plasma products are all considered to be free of infectious HCV (Zuckermann *et al*., 2009).

2.2.7 Therapy and Prophylaxis of Hepatitis C Virus

There is no vaccine or immunoglobulin to prevent HCV infection. The only agents with known activity are type I interferon (alpha and beta). More than half of patients permanently eradicate virus when treated with pegylated (long-acting) interferon and ribavirin for 6 to 12

months. The response and required duration of treatment is highly dependent on viral genotype (Haaheim *et al*., 2002).

2.3 Molecular Structure of HIV

HIV is different in structure from other retroviruses. It is roughly spherical with a diameter of about 120 nm, about 60 times smaller than a red blood cell, yet large for a virus (McGoven *et al.*, 2002). It is composed of two copies of positive single-stranded RNA that codes for the virus's nine genes enclosed by a conical capsid composed of 2,000 copies of the viral protein p24. The single-stranded RNA is tightly bound to nucleocapsid proteins, p7, and enzymes needed for the development of the virion such as reverse transcriptase, proteases, ribonuclease and integrase (Brooks *et al*., 2002).

A matrix composed of the viral protein p17 surrounds the capsid ensuring the integrity of the virion particle. This is, in turn, surrounded by the viral envelope that is composed of two layers of fatty molecules called phospholipids taken from the membrane of a human cell when a newly formed virus particle buds from the cell. Embedded in the viral envelope are proteins from the host cell and about 70 copies of a complex HIV protein that protrudes through the surface of the virus particle. This protein, known as Env, consists of a cap made of three molecules called glycoprotein (gp) 120, and a stem consisting of three gp41 molecules that anchor the structure into the viral envelope (Chan *et al.*, 2009). This glycoprotein complex enables the virus to attach to and fuse with target cells to initiate the infectious cycle. Both these surface proteins, especially gp120, have been considered as targets of future treatments or vaccines against HIV (NIH, 2006).

The RNA genome consists of at least seven structural landmarks (LTR, TAR, RRE, PE, SLIP, CRS, and INS), and nine genes (*gag*, *pol*, and *env*, *tat*, *rev*, *nef*, *vif*, *vpr*, *vpu*, and

sometimes a tenth *tev*, which is a fusion of tat env and rev), encoding 19 proteins. Three of these genes, *gag*, *pol*, and *env*, contain information needed to make the structural proteins for new virus particles (NIH, 2006). For example, *env* codes for a protein called gp160 that is broken down by a cellular protease to form gp120 and gp41. The six remaining genes, *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* (or *vpx* in the case of HIV-2), are regulatory genes for proteins that control the ability of HIV to infect cells, produce new copies of virus (replicate), or cause disease (Chan, *et al*., 2009; NIH, 2006).

The two Tat proteins (p16 and p14) are transcriptional transactivators for the LTR promoter acting by binding the TAR RNA element. The TAR may also be processed into microRNAs that regulate the apoptosis genes ERCC1 and IER3 (Klase *et al*., 2009; Ouellet *et al*., 2008). The Rev protein (p19) is involved in shuttling RNAs from the nucleus and the cytoplasm by binding to the RRE RNA element. The vif protein (p23) prevents the action of APOBEC3G (a cell protein that deaminates DNA: RNA hybrids and/or interferes with the Pol protein). The Vpr protein (p14) arrests cell division at G2/M. The nef protein (p27) down-regulates CD4 (the major viral receptor), as well as the major histocompatibility complex (MHC) class I and class II molecules (Various, 2008).

The vpu protein (p16) influences the release of new virus particles from infected cells.The ends of each strand of HIV RNA contain an RNA sequence called the long terminal repeat (LTR). Regions in the LTR act as switches to control production of new viruses and can be triggered by proteins from either HIV or the host cell. The Psi element is involved in viral genome packaging and recognized by Gag and Rev Proteins. The SLIP element (TTTTTT) is involved in the frameshift in the Gag-Pol reading frame required to make functional Pol (Various, 2008).

2.3.1 HIV Genome

The full HIV genome is encoded on one long strand of RNA. When the virus is integrated into the host's DNA genome (as a provirus) then its information too is encoded in DNA.

The genes in HIV's genome are as follows:

- gag (coding for the viral capsid proteins)
- pol (notably, coding for reverse transcriptase)

(NB. gag and pol together can be expressed in one long strand called "gag-pol")

- env (coding for HIV's envelope-associated proteins)

And the regulatory genes:

- tat
- rev
- nef
- vif
- vpr
- vpu (not present in HIV-2)
- vpx (not present in HIV-1)

The HIV genome also has a "Long Terminal Repeat" (LTR) at each end of its genome – not quite a gene, but a sequence of RNA/DNA which is the same at either end and which serves some structural and regulatory purposes.

2.3.1.1 Gag

Gag is one of the three "main" genes found in all retroviruses (along with envand pol). It contains around 1500 nucleotides, and encodes four separate proteins which form the building blocks for the viral core:

- Capsid protein, CA, p24
- Matrix protein, MA, p17 (this protein isn't actually part of the viral core but the "matrix" which anchors the core to the viral envelope)
- Nucleocapsid protein, NC, p9
- p6

The most significant role of the gag gene is therefore to encode important proteins which will make up the viral core (Various, 2008).

2.3.1.2 Pol

Pol is one of the main retroviral genes. It encodes four proteins, of which the most important is Reverse Transcriptase. Reverse Transcriptase performs a job which is unique to retroviruses, in that it copies the virus' RNA genome into DNA. (Since most organisms and viruses keep their genes in DNA form in the first place, they have no need to perform this task.) The copying of the HIV genome into DNA form is one of the key stages of the HIV life-cycle. The other three products of pol are these:

- Protease – which processes proteins made from HIV's genome so that they can become part of new fully-functioning HIV particles
- RNAse H – which breaks down the retroviral genome following infection of a cell

- Integrase – which integrates the DNA copy of HIV's genome into the host DNA (Various, 2008).

2.3.1.3 Env

The "env" gene in HIV encodes a single protein, gp160. When gp160 is synthesized in the cell, cellular enzymes add complex carbohydrates and turn it from a protein into a glycoprotein hence the name "gp160″ rather than "p160″. gp160 travels to the cell surface, where cellular enzymes again attack it, this time chopping into two pieces – gp120, and gp41. If and when new virus particles bud from the host cell, these two pieces lie opposite of the virus membrane. G120 sits on the outside of the virus particle, forming the virus's spikes, while gp14 sits just on the inside of the membrane each gp41 anchored to a gp120 through the membrane (Various, 2008).

2.3.1.4 Tat

"Tat" is short for "transactivator" – it's a regulatory gene which accelerates the production of more HIV virus. In fact, it's crucial to HIV, because HIV completely fails to replicate itself without it. Tat protein is also toxic, so the large amounts of tat protein released into the blood by HIV-infected cells are no help for the body. Tat works because the protein encoded by tat binds to the start of a new HIV RNAstrand – a part which has been called the "Transactivator Active Region" orTAR. The TAR runs from +1 to +59, that is to say, the first 59 nucleotides of the HIV genome (Various, 2008).

2.3.1.5 Rev

"Rev" is another of HIV's regulator genes. It stimulates the production of HIV proteins, but suppresses the expression of HIV's regulatory genes. The messenger RNAs of HIV can either be sent to the protein-producing part of the cell intact, or they can have bits cut out of them first (splicing). The intact mRNA tends to encode HIV proteins (such as envelope and capsid proteins), while the spliced mRNA encodes regulatory genes such as tatand nef (Various, 2008).

2.3.1.6 Nef

The "negative replication factor" ("nef") gene encodes a protein which hangs around in the cytoplasm of the cell, and retards HIV replication. Possibly it does this by modifying cellular proteins that regulate the initiation of transcription – that is, it affects the proteins which tell the cell whether or not to make RNAcopies of the DNA code (Various, 2008).

2.3.1.7 Vif

The "vif" gene codes for "virion infectivity factor", a protein that increases the infectivity of the HIV particle.The protein is found inside HIV-infected cells, and it works by interfering with one of the immune system's defences – a cellular protein called APOBEC3G. Basically what happens is that vif sticks to APOBEC3G and encourages the cell to degrade it, preventing it doing its job of sneaking into newly-formed virus particles and making them non-productive (Various, 2008).

2.3.1.8 Vpr

"Viral protein R" accelerates the production of HIV proteins. It also facilitates the nuclear localisation of the preintegration complex – the agglomeration of viral RNA and reverse

transcriptase and integrase proteins which must form in order for the HIV genome to be integrated into the host cell's genome. Vpr carries "nuclear localisation signals" (sequences of protein which are recognised by cellular machinery as indicating that it should be transported into the nucleus), and in a sense it mimicks the behaviour of a protein called importin-beta. There also seems to be a role for Vpr in stopping the host cell going through the ordinary "cell cycle" – many cells normally goes through a regular cycle of splitting to create new cells, but Vpr can stop host cells doing this. It seems that a cell which has been stopped during the so-called "G2″ phase of the cell cycle is a nicer environment for HIV replication (Various, 2008).

2.3.1.9 Vpu

"Viral protein U" helps with the assembly of new virus particles, and helps them to bud from the host cell. It's possible for HIV to replicate and bud without this particular protein, but only 10% or 20% as many new virus particles are produced. Vpu also works within the infected cell to enhace the degradation of CD4 proteins. This has the effect of reducing the amount of CD4 sticking out of the infected cell, therefore reducing the likelihood of superinfection. Without the vpu gene, HIV virus actually kills its host cell quicker! A secondary effect of vpu is to delay the cytopathic (cell-killing) effects of virus infection, keeping the cell alive slightly longer so that it can produce more virus particles (Various, 2008)

2.3.1.10 vpx

vpx is found in HIV-2 (and SIV), but not in HIV-1. It is closely related to vpr (if we compare their genetic sequences), which indicates that its existence might have come about as a duplication of the vpr gene (Various, 2008).

2.3.1.11 Long Terminal Repeat

The Long Terminal Repeat is something which is often found in strands of RNAor DNA is the Long Terminal Repeat. At each end of the string is the same sequence of code at each end of the string. Almost like the repeat at the start and finish of these sentences, almost like!

There are two important functions for the LTR:

- Firstly they are "sticky ends" (that's a biochemistry term) which the integraseprotein uses to insert the HIV genome into host DNA.
- Secondly, they act as promoter/enhancers – when integrated into the host genome, they influence the cell machinery which transcribes DNA, to alter the amount of transcription which occurs. Protein binding sites in the LTR are involved with RNA initiation (Various, 2008).

2.3.2 Genetics of HIV

HIV infects cells bearing the $CD4^+$ antigen receptor on their cell membrane for the attachment of the envelop protein, gp120 (Haedicke *et al*., 2009). Cells mostly implicated include T-helper lymphocytes, macrophages, monocytes, glial cells, langerhan cells, B lymphocytes, and other epithelial cell which may express $CD4^+$ molecules at lower density.

HIV enters macrophages and $CD4^+$ T cells by the adsorption of glycoproteins on its surface to receptors on the target cell followed by fusion of the viral envelope with the cell membrane and the release of the HIV capsid into the cell (Zheng *et al.,* 2005). Entry to the cell begins through interaction of the trimeric envelope complex (gp160 spike) and both $CD4^+$ and a chemokine receptor (generally either CCR5 or CXCR4). Gp120 binds to integrin a_4B_7, activating LFA-1, the central integrin involved in the establishment of virological synapses, which facilitate

efficient cell to cell spreading of HIV-1. The gp160 spike contains binding domains for both $CD4^+$ and chemokine receptors (AVLC, 2008). The first step in fusion involves the high-affinity attachment of the $CD4^+$ binding domains for gp120 and $CD4^+$. Once gp120 is bound with the $CD4^+$ protein, the envelop complex undergoes a structural change, exposing the chemokine binding domains of gp120 and allowing them to interact with the target chemokine recptor (AVLC, 2008 and Zheng *et al.*, 2005).

This leads to a more stable two-pronged attachment, which allows the N-terminal fusion peptide gp41 to penetrate the cell membrane. Repeat sequences in gp41, HRI, and HR2 then interact, causing the collapse of the extracellular portion of gp41 into a chapin. This loop structure brings the virus and the cell membranes close together, allowing fusion of the membranes and subsequent entry of the viral capsid (Haedicke *et al*., 2009; AVLC, 2008; Zheng *et al.*, 2005).

After HIV has bound to the target cell, the HIV RNA and various enzymes, including reverse transcriptase, integrin, ribonuclease and protease, are injected into the cell (Brooks *et al.*, 2002). During the microtubule-based transport to the nucleus, the viral single-strand RNA genome is transcribed into double-strand DNA, which is then integrated into a host chromosome (Zheng *et al.*, 2005).

HIV can infect dendritic cells (DC) by this CD4-CCR5 route, but other route mannose specific C-type lectin receptors such as DC-sign can also be used (AVLC, 2008). DC is one of the first cells encountered by the virus during sexual transmission. They are currently thought to play an important role by transmitting HIV to T-cells when the virus is captured in the mucosa by DC. The presence of a brain specific factor, FEZ-1, which occurs naturally in neurons is believed to

prevent the infection of cells by HIV (Haedicke *et al*., 2009). The process of reverse transcription is extremely error-prone, and the resulting mutations may cause drug resistance or allow the virus to evade the body immune system. The reverse transciptase also has ribonuclease activity that degrades the viral RNA during the synthesis of cDNA, as well as DNA-dependent DNA polymerase activity that creates a sence DNA, from the antisence cDNA (Haedicke *et al*., 2009).

Together, the cDNA and its complement form a double-stranded viral DNA that is then transported into the cell nucleus. The integration of the viral DNA into the host cell genome is carried out by another viral enzyme called integrase. The integrated viral DNA may lie domant in the latent stage of HIV infection. To actively produce the virus, certain cellular transcription factors need to be present, the most important being the NF kappa B (NF-$_k$B), which is upregulated when T-cells become activated (Rambaut *et al*., 2004). This implies that the cells most likely to be killed by HIV are those currently fighting infection.

During the viral replication, the integrated DNA provirus is transcribed nto mRNA, which is then spliced into smaller pieces. These small pieces are exported from the nucleus into the cytoplasm, where they are translated into regulatory proteins *Tat* and *Rev* (which encourages new virus production). As the newly produced Rev protein accumulates in the nucleus, it binds to viral mRNA and allows unspliced RNAs to leave the nucleus, where they are otherwise retaianed until spliced. At this stage the structural proteins *Gag* and *Env* are produced from the full-length mRNA. The full-length mRNA is actually the virus genome; it binds to the Gag protein and is packaged into new virus particles (Perelson and Ribeiro, 2008).

The life cycle of HIV can be as short as about 1-5 days from viral entry into a cell, through replication, assembly, and release of additional viruses, to infection of other cells. HIV

lacks *proof reading* enzymes to correct errors made when it converts its RNA into DNA via reverse transcription. Its short life cycle and high error rate cause the virus to mutate very rapidly, resulting in a high genetic variability of HIV. Most of the mutations either are inferior to the parent virus (often lacking the ability to reproduce at all) or convey no advantage, but some of them have a natural selection superiority to their parent and can enable them to slip past defenses such as the human immune system and antiretroviral drugs. The more active copies of the virus the greater the possibility that one resistant to antiretroviral drugs will be made (Fisher *et al.*, 2007). HIV-1 and HIV-2 appear to package their RNA differently; HIV-1 will bind to any appropriate RNA, whereas HIV-2 will preferentially bind to the mRNA that was used to create the *Gag* protein itself. This may mean that HIV-1 is better able to mutate as HIV-1 infection progresses to AIDS faster than HIV-2 infection and is responsible for the majority of global infections (Pope and Haase, 2003).

2.3.3 Pathogenesis of HIV

The major determinant in the pathogenesis and disease caused by HIV is the virus affinity for susceptible cells. HIV infects vital cells in the human immune system such as helper T cells (specifically $CD4^+$T cells), macrophages, and dendritic cells (Cunningham *et al*., 2010). HIV infection leads to low levels of $CD4^+$T cells through a number of mechanisms including: apoptosis of uninfected bystander cells, direct viral killing of infected cells, and killing of infected $CD4^+$T cells by CD8 cytotoxic lymphocytes that recognize infected cells (Garg *et al*., 2012; Kumar, 2012).

The human scavenger receptor gp340 has been identified as binding protein for the viral envelope that is expressed on the cell surface of female genital tract epithelial cells (Cannon *et al*., 2008). This interaction allows such epithelial cells to efficiently transmit infective virus to

susceptible targets and maintain viral infectivity for several days (Miller and Lu, 2003).

Within the context of vaginal transmission, HIV must first traverse a normally protective mucosa containing a cell barrier to reach the underlying T cells and dendritic cells, which propagate and spread the infection. The initial targets of infection in non-traumatic vaginal exposure to HIV have been identified as sub-epithelial T cells and dendritic cells (Miller *et al*., 2005; Miller and Shattonch, 2003). Breaks in the epithelial barrier caused by secondary infection with various STD or the normal physical trauma often associated with vaginal intercourse represent one potential means for viral exposure to sub-mucosal cells and have been shown to significantly increase transmission (Bobardt, 2007; Cohen, 2004). Several host cellular receptors, including DC-specific intercellular adhesion molecule – integrin, galactosyl ceramide, mannose receptor, langerin, heparin sulfate proteoglycans (HSPG), and chondrion sulfate proteoglycans, have been identified as facilitators of disease progression through binding of HIV without being required for fusion and infection (Kumar *et al*., 2006; Miller *et al*., 2005; Vives *et al*., 2005). These host accessory proteins act predominantly through glycosylation–based interactions between HIV envelopes and the host cellular receptors. These different host accessory factors can lead to increased infectivity in *cis* and *trans* or can serve to concentrate and expose virus at sites relevant to furthering its spread within the body (Kumar *et al*., 2006; Miller *et al*., 2005).

The hallmarks of HIV infection include chronic activation of the immune system and loss of $CD4^+$ T cells, which ultimately leaves affected individuals normally susceptible to opportunistic infections. Immune activation is multifaceted, including polyclonal B-cell activation, increased T-cell turnover, increased frequencies of T-cells with an activated phenotype, and increased serum levels of proinflammatory cytokines and chemokines (Picker and Watkins, 2005; Mehandru *et al*., 2004).

Many of the consequences of immune activation are deleterious to the immune and system of HIV-infected individuals. Firstly, high turnover of both $CD4^+$ and $CD8^+$ T cells imposes a strain on T cell homeostatic mechanisms with a decrease in the overall half-life of T cells (Brenchley and Douek, 2006). Secondly, damage to lymphoid tissue results in thymic dysfunction possibly attributed to IFN_a and TGF_B–mediated fibrosis (Mehandru *et al*., 2007). Fibrosis of the lymph nodes have been found to be associated with abnormal retention of effector type T cells while active immune activation results in abnormal T cell trafficking (Picker and Watkins, 2005).

2.3.4 Clinical Signs Associated with HIV Infection

A typical patient who is exposed to HIV goes through an acute viremia stage, followed by a phase of potent immune response during which the patient may do well for many years, until the immune system gives way, the viremia predominates and the patient progresses to AIDS. Non progressors, in contrast, do not suffer an acute phase and retain $CD4^+$ cell counts above 500 (Rowland, 2012).

Virus has been detected in various organs including testes and spleen but most notably in the brain and cerebrospinal fluid of AIDS patients with neoroloic findings (Bobardt *et al*., 2004). It appears that HIV plays a role in the pathogeness of encephalopathy or dementis, or both, in addition to its roles in causing the immune deficiency characteristic of the disease including leucopenia, Lymphopenia and suppressed T cell –functions (Migueles and Connors, 2010).

The serious deficiency in the cell – mediated immunity (CMI) leaves the infected person vulnerable to cancer and pathogens that are normally eliminated by CMI (Anonymous, 2004). Pneumocystis carinin pneumonia is by far the most common opportunistic infection, although tuberculosis, candidiasis, toxoplasmosis, and cytomegalovirus (CMV) infections are seen with

high frequency. About one-third of infected males contract a formally rare cancer called Kaposi sarcoma. The presence of the cancer and opportunistic diseases are often severe and are usually the cause of death in persons with AIDS (Migueles and Connors, 2010).

2.3.5 Laboratory Diagnosis of HIV

Currently, serological tests serve as the mainstay for the diagnosis and management of HIV (UNAIDS/WHO, 2009). Various serological tests for HIV which relies on *in-vitro* reaction between antigens and antibodies have been developed for both screening and confirmatory requirements.

The screening test is based on the identification of the HIV antibody from the serum of an infected person and generally involves the use of enzyme linked immunosorbent assay (ELISA) (Migueles and Connors, 2010). The confirmatory tests on the other hand include the Western blot assay, Radioimmunoassay (RIA), and the latest being molecular technique - Polymerase chain reaction (PCR) (Bartlett, 2006). The radioimmunoassay uses radioisotopes to detect and quantify the antibody-antigen interraction. It combines the specificity of the immune reaction with the sensitivity of radioisotope techniques. The quantity of antigen in a test solution can be determined when a known amount of radiolabelled antigen and a fixed amount of antibody is known and the radioactivity of any of the fractions can be measured (Bartlett, 2006).

Antibody tests may give false negative results because most people show detectable antibodies approximately 30 days after infection depending on the time for seroconversion and the testing population, while vast majority of people (97%), show detactable antibody after three months (Hare *et al*., 2004).

The ELISA combine the specificity of antibodies with the sensitivity of spectrophotometric enzyme assays by using either an antibody or an antigen linked to an enzyme at a site which does

not affect the activity of either compound. The enzyme is easily assayed by adding a substrate that yields a coloured product. The results of ELISA are reported as a number; the most controversial aspect of this test is determining the "Cut-off" point between a positive and negative result (Chou *et al*., 2005). Like the ELISA procedure, the Western blot is an antibody detection test. However, unlike the ELISA, the viral proteins are separated first and immobilized. In subsequent steps, the binding of serum antibodies to specific HIV proteins is visualized (Scand, 2008). ELISA testing alone cannot be used to diagnose HIV. The use of repeatedly reactive enzyme immunoassay followed by confirmatory Western blot or immunoflouorescent assay remains the standard method for diagnosis (Hare *et al*., 2004).

Rapid test kits are commercially available for measuring antibodies through the principle of enzymed-linked immunosorbent assay. These HIV antibody tests kits are simple to perform and give results in 10 minutes and are becoming increasingly used in diagnosis in small scale screening of donor blood. The rapid tests have sensitivities and specificities equal to ELISA and can be read visually without the need for a reader, and interpret with ready-made reagents (Abbas, 2010).

Some of the available commercial kits include Oral Quick which detects HIV 1 and 2 in blood, plasma or saliva; Oralsure which detects HIV in mucosal transudate from the tissues of checks and gums; UNI-Gold which is FDA approved for HIV-1 detection in blood; Home access express HIV -1 test which is the only FDA approved home test; Diagnostics rapid HIV test, as a rapid home test not yet confirmed by FDA and not authorized for sale in the USA; Reveal HIV, a rapid in vitro qualitative test for detecting antibodies to HIV in blood (UNAIDS/WHO, 2009; Owen *et al*., 2008; Chou *et al*., 2005).

Further to the laboratory diagnosis of HIV, other methods such as counting $CD4^+$ T lymphocyte, detection of HIV antigen as well as nucleic acid probes have been found effective. The $CD4^+$ T cell count is not specific HIV test, but rather a procedure where the number of $CD4^+$ T cells in the blood is determined (Armstrong and Taege, 2007). AIDS is officially diagnosed when the count drops below 200 cells/ul or when certain opportunistic infections occur. The value of 200 was chosen because it corresponds with a greatly increased likelihood of opportunistic infection and is associated with a variety of conditions, including many viral and bacterial infection, as well as malnutrition, over-exercise, pregnancy, genetic disorders, blood diseases and other factors (Chou *et al*., 2005).

The p24 antigen test detects the presence of the p24 protein of HIV (also known as CA), the capsid protein of the virus. Monoclonal antibodies specific to the p24 protein are mixed with the person's blood. Any p24 protein in the person's blood will stick to the monoclonal antibody and an enzyme- linked antibody to the monoclonal antibodies to p24 causes a color change if p24 was present in the sample (Hare *et al*., 2004). However, the p24 antigen is not useful for general diagnostics, as it has very low sensitivity and only works during a certain time period after infection before the body produces antibodies to the p24 protein (Hare *et al*., 2004).

2.3.6 Immunity to HIV Infection

Despite urgent need and tremendous scientific effort, researchers are yet to discover a vaccine for HIV that adequately protects humans from infection. Various experimental vaccines contain inactivated virus or protein from the envelope or capsid (Letvin, 2007; Deeks and Walker, 2007). The problem with vaccine development is that the viral antigenicity changes and there is a concern that a vaccine directed to a variable region on the virus would provide only

temporary protection (Deeks and Walker, 2007).

As at 2012 there is no effective vaccine for HIV or AIDS. A single trial of the vaccine RV 144, published in 2009 found a partial efficacy rate of 30% and has stimulated optimism in the research community regarding developing a truly effective vaccine. Further trials of the vaccine are ongoing (Reynell and Trkola, 2012). Many AIDS researchers believe that conquering this epidemic will require more than vaccines and effective chemotherapy. For reasons not completely understood, there are individuals who have developed a natural immunity to the virus without any medical intervention (Rowland, 2012). Immunological studies of people with natural immunity against HIV showed high level of specific cytotoxic T- lymphocyte (CTL) responses to both HIV –1 and 2 peptides. The presence of HIV- specific CTL, able to kill virus – infected cells, in apparently uninfected but repeatedly HIV exposed people indicates possible immunization by exposure to the virus. The finding suggests that cytotoxic T- cell generation may be the most important element in creating protective immunity against HIV. The observation of a naturally occurring protective immunity to HIV provides a new rational for vaccine development and emphasizes the importance of utilizing cytotoxic T- lymphocyte induction in the design pf AIDS vaccines (Rowland, 2012).

2.3.7 Epidemiology of HIV

An estimated 35.3 million people worldwide are living with HIV in 2012 with 2.3 million new cases; inferring a 33% decline in the number of new infections compared with 3.4 million in 2001 (UNAIDS, 2013). Sub-saharan Africa had the highest number of people living with HIV/AIDS (23.5%), followed by Asia (4.8 million); North America, Western and Central Europe had 2.3 million people while Eastern Europe and Latin America had 1.4 million people respectively (UNAIDS, 2012). Current Nigeria HIV prevalence stands at 3.7 million people

living with HIV/AIDS and is reported to be the second largest number of people living with HIV/AIDS in Sub-Saharan Africa (UNAIDS, 2012). The HIV epidemic in Nigeria is reportedly complex and varies widely with region or zones: North central region with the highest prevalence of 7.5% is followed by South South (6.5%), South East (5.1%), North East (4.0%) and South West (2.9%) while the least HIV rates is reported in North West Nigeria with 2.1% (FMOH, 2010). Urban areas in Nigeria like other counties in Sub- Saharan Africa had the highest rates compared with the rural areas. In a study (Bankole *et al*., 2011) among women of child bearing age in South South Nigeria, prevalence of HIV was recorded in 11.7% while 10.2% of pregnant women within these women were living with HIV/AIDS. In a retrospective study involving 10,032 pregnant women in Port Harcourt, South Southern Nigeria, about 5.93% were reported to be living with HIV/AIDS. But eighty percent (80%) of those within the age of 41-45 in that study were positive for HIV (Obi *et al*., 2007). In Southern Africa, where most countries were reported to have a large number of people living with HIV/AIDs, the number of people acquiring HIV has been drastically reduced. For example, between 2001 and 2011, in Malawi, the rate of new HIV infections dropped by 73%; in Botswana 71%, Namibia (68%), and Zambia (58%) while in Zimbabwe a 50% drop in incidence was recorded. In Swaziland where the world's highest HIV prevalence is recorded (26% in the general population, 69.9% in sex workers), reduction in number of new infections has been achieved by 37% (UNAIDS 2012). A recent demographic survey in Botswana reports an overall prevalence of 17.6% which was higher in the cities and towns (20.0%) than in urban villages and rural areas with 16.6% and 16.7% respectively. In South Africa, though HIV prevalence of 29.5% has been maintained consistently from 2007- 2011, the rate of new infections in the country represented by the prevalence rate among 15 to 19 years old has dropped from 14% in 2010 to 12.7% in

2011(Khumalo, 2012). Notable decrease between 2010 and 2011 has been recorded in Kwazulu-Natal, the province with the highest HIV prevalence in South Africa. This decrease from 39.5% (2010) to 37.4% (2012) was attributed to the hard work of the provincial branch of the national AIDS council and success of the government's HIV counseling, testing and circumcision campaigns (Khumalo, 2012).

Furthermore, an estimated 15.7% of Health care workers (HCW) employed in the public and private health facilities were found to be living with HIV/AIDS in the Republic of South Africa (Shisana *et al*., 2004). Infection was found higher in non-professionals (20.3%) than the professionals (13.7%). In another study, slightly lower HIV infection rates (11.5%) were reported among HCW by Connelly *et al.,* (2007) in South Africa and student nurses were recorded with the highest prevalence.

In Uganda, 11.3% of pregnant women in a rural district of Northern Uganda severely affected by civil strife were found living with HIV (Fabiani *et al*., 2006). In 2011, Cameroon demographic and health survey, reported 4.3% million adults to be living with HIV (L'Institut National de la statistique, 2011). They also found higher HIV prevalence in the urban (4.8%) than in the rural areas with 3.8%. In West Africa, Ghana top the list on HIV incidence reduction with 66%, followed by Burkina Faso with 60%. Despite a 25% reduction of HIV prevalence reported for Sub-Saharan Africa, the region accounted for 72% of all new cases of HIV infections worldwide in 2011 (UNAIDS, 2012). In Asia, a drastic reduction in incidence of HIV infections was recorded in Nepal (90%) and 88% in Cambodia. Notwithstanding, the epidemic significantly increased in Bangladesh, Indonesia, Philippines and Sri Lanka (UNAIDS, 2012).

In corroboration with the National (Nigeria) sentinel survey for North central states, HIV prevalence of 27.5% and 11.3% have been reported by Odimayo *et al*., (2010) and Adoga *et al*.,

(2010) among adults in a rural community in Benue state, and a rural population of women of child bearing age in Nasarawa State respectively. Similarly, 5.2% of HIV rates were observed among pregnant women in North east Nigeria; with the age of 30 – 39 years bearing the heaviest burden of 11.8% (Olokoba *et al*., 2009). In South West Nigeria, Imade *et al*., (2010) reported HIV prevalence of 5.2% among antenatal attendees in a tertiary health institution in Benin City, while 7.0% was observed among attendees of an STI clinic (AFRH) in Ibadan. In another study in South West Nigeria, a higher HIV antibody rate (11.7%) was detected in Institute of Human Virology Abeokuta, Ogun State, Nigeria (Motayo *et al*., 2012). In Benue State 27.5% of HIV antibodies detected among adults in a rural community (Odimayo *et al*., 2010), corroborates consistent higher National (Nigeria) HIV sentinel surveys reports for Benue State which is far above other states in the Federation -16.8% in 1999, 13.5% in 2001, 10,05 in 2005, 10.6% in 2008 and 12.7% in 2010 (FMOH, 2010). There is no epidemiological evidence that HIV is transmitted through water or food, sharing eating utensils, coughing or sneezing, toilets, insect bites, shaking of hands or other casual contact. However there is a strong indication that the concentration of HIV in genital secretions determines the probability of transmission (Berlier *et al*., 2005).

2.3.8 Treatment of HIV Infection

Antiretroviral drug treatment guidelines have changed over time. Before 1987, no antiretroviral drugs were available and treatment consisted of treating complications from the immunodeficiency. After antiretroviral medications were introduced, most clinicians agreed that HIV positive patients with low $CD^{+}4$ counts should be treated, but no consensus formed as to whether to treat patients with high $CD^{+}4$ counts. In 1995, "hit hard, hit early" approach with aggressive treatment and multiple antiretrovirals early in the course of the infection was

promoted. Later reviews noted that this approach of "hit hard, hit early" ran significant risks of increasing side effects and development of multidrug resistance, and this approach was largely abandoned (Dybul *et al*., 2002).

Currently, the management of HIV/AIDS normally includes the use of multiple antiretroviral drugs in an attempt to control HIV infection. There are several classes of antiretroviral agents that act on different stages of the HIV life-cycle. The use of multiple drugs that act on different viral targets is known as highly active antiretroviral therapy (HAART). HAART decreases the patient's total burden of HIV, maintains function of the immune system, and prevents opportunistic infections that often lead to death (Dybul *et al*., 2002).

There are several classes of drugs, which are usually used in combination to treat HIV infection. Use of these drugs in combination is generally termed ARTs or Anti-Retroviral Therapy. Anti-retroviral (ARV) drugs are broadly classified by the phase of the retrovirus life-cycle that the drug inhibits. Typical combinations include 2 NRTIs (Nucleoside Reverse Transcriptase Inhibitors) + 1 PI (Protease Inhibitor) or 2 NRTIs + 1 NNRTI (Non-Nucleoside Reverse Transcriptase Inhibitor) (Quashie, 2013).

Entry inhibitors (or fusion inhibitors) interfere with binding, fusion and entry of HIV-1 to the host cell by blocking one of several targets. Maraviroc and enfuvirtide are the two currently available agents in this class. Maravoric works by targeting CCR5, a co-receptor located on human helper T-cells (Sharon and Lieberman, 2008). To prevent fusion of the virus with the host membrane, Fuzeon (T20) can be used. Fuzeon is a peptide drug that must be injected and acts by interacting with the N-terminal heptad repeat of gp41 of HIV to form an inactive hetero six-helix bundle, therefore preventing infection of host cells (Bai Yu, 2013). Nucleoside reverse transcriptase inhibitors (NRTI) and nucleotide reverse transciptase inhibitors (NtRTI) are

nucleoside and nucleotideanalogues which inhibit reverse transcription. NRTIs are chain terminators such that once incorporated, work by preventing other nucleosides from also being incorporated because of the absence of a 3' OH group; they both act as competitive substrate inhibitors. Examples of NRTIs include deoxythymidine, zidovudine, stavudine, didanosine, zalcitabine, abacavir, lamivudine, emtricitabine, and tenofovir (Kalyan, 2013).

Non-Nucleoside reverse transcriptase inhibitors (NNRTI) inhibit reverse transcriptase by binding to an allosteric site of the enzyme; NNRTIs act as non-competitive inhibitors of reverse transcriptase. NNRTIs affect the handling of substrate (nucleotides) by reverse transcriptase by binding near the active site and causing "molecular arthritis". NNRTIs can be further classified into 1st generation and 2nd generation NNRTIs. 1st generation NNRTIs are more rigid in structure and resistance can quickly be developed against them. Because 2nd generation NNRTIs have a more flexible structure, they can adjust more readily and resist mutation more effectively. NNRTIs, for example, include nevirapine, delavirdine, efavirenz, and rilpivirine (Kalyan, 2013).

Integrase inhibitors inhibit the enzyme integrase, which is responsible for integration of viral DNA into the DNA of the infected cell. There are several integrase inhibitors currently under clinical trial, and raltegravir became the first to receive FDA approval in October 2007. Raltegravir has two metal binding groups that compete for substrate with two Mg^{2+} ions at the metal binding site of integrase. Another clinically approved integrase inhibitor is Elvitegravir (Quashie, 2013).

Antiretroviral combination therapy defends against resistance by suppressing HIV replication as much as possible. Combinations of antiretrovirals create multiple obstacles to HIV replication to keep the number of offspring low and reduce the possibility of a superior mutation. If a mutation that conveys resistance to one of the drugs being taken arises, the other drugs

continue to suppress reproduction of that mutation. With rare exceptions, no individual antiretroviral drug has been demonstrated to suppress an HIV infection for long; these agents must be taken in combinations in order to have a lasting effect. As a result, the standard of care is to use combinations of antiretroviral drugs. Combinations usually comprise two nucleoside-analogue RTIs and one non-nucleoside-analogue RTI or protease inhibitor. This three drug (3-drug) combination therapy is commonly known as a triple cocktail (DHHS, 2009 and USDHHS, 2004).

2.3.9 Prevention and Control of HIV Infection

Currently, no cure for HIV/AIDS exists. The most universally recommended method for the prevention of HIV/AIDS is to avoid blood-to-blood contact between people and to otherwise practice safe sex. Many governments and research institutions participate in HIV/AIDS control and prevention. This research includes behavioral health interventions, such as research into sex education, and drug development, which include microbicides for sexually transmitted diseases, HIV vaccines, and antiretroviral drugs.

Other medical research areas include the topics of pre-exposure prophylaxis, post-exposure prophylaxis, circumcision and use of condoms (Todar, 2009). Of these, the only universally medically proven method for preventing the spread of HIV during sexual intercourse is the correct use of condoms, and condoms are also the only method promoted by health authorities worldwide (Crosby and Bounse, 2012).

Increased risk of contracting HIV often correlates with infection by other diseases, particularly other sexually transmitted infections. Medical professionals and scientists recommend treatment or prevention of other infections such as herpes, hepatitis A, hepatitis B,

hepatitis C, human papillomavirus, syphilis, gonorrhea, and tuberculosis as an indirect way to prevent the spread of HIV infection. For HIV positive mothers wishing to prevent the spread of HIV to their child during birth, antiretroviral drugs have been medically proven to reduce the likelihood of the spread of the infection. Early treatment of HIV-infected people with antiretrovirals protected 96% of partners from infection (Anglemyer *et al*., 2011). Pre-exposure prophylaxis with a daily dose of the medications tenofovir with or without emtricitabine is effective in a number of groups including: men who have sex with men, by couples where one is HIV positive, and by young heterosexuals in Africa (Siegfried *et al*., 2009).

A course of antiretrovirals administered within 48 to 72 hours after exposure to HIV positive blood or genital secretions is referred to as post-exposure prophylaxis. The use of the single agent zidovudine reduces the risk of subsequent HIV infection five-fold following a needle stick injury (Siegfried *et al*., 2009). Treatment is recommended after sexual assault when the perpetrator is known to be HIV positive but is controversial when the HIV status is unknown (Young *et al*., 2007). Current treatment regimes typical use lopinavir or ritonavir and lamivudine or zidovudine or emtricitabine/tenofovir and may decrease the risk further (Siegfried *et al*., 2009).

Social strategies do not require any drug or object to be effective, but rather require persons to change their behavior in order to gain protection from HIV. Some social strategies which people consider include the following: sex education, needle-exchange programmes, safe injection sites, safe sex, sexual abstinence, and immigration regulation. Populations which receive HIV testing are less likely to engage in behaviors with high risk of contracting HIV. HIV testing is almost always a part of any strategy to encourage people to change their behavior to become less likely to contract HIV. Over 60 countries impose some form of travel restriction,

either for short or long term stays, for people infected with HIV (Crosby and Bounse, 2012).

Circumcision in sub-Saharan Africa reduced the risk of HIV infection in heterosexual men by between 38 – 66% over two years. Based on these studies, the World Health Organization and UNAIDS both recommended male circumcision as a method of preventing female-to-male HIV transmission in 2007 (WHO, 2007). Whether it protects against male-to-female transmission is disputed (Kim and Goldstein, 2010), and whether it is of benefit in developed countries and among homosexuals is undetermined (Templeton *et al*., 2010). Some experts fear that a lower perception of vulnerability among circumcised men may result in more sexual risk-taking behavior, thus negating its preventive effects. Women who have undergone female genital cutting have an increased risk of HIV (Billing and Kentenich, 2008).

Universal precautions within the health care environment are believed to be effective in decreasing the risk of HIV. Intravenous drug use is an important risk factor and harm reduction strategies such as needle-exchange programmes and opioid substitution therapy appear effective in decreasing this risk. Needle exchange programs (also known as syringe exchange programs) are effective in preventing HIV among IDUs as well as in the broader community (WHO, 2012).

Programs to prevent the transmission of HIV from mothers to children can reduce rates of transmission by 92-99% (WHO, 2012 and Seigfried *et al*., 2011). This primarily involves the use of a combination of antivirals during pregnancy and after birth in the infant. It also potentially include ceasarean section, and bottle feeding rather than breastfeeding (Seigfried *et al*., 2011). The current WHO recommended regimen is as follows: Zidovudine (AZT) from 28 weeks or as soon as possible thereafter, with Nevirapine (NVP) when entering labour, and AZT+3TC for one week following delivery. The child should be given single dose Nevirapine immediately after delivery and daily Zidovudine until one week old (Coutsoudis *et al*., 2010).

Three quarters of people with HIV infection are in their reproductive years and may consider pregnancy planning. In cases where the woman is HIV negative and the man is HIV positive, the primary assisted reproductive method used to prevent HIV transmission is sperm washing followed by intrauterine insemination (IUI) or *in vitro* fertilization (IVF). There is no described case of seroconversion in women or their offspring after such sperm washing, but it is yet not certain whether the method is completely safe (Macaline *et al*., 2009). For cases where the woman is HIV positive and the man is HIV negative, the usual method is artificial insemination. With appropriate treatment the risk of mother-to-child infection can be reduced to about 1% (Macaline *et al*., 2009).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study was conducted in parts of the South East geo-political zone of Nigeria, comprising five (5) States namely: Abia, Anambra, Ebonyi, Enugu and Imo. It has a land area of about 41,440 km^2 with an estimated population of 16,381,729 (United Nations Department of Economic and Social Affairs, 2012). The study area is located in 5^o00'N, 7^o00'E and 7^o30'N, 8^o30'E (Figure 3.1). The climate of the study area has two main regimes; dry (November-February) and rainy or wet (March-October) seasons. Rainfall in the study area is between 1800 - 2700 mm and average temperature of 28±2^{o}C (Felix and Adebayo, 2013). The study area has inhabitants who are predominantly farmers, traders, civil servants, cyclist riders and students.

3.2 Study Population and Sample Size

The study was a hospital based type conducted within the period of February, 2016 to November, 2017. The study population included 2,500 HIV patients on Highly Active Antiretroviral Therapy (HAART) who attended Federal Medical Centre Umuahia, Federal Medical Centre Owerri, Federal Teaching Hospital Abakaliki, Enugu State University Teaching Hospital and Chukwuwemeka Odumegwu Ojukwu University Teaching Hospital Awka during the period of the study. Five hundred samples were sought from each of the hospitals mentioned. A Cross sectional study was carried out from 1,400 males and 1,100 females in this study. In order to allow for valid analyses and to provide the desired level of accuracy in estimates of proportions, the sample size was determined according to Araoye (2004) using the formula;

$$N = Z^2 pq/d2$$

Where: N is the sample size, Z= standard deviation at 95% confidence interval (1.96), p is the proportion to be used on estimation (12.5%) (Alo *et al*., 2013), d = degree of accuracy/precision expected (0.05), q=1-p

3.3 Experimental Design

A Cross sectional study was carried out from 2,500 HIV patients attending the above mentioned Federal and State owned Government hospitals. Samples were collected from Monday to Fridays in the morning hours of 8:00 a.m. – 11:00 a.m. except on public holidays and weekends. During this research, sample collection and analysis were done for a period of 21 months ranging from February, 2016 to November, 2017. These samples were analyzed for HBV, HCV single infections, HBV/HCV co-infections as well as the associated risk factors obtained from responses to the structured questionnaires. Samples of the same patients were collected three times over a period of 18 months for determination of haematological and biochemical baseline. In this sample population, HBV-negative and and HCV-negative subjects were included as controls.

Plate 3.1: Map showing South East of Nigeria

Source: http://maps.google.com

3.4 Ethical Approval

The study was approved by the Ethical and Research Committees of the various Hospitals used in the study (see appendix iv-viii). Informed consent was also obtained from all participating patients. For subjects under 18 years, parental consent was sought and obtained.

3.5 Inclusion and Exclusion Criteria

3.5.1 Inclusion Criteria

HIV patients of <10 - >70 years of age, male and female sexes who attended the hospital during the course of the study, after given a verbal consent to participate in the study were included. HIV patients, who are on Highly Active Antiretroviral Therapy (HAART), were included in this study.

3.5.2 Exclusion Criteria

HIV patients who refused consent and, also not on Highly Active Antiretroviral Therapy HAART were excluded from this research.

3.6 Questionnaire Administration

Questionnaires were used in this study to collect data from each patient (see appendix i). All the patients who fulfilled the inclusion criteria were interviewed in detail and the data was recorded on a prescribed questionnaire. The data consisted of participant's demographic variables as (Age, sex, marital status, occupational status), and predisposing risk factor (previous history of blood transfusion, tribal mark/tattoos, intravenous drug users and educational status) for contracting both hepatitis B and C virus.

3.7 Collection of Blood Samples

A 15ml specimen of blood was aseptically collected from each patient using a sterile vacutainer and from a prominent vein situated at the antecubital fossa (venipuncture) of each patient. The blood specimen were transported in insulated containers containing ice packs to Safety Molecular and Pathology Laboratory Enugu where the plasma and serum of each sample was separated from the whole blood and placed in non-EDTA bottle. It was stored in an industrial freezer for preservation.

3.8 Confirmation of HIV Status

3.8.1 Assay Procedure

All reagents and samples were allowed to reach room temperature (28±2^{o}C) for at least 15-30 minutes. The Wash buffer was checked for concentrate, for the presence of salt crystals. If crystals had formed in the solution, resolubilization by warming was done at 37°C until crystals dissolved. Wash Buffer was diluted, 1 in 20 with distilled or deionized water.

The strips needed were set in strip-holder and it was numbered sufficiently including three for the Negative controls (e.g. B1, C1. D1), two for the Positive controls (e.g. E1, F1) and one Blank (e.g. A1, neither samples nor HRP- Conjugate was added into the Blank well). 100 µl of Positive controls, Negative controls, and Specimen were added into their respective wells. The plates were covered with the plate cover and incubated for 30 minutes at 37°C. At the end of the incubation, the plate cover was removed and discarded. Each well was washed 5 times with diluted Wash buffer. Each time, the microwells were allowed to soak for 30-60 seconds. After the final washing cycle, the plates were turned down on a clean towel, and tapped as to remove any remaining liquids. 100µl of HRP- Conjugate was added into each well except in the Blank. The plates were covered with the plate cover and incubated for 30 minutes at 37°C.

At the end of the incubation, the plate cover was removed and discarded. It was washed well 5 times with diluted Wash buffer. 50µl of Chromogen A and 50µl Chromogen B solution was added into each well including the Blank, covered with a plate cover and mixed by tapping the plate gently. It was incubated at 37°C for 15 minutes avoiding light. The enzymatic reaction between the Chromogen solutions and the HRP-Conjugate produced blue color in positive control. Plate cover were removed and discarded. Using a multichannel pipette, 50µl of Stop Solution was added into each well and mix gently. Intensive yellow color develops in Positive control and HIV positive sample wells. The plate reader were calibrated with the Blank well and read at absorbance of 450nm.

3.9 Determination of Immunological Profile of Patients

3.9.1 $CD4^+$ T-cell Counts

$CD4^+$ count was determined using CD4 Easy count kit, Partec code no. 05-8401, and CyflowCounter (Partec, GmBH. Otto. Hahn Starbe. D- 48161, Munster- Germany). The test kit consists of monoclonal antibodies which are specific for $CD4^+$ antigen, No lyses buffer, test tubes (Partec code: 04-2000), decontaminating fluid, clean solution and sheath fluid. The counting and identification of $CD4^+$ T Lymphocytes (subsets of white blood cells) in whole blood samples was carried out for each HIV infected blood sample.

Whole blood collected from patients who tested HIV positive were put into vacutainers which were placed on rollers to mix and homogenize. After this, 20µl of the whole blood was dispensed into the Partec test tube changing pipette tip for each sample. Then 20µl of CD4 monoclonal antibodies PE (incooporated with chromogens) were added to each specimen, so that CD4 antigens will bind, thereby forming a complex. After gently tapping the mixture it was placed in a carton to protect it from sunlight and incubated for 15minutes at room temperature

(~25^{0}C). Then 800µl of no lyse buffer was added and the mixture was shaken vigorously. The preparation yielded 840µl of the mixture comprising of no lyse buffer, specimen and monoclonal antibodies. Observing peaks generated on the screen, the CD4+ count value was read directly on the machine and recorded. Based on a preset dilution factor in counts / ul, absolute CD4+ T-Lymphocyte count was expressed in cells/ul. The count results in cells/µl are the concentration of the CD4+ lymphocytes per µl of the original whole blood samples.

3.10 Determination of Haematological Profile of Patients

3.10.1 Haemoglobin Test (Hb Test)

The test was done using Haemocue Haemoglobin meter 301. 10 µl of venous blood was pipetted and added into the haemocue Hb 301 micro-cuvette. The micro-cuvette was placed in the cuvette's holder, after 40-60 seconds the readings were displayed in gram per decilitre (g/dl).

3.11 Alanine Aminotransaminase (ALT) Activity

The activity of (ALT) was determined by the Reitman-Frankel colorimetric method (Reitman and Frankel, 1957) for *in vitro* determination of GPT/ALT in serum using a Quimica Clinica Applicada (QCA) test kit.

3.11.1 Test Principle

Alanine aminotransaminase also called glutamic-pyruvate transaminase (GPT) catalyses the transfer of α-amino group from alanine to α-ketoglutarate with the release of pyruvate and glutamate.

L- alanine + α-oxologlutarate $\xrightarrow{ALT}$ pyruvate + L – glutamate - - - -I

Pyruvate + Reduced Cofactor + H^+ → Lactate + Cofactor - - - - II

ALT activity was measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine which is proportional to its concentration at 546 nm.

3.11.2 Reagents

GPT substrate solution (Reagent A): containing phosphate buffer pH 7.4 and α- ketoglutaric acid and L-alanine.

Colour developer (Reagent B): containing 2, 4–dinitrophenylhydrazine (DNPH)

NaOH 4N- This was diluted 1/10 with deionized water prior to use (Reagent C).

Standard (Reagent D): aqueous solution of sodium pyruvate.

3.11.3 Methodology

To each of the test tubes were added 0.5 ml of Reagent A (ALT substrate solution) and incubated for 5 min at 37℃. After incubation 0.1 ml of each of the serum samples was then added. The test tubes were incubated for 30 mins at 37°C. The standards were prepared as follows;

Tube 1 – 0.1 ml deionised water + 0.5 ml reagent A

Tube 2 - 0.1 ml deionised water + 0.45 ml reagent A+ 0.05 ml of standard

Tube 3 - 0.1 ml deionised water + 0.40 ml reagent A+ 0.10 ml of standard

Tube 4 - 0.1 ml deionised water + 0.35 ml reagent A+ 0.15 ml of standard

Tube 5 - 0.1 ml deionised water + 0.30 ml reagent A+ 0.20 ml of standard

A 0.5 ml of colour developer (Reagent B) was then added to both sample tubes and the standards. They were allowed to stand for 20 min at room temperature. After, 5 ml of NaOH working solution (diluted reagent C) was added and they were left to stand for 15 min at room temperature. The absorbencies of both samples and standards were read at 546 nm against deionised water blank within 1 hour. To obtain ALT activity/ value of the samples were

intrapolated in the calibration curve made from thestandards; the results were expressed in SI units [International units per litre (IU/L).

3.12 Aspartate Aminotransferase Activity (AST)

AST activity was determined by the Reitman – Frankel colorimetric method (Reitman and Frankel, 1957) for *in vitro* determination of GOT/AST in serum using a Quimica Clinica Applicada (QCA) test kit

3.12.1 Principle

Aspartate aminotransferase (AST) formerly called glutamate–oxaloacetate transaminase (GOT) is measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4-dinitrophenylhydrazine. The enzyme catalyzes the transfer of the α- amino group from aspartate to α- ketoglutarate with the release of oxaloacetate and glutamate

L-aspatate + α- ketoglutarate $\xrightarrow{AST}$ oxaloacetate + L- glutamate- - - -I

Oxaloacetate + NADH + H^+ → Malate+ NAD^+ - - - - -II

AST activity was measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4 – dinitrophenylhydrazine spectrophotometrically at 546 nm.

3.12.2 Reagents

AST substrate solution (Reagent A): containing phosphate buffer pH 7.4 and α- ketoglutaric acid and L-Aspartatic acid.

Colour developer (Reagent B), containing 2, 4–denitrophenylhydrazine (DNPH)

NaOH (4N) – This was diluted 1/10 with deionized water prior to use (Reagent C).

Standard (Reagent D): aqueous solution of sodium pyruvate.

3.12.3 Methodology

To each of the test tubes were added 0.5 ml of Reagent A (AST substrate solution) and incubated for 5 min at 37°C. After incubation 0.1 ml of each of the serum samples was then added. The test tubes were incubated foe 60 mins at 37℃. The standards were prepared as follows;

Tube 1 – 0.1 ml deionised water + 0.5 ml reagent A

Tube 2 - 0.1 ml deionised water + 0.45 ml reagent A+ 0.05 ml of standard

Tube 3 - 0.1 ml deionised water + 0.40 ml reagent A+ 0.10 ml of standard

Tube 4 - 0.1 ml deionised water + 0.35 ml reagent A+ 0.15 ml of standard

Tube 5 - 0.1 ml deionised water + 0.30 ml reagent A+ 0.20 ml of standard

A 0.5 ml of colour developer (Reagent B) was then added to both sample tubes and the tandards. They were allowed to stand for 20 min a troom temperature. After, 5 ml of NaOH working solution (diluted reagent C) was added and they were left to stand for 15 min at room temperature. The absorbencies of both samples and standards were read at 546 nm against deionised water blank within 1 hour. To obtain AST activity/ value of the samples were intrapolated in the calibration curve made from the standards; the results were expressed in SI units [International units per litre (IU/L).

3.13 Alkaline Phosphatase (ALP) Activity

Phenolphthalein monophosphate method (Klein *et al*., 1960), for the *in vitro* determination of alkaline phosphatase in serum, using Quimica Clinica Applicada (QCA) test kit.

3.13.1 Principle

Alkaline phosphatase acts upon the AMP-buffered sodium thymolphthalein monophosphate. Addition of the alkaline reagent stops the enzyme activity and simultaneously develops a blue chromagen which can be measured photometrically at wavelength of 550 nm.

3.13.2 Reagents

Alkaline phosphatase chromogenic substrate, colour developer, standard solution of alkaline phosphatase in water (equivalent to 30 IU/L).

3.13.3 Methodology

The colour developer was prepared by adding one vial of colour developer salt to 250 ml of deionized water. Deionized water 0.1 ml was added to a clean test tube, one drop of chromogenic substrate was added, mixed and incubated at 37^0C for 5minutes. Serum sample (0.1ml) was added to the test tube, mixed and incubated at 37^0C for 20 minutes. 5ml of colour developer was added. The absorbance was read against a water blank at wavelength of 550nm.

For the standard, 1 ml of water was added to a test tube and one drop of the chromogenic substrate added. It was mixed and incubated at 37^0C for 20 minutes. Colour developer (5ml) was added and absorbance read at 550 nm.

3.14 Determination of Hepatitis B Seromarker (HBsAg)

3.14.1 Test Principle for HBsAg

It uses antibody "Sandwich" ELISA method in which, polystyrene microwell strips are pre-coated with monoclonal antibodies specific to HBsAg. Patient's plasma sample is added to the microwell together with a second antibody conjugated the enzyme horseradish peroxidase (the HRP-Conjugate) and directed against a different epitope of HBsAg. During incubation, the specific immunocomplex formed in case of presence of HBsAg in the sample, is captured on the solid phase. After washing to remove sample serum proteins and unbound HRP-conjugate, Chromogen solutions containing tetramethyl-benzidine (TMB) and urea peroxide are added to the wells. In presence of the aniibody-antigen-antibody (HRP) "sandwich" immunocomplex, the colorless Chromogens are hydrolyzcd by the bound HRP-conjugatc to a blue-colored product. The blue color turns yellow after stopping the reaction with sulfuric acid. The amount of color

intensity can be measured and it is proportional to the amount of antigen captured in the wells, and to its amount in the sample respectively. Wells containing samples negative for HBsAg remain colorless.

3.14.2 Assay Procedure

The reagents and samples were allowed to reach room temperature (18~30^{0}C) for at least 15-30minutes. The Wash buffer was checked for the presence concentrate of salt crystals. If crystals were formed in the solution, resolubilization by warming at 37^{0}C was done until crystals dissolve. The stock wash Buffer was diluted in 1 to 20 with distilled or deionized water. Only clean vessels were used to dilute the buffer. The strips needed were set in strip-holder, and number sufficient number of wells including three for the Negative control (e.g. B1, C1, D1), two for the Positive control (e.g. E1**,** F1) and one Blank (e.g. A1, neither samples nor HRP-Conjugate was added into the Blank well). 50µl of Positive control was added. Negative control and specimen were added into their respective wells. Note: a separate disposal pipette tip was used for each specimen, Negative and Positive Control as to avoid cross-contamination). 50µl HRP-Conjugate were added in to each well except the Blank, and mixed by tapping the plate gently.The plate was incubated by covering with the plate cover and incubated for 60 minutes at 37°C. At the end of the incubation, the plate cover was removed and discarded. Each well was washed 5 times with diluted Wash buffer. Each time plates were allowed to soak for 30-60 seconds. After the final washing cycle, it was turned down on to blotting paper or clean towel, and tapped to remove any remainders. 50µl of Chromogen A and 50µl Chromogen B solution were dispensed into each well including the Blank, and mixed by tapping the plate gently. The plates were incubated at 37°C for 15 minutes avoiding light. The enzymatic reaction between the Chromogen solutions and HRP Conjugate produced blue color in positive control and

HBsAg positive specimen wells. 50µl Stop Solution was added into each of the well using a multichannelled pipette and mixed gently. Intensive yellow color developed in Positive control and HBsAg positive sample wells. The plate reader was calibrated with the Blank well and read at the absorbance of 450nm. The Cut-off values were calculated and the results recorded. (Note: the absorbance was read within 10 minutes after stopping the reaction).

3.15 Determination of Hepatitis B Seromarker (Anti-HBcIgM)

3.15.1 Test Principle

The assay is based on an "Immuncapture Sandwich" principle. The wells of microelisa have been coated with anti-human IgM. A positive test sample incubated in such well, all IgM-class Abs will bind to the solid phase, upon the addition of conjugate (HBcAg anti-HBs labeled with HRP). After incubation and washing to remove unbound material, an enzyme substrate solution containing a chromogen is added that will develop to a blue color if the sample is positive, which turn to yellow when the reaction is blocked.

3.15.2 Assay Procedure

An aliquot of 100µl of samples (negative, positive control and calibrator) was dispensed into assigned wells and incubated at 37 ºC for 120 minutes, then washing was carried out 5 times with washing buffer. Thereafter, 100µl of conjugate was dispensed into each well. After covering and mixing gently, the plate was incubated at 37 ºC for 60 minutes, and then the plate was washed 5 more times. Then, 100µl of substrate-chromogen solution was added into each well, the plate was incubated at 20-25 ºC for 30 minutes, and the reaction was blocked with 100µl of 1N H_2SO_4. The photometric reader was set at 450 nm.

3.16 Determination of Hepatitis B Seromarker (Anti-HBs)

3.16.1 Test Principle

The bioelisa anti-HBs kit is an ELISA test for detection of antibodies to HBsAg in human serum or plasma. It is a direct immunoenzymatic method of the "Sandwich" type. The sample was incubated in microplate coated with HBsAg (ad and ay subtypes). If a sample contains anti-HBs, it will bind to the HBsAg after incubation. When the HBsAg conjugate to peroxidase it will bind to antigen- antibody complex formed during first incubation. After second incubation and washing, an enzyme substrate containing chromogen is added then a blue color is developed if the sample is positive, the color changes after blocking the reaction with sulphuric acid. The intensity of the color is proportional to the concentration of anti-HBs present in the sample.

3.16.2 Assay Procedure

An aliquot of 100µl of each sample and controls were dispensed into assigned well, and then the plate was incubated at 37 ºC for 60 min. The plate was washed with the diluted washing solution 4 times. Then, 100µl of conjugate was added to each well except the blank well; the plate was incubated at 37 ºC for 30 min., and then re-washed 4 times. Thereafter, 100µl of substrate-TMB was added to each well, including blank, and the plate was incubated at 20-25ºC for 30 mins, then the reaction was blocked by adding 100µl of 1mol / N H_2SO_4.The photometric reader was set at 450 nm.

3.17 Determination of Hepatitis B Seromarker (Anti-HBc Total)

3.17.1 Test Principle

Bioelisa anti-HBc is an ELISA test for detection of total antibody to HBcAg in human serum or plasma. The assay is based on a competition between human antibodies present in the

sample and rabbit IgG anti-HBc conjugate to peroxidase (HRP) when simultaneously incubated in a well coated with recombinant HBcAg. After incubation an enzyme substrate solution containing a chromogen is added, that will develop a blue color if the sample is negative. The blue color changes to yellow after blocking the reaction with sulphuric acid. The assay can be summarized as follow:

3.17.2 Assay Procedure

An aliquot of 50µl (serum samples, negative control and positive control) were added into assigned wells and 50µl of conjugate were dispensed into each well, except one reserved for blank. After covering and mixing gently, the plate was incubated at 37 ºC for 60 min., and then washed 4 times. Thereafter, 100µl of substrate-TMB was pipetted into each well then was incubated at 20-25ºC for 30 min. Then 100µl of stopping solution (1N H_2SO_4) was added. The photometric reader was set at 450 nm.

3.18 Determination of Hepatitis C Seromarker (Anti-HCV)

3.18.1 Test Principle Anti-HCV

This kit is a two-step incubation enzyme immunoassay, which uses polystyrene microwell strips pre-coated with recombinant HCV antigens expressed in *E.coli* (recombinant Core and NS3/4/5). Patient's serum or plasma sample is added together with biotin-conjugated HCV antigens. During the first incubation step, the specific HCV antibodies, if present, will be captured inside the wells as a double antigen "sandwich" complex comprising of the coated, and the biotin-conjugated HCV antigens. The microwells arc then washed to remove unbound serum proteins. During the second incubation step, the captured HCV antibodies are detected by adding of HRP-Conjugate. The microwells are then washed to remove unbound conjugate, and Chromogen solutions are added to the wells. In wells positive for HCV antibodies, the colorless

Chromogens are hydrolyzed by the bound HRP conjugate to a blue colored product. The blue color turns yellow after stopping the reaction with sulfuric acid. The amount of color intensity can be measured and is proportional to the amount of antibodies captured in the wells, and to the sample respectively. Wells containing samples negative for anti-HCV remain colorless.

3.18.2 Assay Procedure

The reagents and samples were allowed to reach room temperature (18-30°C) for at least 15-30 minutes. (Wash buffer were checked for concentrate, for the presence of salt crystals. If crystals had formed in the solution, resolubilization by warming at 37°C was done until crystals dissolve). The stock wash Buffer was diluted to 1 to 20 with distilled or deionized water. Only clean vessels were used to dilute the Wash buffer. Strips needed were placed in strip-holder and number sufficient number of wells including three Negative controls (e.g. B1, C1, D1), two Positive control (e.g. E1, F1) and one Blank (Al, neither samples nor HRP-Conjugate was added into the Blank well). 50µl of biotin conjugated HCV antigens was added into each well except in the Blank. Thereafter 50ul of Positive control was added. Negative control and Specimen was also added into their respective wells. The plate was covered with the plate cover and incubated for 60 minutes at 37°C. At the end of the incubation the plate cover was removed and discarded. Each well was washed 5 times with diluted Wash buffer. Each time, the microwell was allowed to soak for 30-60 seconds. After the final washing cycle, the strip plates were turned onto blotting paper or clean towel, and tap it to remove any remainders. 100µl HRP-Conjugate was added to each well except the Blank. The plates were covered with the plate cover and incubated for 30 minutes at 37°C. At the end of the incubation, plate covers were removed and discarded. Each well was washed 5 times with diluted Wash buffer. 50µl of Chromogen A and 50µl Chromogen B solution was added into each well including the Blank and mixed by tapping the

plate gently. The plate was incubated at 37°C for 30 minutes avoiding light. The enzymatic reaction between the Chromogen A/B solutions produced blue color in Positive control and anti-HCV positive sample wells. Using a multichannelled pipette, 50µl of Stop Solution was added into each well and mixed by tapping the plate gently. Intensive yellow color developed in Positive control and anti-HCV positive sample wells. The plate reader was calibrated with the Blank well and read at the absorbance of 450nm.

3.19 Determination of Hepatitis D Seromarker (HDV IgG)

3.19.1 Principle of the Assay

The HDV IgG ELISA kit employs the solid phase, two-step incubation, indirect ELISA method. Recombinant HDV antigens are pre-coated on the polystyrene microwell strips. Anti-HDV specific antibodies, if present, will appear during the first incubation stage - and be bound to the solid phase pre-coated HDV antigens. After this step, the wells are washed to remove unbound serum proteins. Next it is important to add anti-human IgG antibodies (anti-IgG) conjugated to the enzyme horseradish peroxidase (HRP-Conjugate). These HRP-conjugated antibodies, during the second incubation stage, will be bound to any antigen-antibody (IgG) complexes that were formed before. At this point, the unbound HRP-conjugate is then removed by washing. Added to the wells after this are both the chromogen solutions containing Tetramethylbenzidine (TMB) and urea peroxide. During this stage of the presence of the antigen-antibody-anti-IgG (HRP) immunocomplex, a blue-colored product appears, which is the result of colorless chromogens hydrolyzed by the bound HRP conjugate. After stopping the reaction with sulfuric acid, the blue color turns yellow. The color intensity can be gauged proportionally to the amount of antibody captured in the wells, and the amount of antibody in the sample, respectively. Colorless wells appear when samples are negative for HDV IgG.

3.19.2 Assay Procedure

The reagents were allowed to reach room temperature (18-30°C) for at least 15-30minutes. The strips needed were set in strip-holder and sufficient number of wells were numbered including three Negative controls (e.g. B1, C1, and D1), two Positive controls (e.g. E1, F1) and one Blank e.g. A1, Neither samples nor HRP-Conjugate was added into the Blank well). 100µl of Sample diluent were dispensed into each well except in the Blank well.10µl specimen, 10µl Negative controls, and 10µl Positive controls were added into their respective wells. The plates were covered with the plate cover and incubate for 30minutes at 37^0C. It was recommended to use thermostat-controlled water tank to assure the temperature stability and humidity during the incubation. Each well was washed 5 times with diluted Washing buffer. Each time, the microwell was allowed to soak for 30-60 seconds. After the final washing cycle, the plates were turned down onto blotting paper or clean towel, and it was tapped to remove any remaining liquids. 100µl of antibody HRP-Conjugate was added to each well except the blank. It was incubated for 50 minutes at 37^0C. Each well was washed 5 times with diluted Washing buffer. Each time, the microwells were allowed to soak for 30-60 seconds. After the final washing cycle, the plate was turned down onto blotting paper or clean towel, and tapped to remove any remaining liquids. 50µl of Chromogen A and 50µl Chrornogen B solution were added into each well and covered with a plate cover which is also mixed gently. It was incubated at 37^0C for 15minutes avoiding light. The enzymatic reaction between Chromogen solutions and the HRP-Conjugate produced blue color in Positive control and HDV-IgM positive sample wells.

The plate cover was removed and discarded; using a multichannel pipette 50µl of Stop solution was added into each well and mixed gently. Intensive yellow color developed in Positive control and HDV IgG positive sample wells. It was read at 450nm of wavelength.

3.20 Determination of Hepatitis D Seromarker (HDV Total Antigen)

Again the procedure is the same with the above ELISA method except that the polystyrene strips in the microwells were precoated with monoclonal antibodies reactive to HDVAg. Approximately 50µl of the negative control, positive control and the serum samples were added into their respective wells as described previously. Extraction solution was then added into each well except the blank using a separate pipette tip for each sample. The microplates were sealed, shake and incubated, using an automated microplate incubator with a shaker and a timer (Model: STAT FAX 2200, Awareness Technology Inc, USA) at a steady temperature of 37^{o}C for 30 min. After incubation, the seal were removed and each plate was washed 5 times with diluted wash buffer as previously described. After the final cycle of washing, the plate was inverted on a blotting paper and tapped to drain the wash solution. Then 100ul HPR- conjugates (horseradich peroxidase conjugated to anti-HDV antibodies) was added to each well, except the blank, the microplates were sealed, mixed and incubation resumed for 30min at 37^{o}C. After the second incubation, the procedure was as previously described.

3.21 Extraction of Viral RNA or Viral DNA Using Roche High Pure Viral Nucleic Acid Kit

3.21.1 Principle of the Procedure

The High Pure Viral Nucleic Acid Kit provides a rapid and efficient method to simultaneously purify viral RNA/DNA from fresh or frozen cell-free biological fluids (plasma, serum, cerebrospinal fluid) and cell culture supernatants. The kits allow efficient lysis of viral particles at elevated temperatures using Proteinase K and selective binding of viral nucleic acids to the silica matrix under highly denaturing conditions. High quality viral nucleic acids from a variety of RNA and DNA viruses are isolated with 45 minutes using low elution volumes that allow sensitive downstream analysis such as viral load quantitation and viral detection.

3.21.2 Assay Procedure

Tubes of 2 ml were used, it was numbered for samples and control, into each tube 50ul of Proteinase K solution was added. Thereafter 200 µl of sample and control were added into appropriate tubes. Then 200 µl of the Lysis Binding Buffer (LBB) was also added**,** the tubes were closed. It was mixed by vortexing for 15 seconds and incubated at 72°C for 10 minutes. After which 100 µl of Binding Buffer (BB) was added to each tube, closed and also mixed by vortexing for 15 seconds. The lysate was incubated for 3 minutes at room temperature. At this point an aliquot of elution buffer was placed into the heating block at 72°C to pre-warm it. Spin columns (High Pure Filter Tubes) were selected and labeled. Lysates were transferred into the corresponding spin columns. It was centrifuged at 13000 rpm for 1 minute. The flow through was discarded. The Spin Column was placed into a new collection tube. The Column was washed with 500µl of Inhibitor Removal Buffer. It was centrifuged at 13000 rpm for 1 minute and the flow through was discarded. The column was washed with 500µl of the Wash Buffer**.** The flow through was also discarded and the column into was put into a new collection tube. Washing was also repeated of the column with 500µl of the Wash Buffer. The flow through was discarded and the column was put into a new collection tube, The Spin Column was placed in a clean Collection tube, centrifuged at 14000 rpm for 1 minute to remove residual Wash Buffer. Spin Column was placed into a clean 1.5 ml tube (Recovery tube). 55µl of sterile RNAse-free water was added into each tube and incubated at room temperature for 1 minute. It was later centrifuged at 13000 rpm for 1 minute. The purified viral RNA or DNA was labeled and stored at -80 °C and was later used for testing.

3.22 Measurement of Hepatitis B Viral Load

3.22.1 Principle of the Procedure

This is a Real Time PCR method. It uses probe master mix and a validated in-house primer-probe mix. Two different commercial Probe Master Mixes have been validated on this system: Promega UK Probe Master and Applied Biosystem Universal Master mix. This test is used for detection and quantitation of Hepatitis B virus (all genotypes are detectable). This test is validated on ABI Step One plus Real Time PCR system (96 well) and ABI Step One Real Time PCR system (48 well).

3.22.2 Assay Procedure

HBV mix was thawed and pipettes were cleaned with both water and 70 % ethanol. For each sample and standard the reagents were pipetted as follows into each well of the plate:

2x Probe Master Mix: 25 µl

HBV Mix: 15 µl

Viral DNA sample: 10 µl

Total reaction volume been 50 µl.

The machine and the computer were turned on and the software versions 2.3 were opened. From the New Experiment Menu on the desktop, it was clicked and select Run Templates was opened, and finally HBV DNA was selected. This HBV DNA method was loaded. Once loaded, Set UP was highlighted and clicked on Experiment Properties. The title of the run was entered e.g., 0104 HBV DNA and then sample details was entered in the Plate Set Up. Assign the wells accordingly. Once the wells were assigned properly including the standards, the machine was opened and the plates placed properly following the orientation in the machine. Run icon was clicked on. The HBV run takes about 1 hour 20 minutes. When the run was completed,

amplification plots and standard curve were checked. The machine does auto-threshold using the Graph Pad Prism Version 6.02. The values of slope, R^2, intercept, efficiency, threshold value and plateau of the amplification plot were displayed. After checking for performance characteristics, results were exported to excel and transfer. Results are reported in IU/ml.

3.23 Detection of HBV S Region by Singleplex PCR

3.23.1 Principles of detecting of HBV S Region by Singleplex PCR

This singleplex PCR relies on primers that amplify a much conserved portion of the S region in all the eight HBV genotypes (A-H). The amplicon size is 116 bp.

3.23.2 Singleplex PCR Procedure

Set up the HBV S PCR was follows-

Appropriate number of PCR tubes or wells in a plate was selected, there were 2 controls. Tubes were used when the number of samples was less than 10 and they were labelled accordingly.

12.5 µl of the 2x HS Biolab PCR Master Mix was dispensed into each tube or well. After 7.5 µl of HBV S 116 mix was added into each tube or well and 5 µl of the DNA sample or control to the corresponding tube or well. Briefly it was mixed by quick spin on the minifuge for 5 seconds. The tubes were transferred into the thermal cycler and positioned properly, the lid was closed and run programme titled '55555' in PAUL or 'KLK-va' in Barnabas [PAUL and BARNABAS are thermal cyclers in the lab]. The thermal profile was 58^oC for 30 seconds. Electrophoresis was carried out, 3.0% agarose gel in 0.5x TBE buffer (100 ml) was prepared; 20 µl of Ethidium bromide (dye) were added per 100 ml of agarose solution. The ready reaction set up was run on the electrophoretic machine at 100 V for 1 hr and viewed in UV light. 50 and 100 bp DNA ladder (marker) was used as PCR sizer; it was added to the first well of the agarose gel.

3.24 Determination of Hepatitis B Virus Genotypes Using Multiplex PCR

3.24.1 Principles of Hepatitis B Virus Genotyping

This PCR is designed to type for A-H genotypes. The PCR involves two multiplex PCR reactions: N1 and N2 reactions in single rounds. The primers are based on C, Pre-S2, and S regions of the HBV.

3.24.2 Multiplex PCR Procedure

Multiplex PCR uses two set up of reactions N1 and N2 reactions. N1-ABFGH which has 6 primer mixes and N2-CDE which has 4 primer mix.N1 set up reactions had a total elution volume of 50µl comprising of Platinium Multiplex Master mix of 25 µl, N1-ABFGH mix of 15 µl (containing 6 primer mixes) and viral DNA elute of 10 µl. N2 set up reactions had a total elution volume of 50 µl containing Platinum Multiplex Master mix of 25µl N2-CDE mix of 15 µl (containing 4 primer mixes) and viral DNA elute of 10 µl. Both N1 and N2 were set up the same time. The Thermal Profile was set in the Eppendorf Machine as 'HBVmultiplex'. The profiles wereas follows:

a. 94^{o}C for 5 min
b. 94^{o}C for 1 min
c. 59.5^{o}C for 1min
d. 72^{o}C for 1 min
e. 72^{o}C for 1 min

b-d are 40 cycles

After the 40 cycles of the set up reactions Electrophoresis was carried out. 3.0% agarose gel in 0.5x TBE buffer (100 ml) was prepared; 20 µl of Ethidium bromide (dye) were added per

100 ml of agarose solution. The ready reaction set up was runon the electrophoretic machine at 100 V for 1 hr and viewed in UV light. 50 and 100 bp DNA ladder (marker) was used as PCR sizer; it was added to the first well of the agarose gel.

3.24.3 Interpretation of Results

From N1-ABFGH reaction

a. **508** bp for HBV Genotype A

b. **734** bp for HBV Genotype B

c. **259** bp for HBV Genotype F

d. **228** bp for HBV Genotype G

d. **259 bp** and **936 bp** for HBV Genotype H

From N2- CDE reaction

d. **258** bp for HBV Genotype C

e. **867** bp for HBV Genotype D

f. **666** bp for HBV Genotype E

3.25 Determination of Hepatitis C Virus Genotypes

3.25.1 Polymerase Chain Reaction Analysis

Primary and secondary PCR were performed on the extracted RNA following a nested PCR approach. Reverse transcription and primary PCR were done in a 'one step' reaction using the Qiagen Onestep RT-PCR Kit (Qiagen, Hilden, Germany) which uses specially formulated reverse transcriptase and DNA polymerase enzymes for reverse transcription and PCR amplification, respectively. Secondary PCR was done using the Qiagen Taq PCR master mix kit (Qiagen, Hilden, Germany). Components for both primary and secondary PCR and the

thermocyling conditions that were used are outlined in tables 3.1 and 3.2. The thermo cycling program recommended in the kit insert was used.

Table 3.1: Reaction components for RT, primary and secondary PCR mixtures

Component	**Volume/reaction (**µl)	**Final concentration**
RT & Primary PCR		
Water	8.3	-
5X Qiagen OneStep RT-PCR buffer containing 12.5Mm $MgCl_2$	10.0	1X
dNTP mix (containing 10mM of each dNTP)	2.0	400µM of each dNTP
Forward primer (50µM)	0.6	0.6 µM
Reverse primer (50µM)	0.6	0.6 µM
Qiagen OneStep RT-PCR Enzyme Mix	2.0	-
RNase inhibitor (5units/µl)	1.5	7.5units/reaction
RNA template	25.0	-
Total Volume	**50.0**	-
Secondary PCR		
Master Mix	50.0	-
Water	43.0	-
DNA template from 1^0 PCR	5.0	-
Forward primer (50µM)	1.0	0.5µM
Reverse primer (50µM)	1.0	0.5µM
Total Volume	**100**	-

Table 3.2: Thermo cycling conditions used for RT, primary and secondary PCR

Process	Temperature (^{0}C)	Time
RT & Primary PCR		
Reverse Transcription	50	30 minutes
Initial PCR activation step	95	15 minutes
3- Step Cycling 1 (5 cycles)		
Denaturation	94	30 seconds
Annealing	62	45 seconds
Extension	72	1 minute
3- Step Cycling 2 (30 cycles)		
Denaturation	94	30 seconds
Annealing	55	45 seconds
Extension	72	1 minute
3- Step Cycling 3 (5 cycles)		
Denaturation	94	30 seconds
Annealing	52	45 seconds
Extension/Elongation	72	1 minute
Final Extension	72	10 minutes
Hold	4	1-16 hours
Secondary PCR		
Denaturation	95	5 minutes
3-step cycling (35 cycles)		
Denaturation	95	30 seconds
Annealing	55	30 seconds
Extension	72	30 seconds
Final extension/Elongation	72	10 minutes
Hold	4	1-16 hours

3.25.2 Visualisation of the PCR products

The PCR amplicons were visualised using a 1.5% agarose gel stained with 5µl ethidium bromide. The gel was prepared by dissolving 1.5 grams of agarose powder in 100ml of 0.5 X Tris-Borate-EDTA buffers (pH8). The solution containing the partially dissolved powder was heated in a microwave oven for 3 minutes to enable complete dissolution and then 5µl of ethidium bromide (10mg/ml) added to it and gently swirled. The solution was then poured onto an electrophoresis tray. Combs were then inserted into the liquid gel to create wells for sample addition. After solidification of the gel, the combs were removed and approximately 100ml of the 0.5 X Tris-Borate-EDTA buffers poured onto the electrophoresis tray completely immersing the gel. A mixture of 8µl of sample and 2µl of gel loading buffer dye (5X) was then loaded into the agarose gel wells using a micropipette. In addition to sample, positive and negative control samples obtained from the WHO HCV reference laboratory were also added in the same manner as the samples. A 100bp DNA molecular weight marker (New England Biolabs) was added to an adjacent well to enable size estimation of the resolved bands. The gel was left to run for 45 minutes at 100 volts. At the end of the 45 minutes, the gel was placed on an ultra violet (UV) illuminator to visualise the bands and a digital image of the gel captured.

3.26 Measurement of Hepatitis C Viral Load Using 5x Quantitect Virus Master Mix

3.26.1 Principle of Hepatitis C Virus Load Test

This is a Real Time PCR method based on TaqMan chemistry. It uses TaqMan primer-probe mix, qiagen's 5x quantitect virus master mix and quantitect virus real time mix. Two different FAM labeled TaqMan probes: HCV-84-Pr and HCV-108-Pr are used in the reaction.

3.26.2 Procedure for HCV Load Test

All reagents were thawed.The under listed primers and probe mix were used at 10µM concentrations

Table 3.3: Primers and Probe mix used at 10 µM concentrations

Primers and Probe	Per reaction	100 Reactions	50 reactions
HCV-S4-F (10 µM)	1	100	50
HCV-S4-R (10 µM)	1	100	50
HCV-84-Pr (10 µM)	0.5	50	25
HCV-108-F (10 µM)	1	100	50
HCV-108-R (10 µM)	1	100	50
HCV~108-Pr (10 µM)	0.5	50	25
RNAse Free Water	4.5	450	225
Total volume	9.5	950	475

After the 9.5µl prime probe mix were added into the PCR plates, 5µl of 5xquantitect virus master mix and 0.5µl of quantitect virus real time mix were also added, it was gently mixed. The total volumes of the reagents were 15µl. Then 10µl of HCV RNA was added, giving a total volume of 25µl.

Elution buffer was used as Negative control and previously tested positive sample as Positive Control. The tubes sealed and put into ABI real time PCR machine. The machine and the computer were turned on. The software versions 2.3 were opened. From the New Experiment Menu on the desktop, click and select Run Templates, and finally select HCV

quantitation. This will load the HCV method. Once loaded, go to Set up and click on Experiment Properties. Enter title of the run e.g., 0104 HCV quantity and then enter sample details in the plate set up. Click on the Run icon. The HCV quantitation run takes about 2 hours 10 minutes. When the run is completed, check the amplification plots and standard curve. The thermal profile used 50°C for 20 minutes, 95 °C for 5 min, 95 °C for 15 seconds and 60 °C for 45 seconds. Number of cycles should be 45. When the run was completed, the amplification plots were checked and standard curve. Check for the values of slope, R^2, intercept, efficiency, threshold value and plateau of the amplification plot were also done.The machine does auto-threshold but this can be manually set to correct for amplification patterns. Results were reported in IU/ml.

3.27 Detection of HBV-DNA by Nested PCR (PCR Amplification)

The presence of HBV-DNA was examined in all samples using a routine diagnostic PCR in the reference labs. Primer pairs were designed from the highly conserved overlapping regions of the S and P regions of the HBV genome. A nested PCR was performed with the following primers as shown in the table below:

Name	Position	Nucleotide Sequence Orientation
230F	231 - 249	3'–TCA CAA TAC CGC AGA GTC T – 5' Sense
800R	801 - 782	5'– AAC AGC GGT ATA AAG GGA CT – 3' Antisense
P7	256 - 278	3'–GTG GTG GAC TTC TCT CAA TTT TC – 5' Sense
P8	796 - 776	5'–CGG TAW AAA GGG ACT CAM GAT – 3' Antisense

PCR amplifications were carried out in 25 µl reaction volumes with 5 ng of genomic DNA, 10x PCR buffer (20mM Tris-HCl pH 8.4, 50 mMKCl; Qiagen), 2mM of dNTPs, 50 ng of each primer and 1U AmpliTaq gold DNA polymerase (Applied Biosystems) on a PTC 200 cycler

(Peltier Thermal cycler Watertown, Massachusetts, USA). Thermal cycling parameters were: initial denaturation at 94°C for 2 min, followed by 35 cycles of 30sec at 94°C denaturation, 30 sec at 52°C annealing temperature, 45 sec at 72°C extension, followed by a final extension of 5 min at 72°C. Thermal cycling parameters remained the same as in the first PCR round except for the number of cycles which were increased to 40 cycles in the subsequent amplification. The second round of amplification added 5 µl of first round product to 45 µl of a mixture containing same above but with p7 and p8 primers. Amplification was performed in a programmable thermocycler (Techne) as follows: denaturation at 94 °C held for 45 s, annealing at 53 °C held for 60 s and elongation at 72 °C held for 90 s for a total of 40 cycles with a final extension at 72 °C held for 10 min. Each PCR product (5 μL) was analysed by electrophoresis in 2% agarose gels.

3.28 Determination of Genetic Relatedness of Hepatitis B Genotypes (Homology with Consensus Sequence).

3.28.1 Procedure

HBV DNA genotyping by DNA sequencing involved two key steps: Nested PCR to amplify a 2.7 kb region of HBV DNA and sequencing using Big Dye Terminator chemistry in a Capillary Electrophoresis Genetic Analyzer, ABI 3130. Sequenced data are aligned and search in Genomic Database (NCBI). The targeted amplicon was the P region of 2.7 kb size. The Primers for Ist round was5'--GAGTATTTGGTGTCTTTTGGAGTGTGGATT--3'(P-F1) (nt2287--2316) sense which was the forward primer and 5'- GAAGTATGCCTCAAGGTCGGTCGTT-3’ (P-R1) (nt1710--1686) antisense was the reverse primer. The 2nd Round primers used were 5'-CACCAAATGCCCCTATCTTATCAACACT-3'(P-F2) (nt2339--2366) sense 5’-GGCGTTCACGGTGGTCTCCAT-3’ (P-R2) (nt1629--1609) antisense.

All primers used were synthesized by Eurofins, Germany. Lyophilized primers were reconstituted in 1x TE buffer from IDT Europe.

Big Dye Terminator chemistry (Thermofisher, UK) was used. Genomic DNA were first amplified using the Ist round primers, following by second PCR using the second-round primers and PCR product of first round (Nested PCR).

The sequencing primer, 5'- CACCAAATGCCCCTATCTTATCAACACT-3' was used at a final concentration of 0.1 µM. Sequencing was done in Genetic Analyzer AB3130 (Applied Biosystems).

Sequencing data was aligned in the SeqMan Pro software of DNA Star. Aligned data were searched in BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for genotype identification using the HBV taxid (Accession No) 10407 as consensus sequence (see appendix ii for proof).

3.29 Determination of Genetic Relatedness of Hepatitis C Genotypes (Homology with Consensus Sequence).

3.29.1 Procedure

HCV RNA genotyping by DNA sequencing involved three key steps: Reverse transcription of HCV RNA into cDNA, Nested PCR to amplify a 266 bp 5' UTR (Untranslated Region) and sequencing using Big Dye Terminator chemistry in a Capillary Electrophoresis Genetic Analyzer, ABI 3130. Sequenced data are aligned using DNAStar and searched in Genomic Database (NCBI). The targeted amplicon was 5' UTR, 266 bp**.** The Primers for 1st round were 5'-CCCCTGTGAGGAACTWCTGTCTTCACGC-3' (Forward primer) and 5'-AGGTTTAGGATTTGTGCTCAT-3 (Reverse primer)**.** The 2nd Round primers were 5'-GAAAGCGYCTAGCCATGGCGTTAG-3' (Forward primer) and

5’-ACGGTCTACGAGACCTCCCGGGGC-3’ (Reverse primer). All primers used were synthesized by Eurofins, Germany. Lyophilized primers were reconstituted in 1x TE buffer from IDT Europe. Big Dye Terminator chemistry (Thermofisher, UK) was used. HCV RNA was extracted using Zymo viral RNA kit; cDNA was made using Thermofisher First Strand DNA synthesis. HCV targets were first amplified using the Ist round primers, following by second PCR using the second-round primers and PCR product of first round (Nested PCR) in Eppendorf Nexus Gradient Master Cycler. The sequencing primer, 5’GAAAGCGYCTAGCCAT GGCGTTAG -3’ was used at a final concentration of 0.1 µM.

Sequencing was done in Genetic Analyzer AB3130 (Applied Biosystems). Sequencing data was aligned in the SeqMan Pro software of DNA Star. MegAlign was used searching BLAST(https://blast.ncbi.nlm.nih.gov/Blast.cgi) for genotype identification using the HCV taxid (Accession No) 11103 as consensus sequence (see appendix iii for proof).

3.30 Statistical Analysis

The percentage frequency of occurrence of the hepatitis infection (HBV and HCV) in HIV patients in South East Nigeria were calculated using Frequency (%)$=\frac{n}{N}\times\frac{100}{1}$. Where n = Number of positive occurrences of hepatitis infection, N = Total number of individuals tested. Results of the study were analysed using Statistical Package for Social Sciences (SPSS) version 20.0 for windows. The data were expressed as mean plus or minus standard error of mean (Mean ± SEM, n=3). ANOVA and Student’s t-test used to compare mean and values at p less than 0.05 ($p<0.05$) were considered significant.

CHAPTER FOUR

RESULTS

Out of the 2,500 HIV patients sampled for this study within the South East, 256 (10.2%) were positive for HBsAg, also P- value of HBsAg was highly significant with P Value of 0.000 hence are positive to Hepatitis B infection. Enugu State showed the highest, 62 (12.4%) seroprevalence rate followed by Anambra, Imo and Abia States with the corresponding number (%): 57 (11.4%), 50 (10.0%) and 45 (9.0%) respectively, Ebonyi State recorded the least seroprevalence rate, 42 (8.4%) as shown in Table 4.1.

Only 850 (34.0%) were positive for Anti-HBs, either from vaccination or from past infection hence were positive for Anti-HBs from the total 2,500 HIV patients sampled. P value of Anti-HBs was not significant with P=0.060. The highest prevalence was observed in HIV patients from Enugu State (50.0%), followed by Anambra State (40.0%), Imo State (30.0%) and Abia State (30.0%), while the least was observed with HIV patients in Ebonyi State (20.0%) as also indicated in Table 4.1.

A total of 520 (20.8%) were positive for Anti-HBcIgM of the overall 2,500 HIV patients sampled in this study with P- value at 0.215, showing they are not statistically significant. Detection of Anti-HBcIgM is an indication of an acute and chronic HBV infection. The highest was recorded in Anambra State (30.0%), followed by Enugu State (24.0%), Abia and Imo States (20.0%), and with least recorded in Ebonyi State, 10.0% (Table 4.1).

Out of 2,500 HIV patients sampled in this study, 175 (7.0%) were positive for Anti-HCV, with P=0.191. The highest seroprevalence rate was recorded in Anambra and Enugu States (10.0%) respectively, followed by Abia State (6.0%) and Ebonyi State (5.0%); Imo state recorded the least (4.0%) as shown in Fig. 4.1.

Table 4.1: Seroprevalence of HBV Markers amongst HIV Patients in SouthEast, Nigeria

States	No. of Individuals Tested	Hepatitis B Viral Markers (%)		
		HBsAg^{+}	Anti-HBs^{+}	Anti-HBcIgM
Anambra	500	57 (11.4)	200 (40.0)	150 (30.0)
Enugu	500	62 (12.4)	250 (50.0)	120 (24.0)
Abia	500	45 (9.0)	150 (30.0)	100 (20.0)
Imo	500	50 (10.0)	150 (30.0)	100 (20.0)
Ebonyi	500	42 (8.4)	100 (20.0)	50 (10.0)
Total	**2500**	**256 (10.2)**	**850 (34.0)**	**520 (20.8)**
P- Value		**0.000**	**0.060**	**0.215**

Key:

HBV: Hepatitis B Virus

HIV: Human Immunodeficiency Virus

HBsAg+: Hepatitis B Surface Antigen

Anti-HBs+: Hepatitis B Surface Antibody

Anti-HBcIgM: Hepatitis B Core Immunoglobulin M

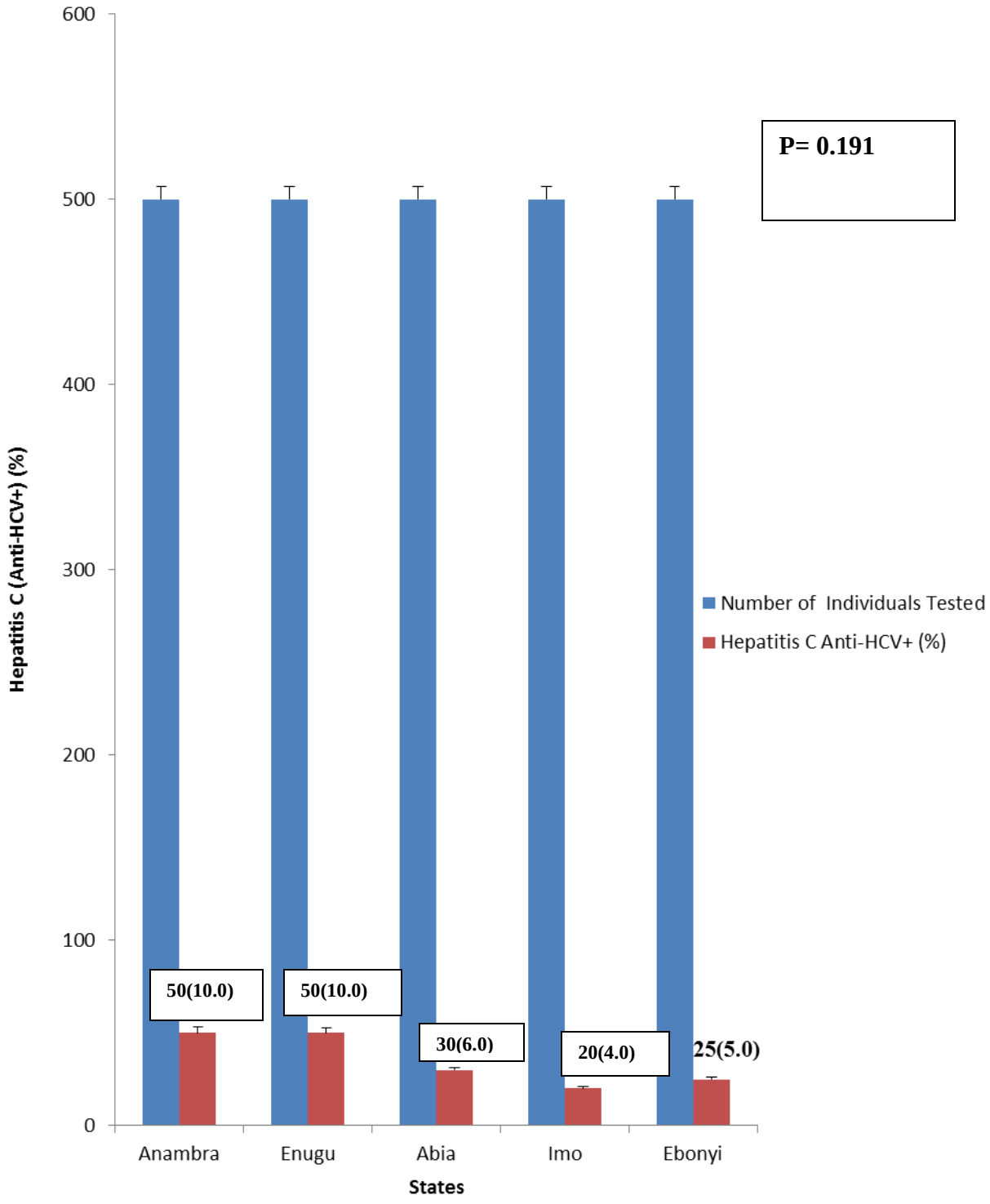

Figure 4.1: Seroprevalence of HCV Markers amongst HIV Patients in SouthEast, Nigeria

Key: HCV: Hepatitis C Virus, HIV: Human Immunodeficiency Virus and Anti- HCV$^{+:}$ Hepatitis C Antibody positive

Only 25 (1.0%) HIV patients were recorded positive for HBV/HCV co-infection of the total 2,500 HIV patients sampled in this study, with P-value 0.064. Anambra and Abia States had (2.0%) which recorded the highest for HBV/HCV Co-infection, followed by Enugu State (1.0%). Imo and Ebonyi States (0.0%) showed no seroprevalence of HBV/HCV co-infection among HIV patients as presented in Fig 4.2.

The distribution of sero-markers ($HBsAg^+$, Anti-HBs+ and Anti-HBcIgM**)** among HIV patients in South East with respect to demographic parameter (age) is shown in Table 4.2. Age range of between 31-40 years showed highest $HBsAg^+$(17.5%) among the 256 (10.24%) positive, followed by 11-20 years (15.0%), 41-50 years (13.3%), 21-30 years (11.6%), 51-60 years (6.0%), 61-70 years (4.0%), <10 years (5.0%) and >70 years (2.4%), recorded the least. HBsAg was not statistically significant with P value 0.356 with respect to age.

Age range between 31-40 years (52.5%) showed the highest Anti-HBs^+, followed by 41-50 years (50.0%), 21-30 years (40.0%), 11-20 years (25.0%), 51-60 years (25.0%), 61-70 years (24.0%) and <10 years (10.0%), while >70 years (8.0%) recorded the least (Table 4.2). Anti-HBs^+, had P- value 0.003 showing statistical significance

31-40 years age range showed the highest Anti-HBcIgM (35.0%), followed by 51- 60 years (30.0%), 41-50 years (30.0%), 11-20 years (27.5%), 21-30 years (20.0%), 61-70 years (10.0%) and >70 years (6.0%), <10 years (5.0%) recorded the least. Anti-HBcIgM had P- value of 0.000, highly significance.

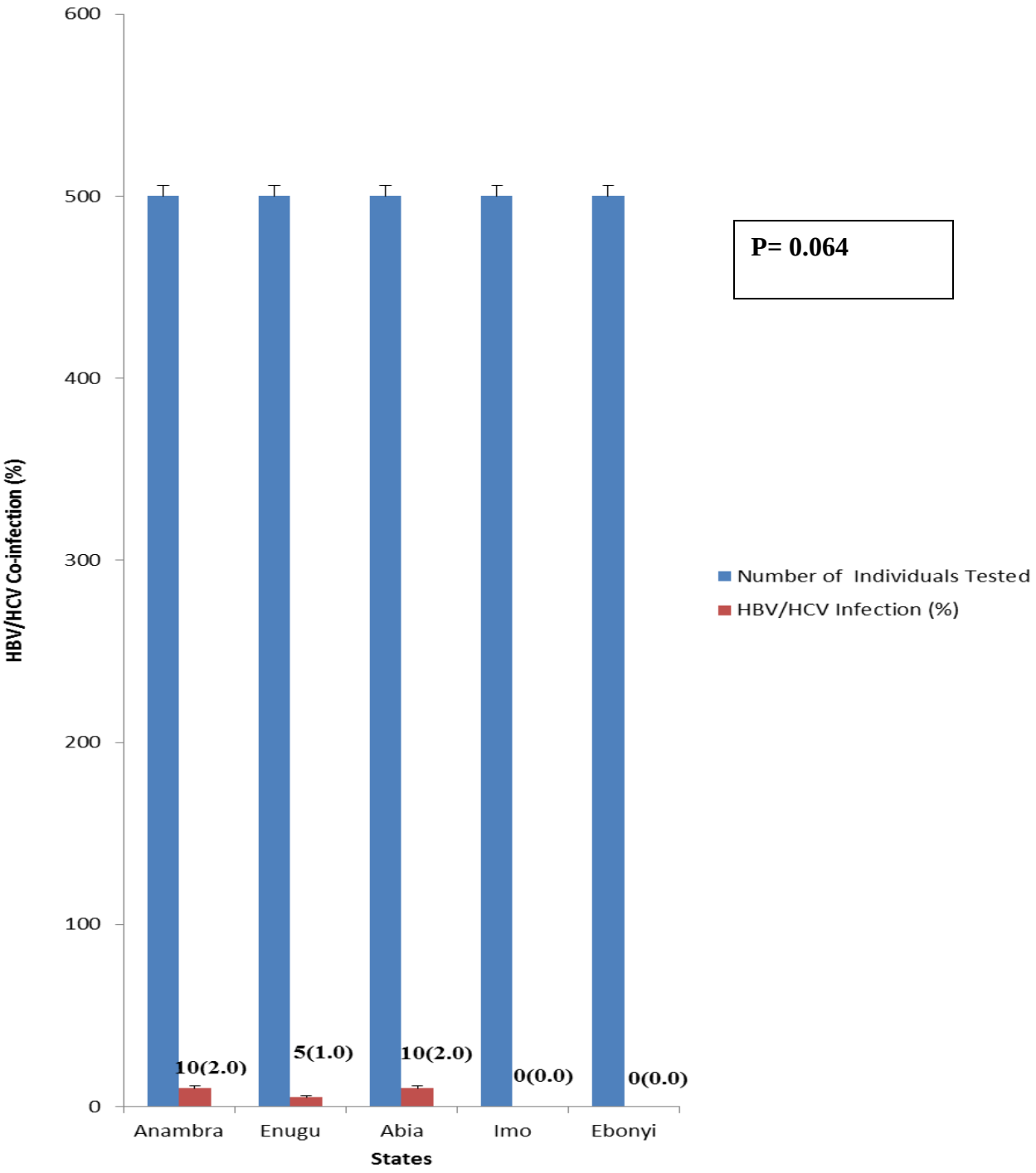

Figure 4.2: Seroprevalence of HBV/HCV Co-infection amongst HIV Patients in SouthEast, Nigeria

Key:
HBV: Hepatitis B Virus
HCV: Hepatitis C Virus
HIV: Human Immunodeficiency Virus

Table 4.2: Distribution of HBV Sero-markers amongst HIV Patients by Age in SouthEast, Nigeria

Age Range	No. Tested	Viral Markers		
		HBsAg+ (%)	Anti-HBs+ (%)	Anti-HBcIgM (%)
<10	100	5 (5.0)	10 (10.0)	5 (5.0)
11-20	200	30 (15.0)	50 (25.0)	55 (27.5)
21-30	500	58 (11.6)	200 (40.0)	100 (20.0)
31-40	400	70 (17.5)	210 (52.5)	140 (35.0)
41-50	400	53 (13.3)	200 (50.0)	120 (30.0)
51-60	400	24 (6.0)	100 (25.0)	60 (15.0)
61-70	250	10 (4.0)	60 (24.0)	25 (10.0)
>70	250	06 (2.4)	20 (8.0)	15 (6.0)
Total	**2500**	**256 (10.24)**	**850 (34.00)**	**520 (20.80)**
P- value		**0.356**	**0.003**	**0.000**

Key:

HBV: Hepatitis B Virus

HIV: Human Immunodeficiency Virus

HBsAg+: Hepatitis B Surface Antigen

Anti-HBs+: Hepatitis B Surface Antibody

Anti-HBcIgM: Hepatitis B Core Immunoglobulin M

The age range of 31-40 years (17.5%) showed the highest anti-HCV^+ seroprevalence rate, followed by 21-30 years (8.0%), 41-50 and 11-20 years (5.0%), 61-70 and > 70 years (4.0%), 51-60 years (3.7%) and <10 years (0.0%) showed the least as shown in Fig 4.3. P- value is not statistically significant with value of 0.561.

On the basis of the demographic parameter (sex), in the study population, male showed highest sero-markers ($HBsAg^+$, anti-HBs^+ and anti-HBcIgM) with the corresponding sero-prervalence rate of 11.8%, 35.7% and 22.1% respectively, while female showed the lower seroprervalence rate of $HBsAg^+$ (8.2%), anti-HBs^+ (31.8%) and Anti-HBcIgM (19.1%) as shown in Fig 4.4. With respect to sex $HBsAg^+$, anti-HBs^+ and Anti-HBcIgM had P values of 0.245, 0.739 and 0.001 respectively. Anti-HBcIgM showed statistical significance.

Female showed the higher seroprevalence rate of anti-HCV^+ 8.2% and male (6.1%) as shown in Fig 4.5, with p value of 0.696.

Out of the total 776 Acute Hepatitis B (AHB) patients observed in this study, 520 (67.0%) HIV patients revealed the presence of anti-$HBcIgM^+$, followed by $HBsAg^+$ obtained in 256 (33.0%) HIV patients and no HIV patients were detected with anti-HBs^+ (Fig 4.6), p values of the sero markers showed high statistical significance of P= 0.000.

A total of 256 HIV patients which were positive for Chronic Hepatitis B (CHB) virus infections were all positive for $HBsAg^+$with 100% seroprevalence rate. Other serological markers (anti HBs^+ and anti-$HBcIgM^+$) were not detected in the CHB patients as shown in Fig 4.7, p values of the sero markers showed high statistical significance of P= 0.000.

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Out of the 776 Acute Hepatitis B (AHB) virus infected patients, none (0%) showed the presence of the serological markers (Anti HDV IgM and Anti-HDV). In the same vein, the serological markers (Anti HDV IgM and Anti-HDV) were also not detected in Chronic Hepatitis B (CHB) patients as shown in Fig 4.8 below, p values of the sero markers showed high statistical significance of P= 0.000.

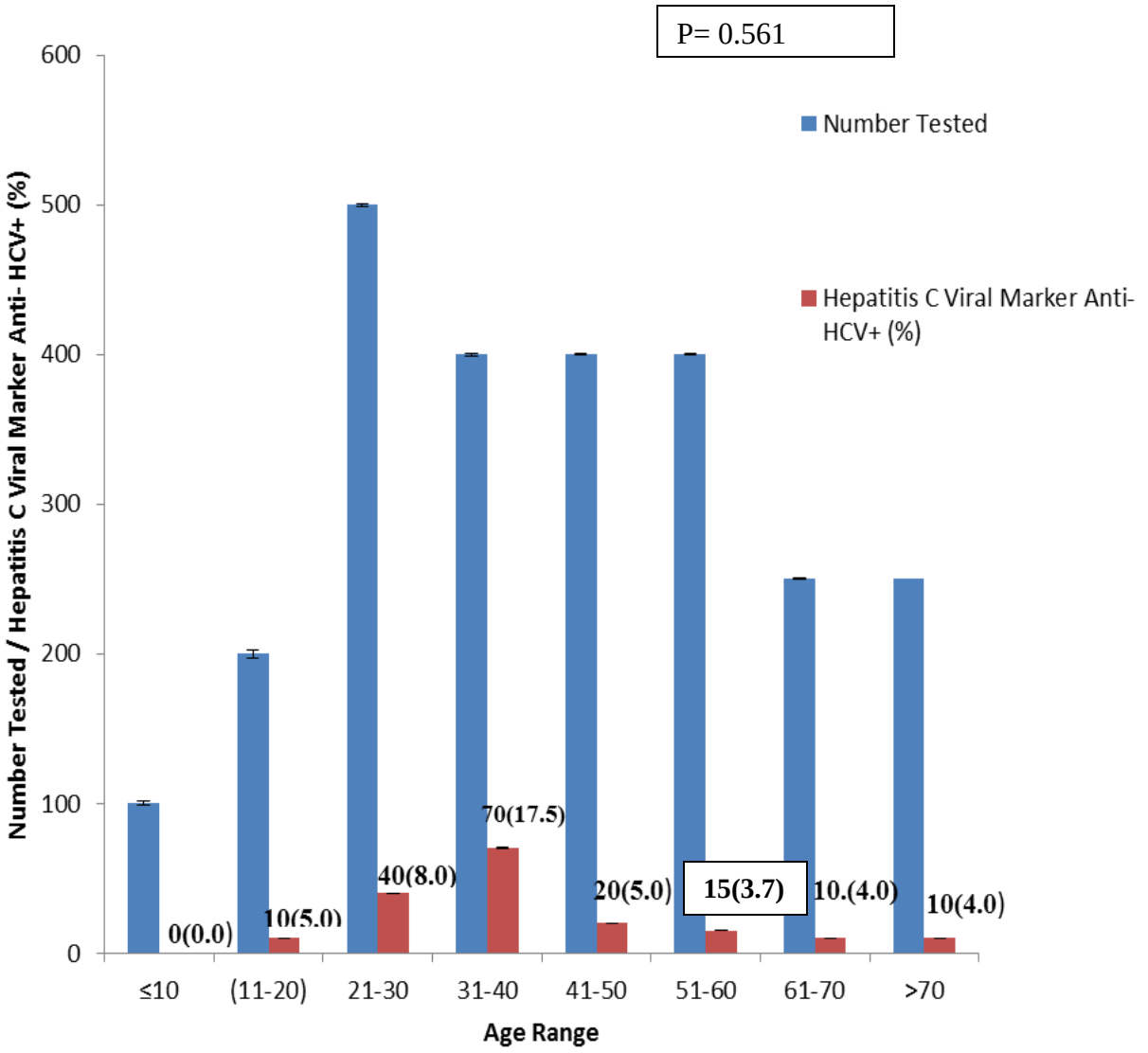


Figure 4.3: Distribution of HCVsero-markers amongst HIV Patients by Age in SouthEast, Nigeria

Key:

HCV: Hepatitis C Virus
HIV: Human Immunodeficiency Virus
Anti- $HCV^{+:}$ Hepatitis C Antibody positive

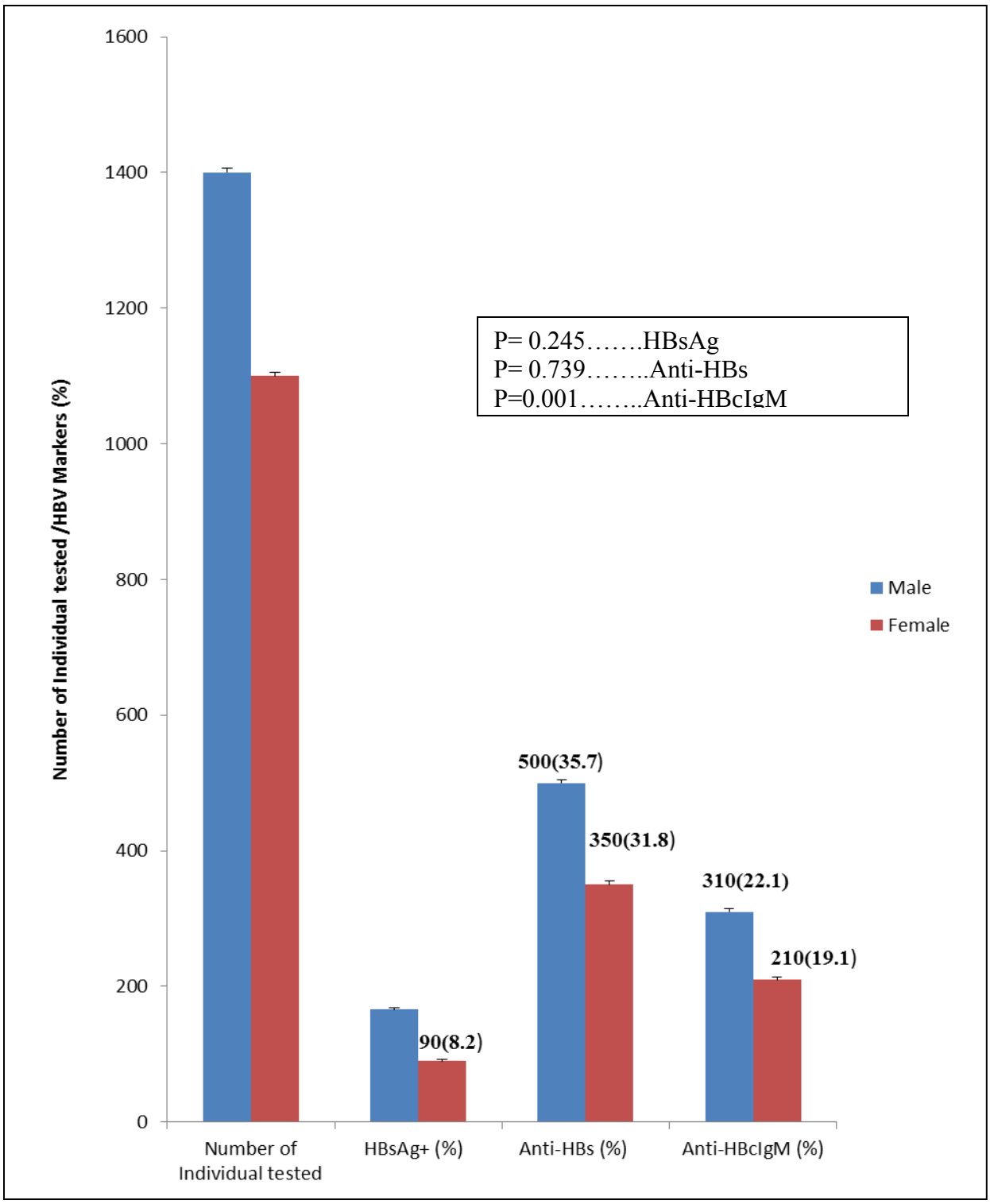


Figure 4.4: Distribution of HBV Sero-markers amongst HIV patients by Sex in SouthEast, Nigeria

Key:
HBV: Hepatitis B Virus
HIV: Human Immunodeficiency Virus
HBsAg+: Hepatitis B Surface Antigen
Anti-HBs+: Hepatitis B Surface Antibody
Anti-HBcIgM: Hepatitis B Core Immunoglobulin M

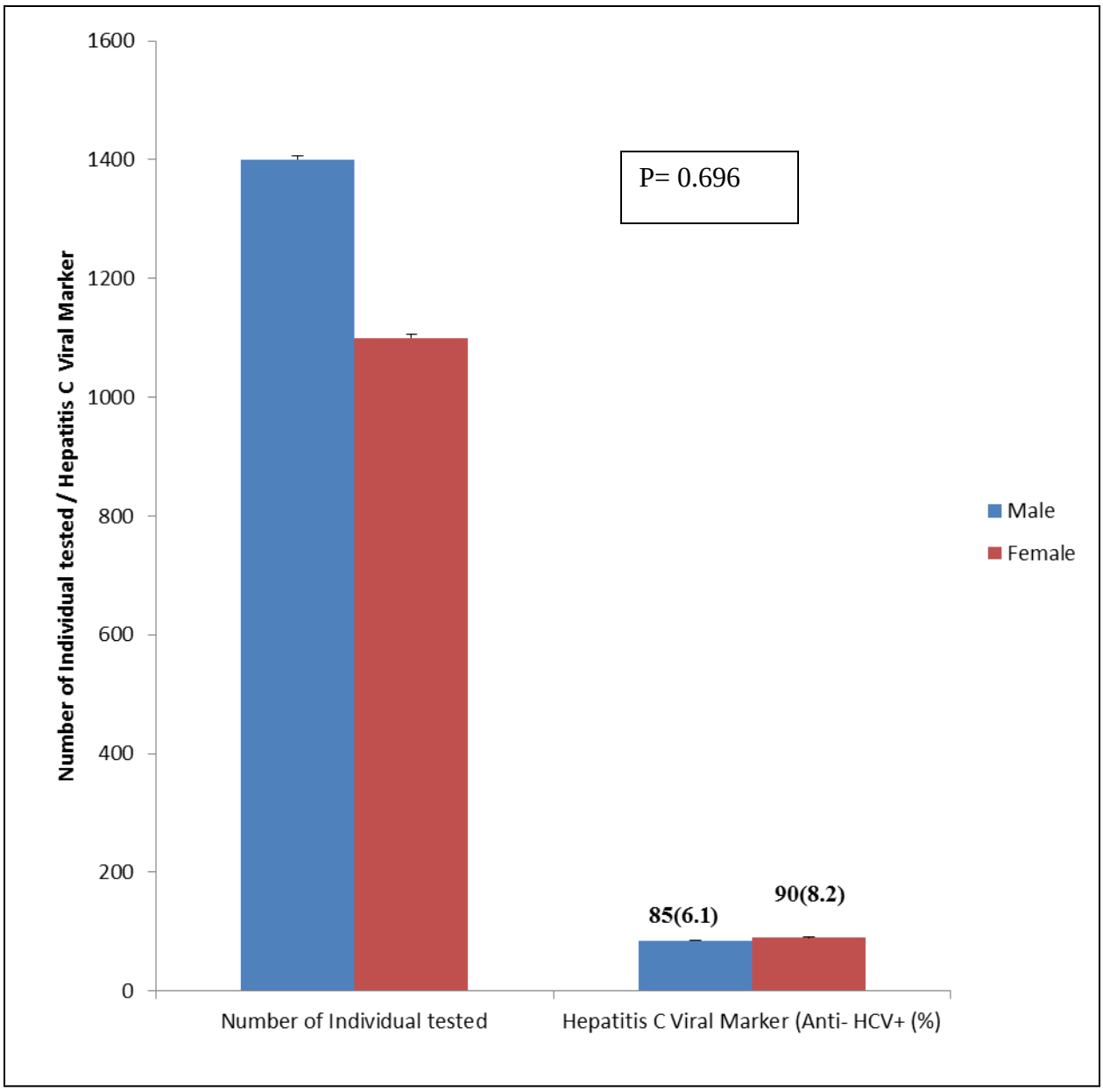


Figure 4.5: Distribution of HCV Sero-markers amongst HIV Patients by Sex in SouthEast, Nigeria

Key:

HCV: Hepatitis C Virus
HIV: Human Immunodeficiency Virus

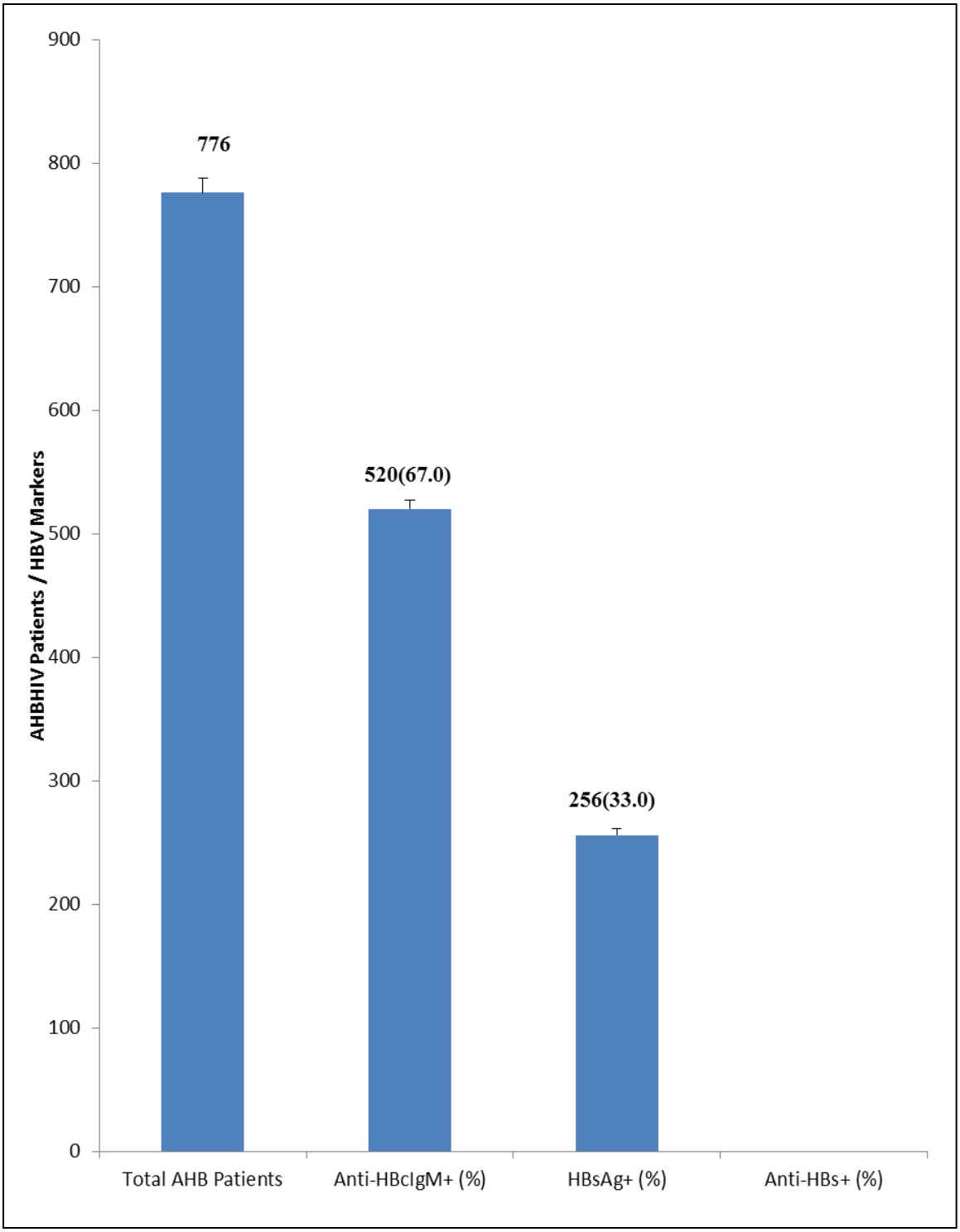


Figure 4.6: Seroprevalence of HBV Serological Markers in AHB HIV Patients

Key:

HBV: Hepatitis B Virus
Anti-HBs+: Hepatitis B Surface
AHB: Acute Hepatitis B Virus infection
HBsAg+: Hepatitis B Surface Antigen
Anti-HBcIgM: Hepatitis B Core Immunoglobulin M
HIV: Human Immunodeficiency Virus

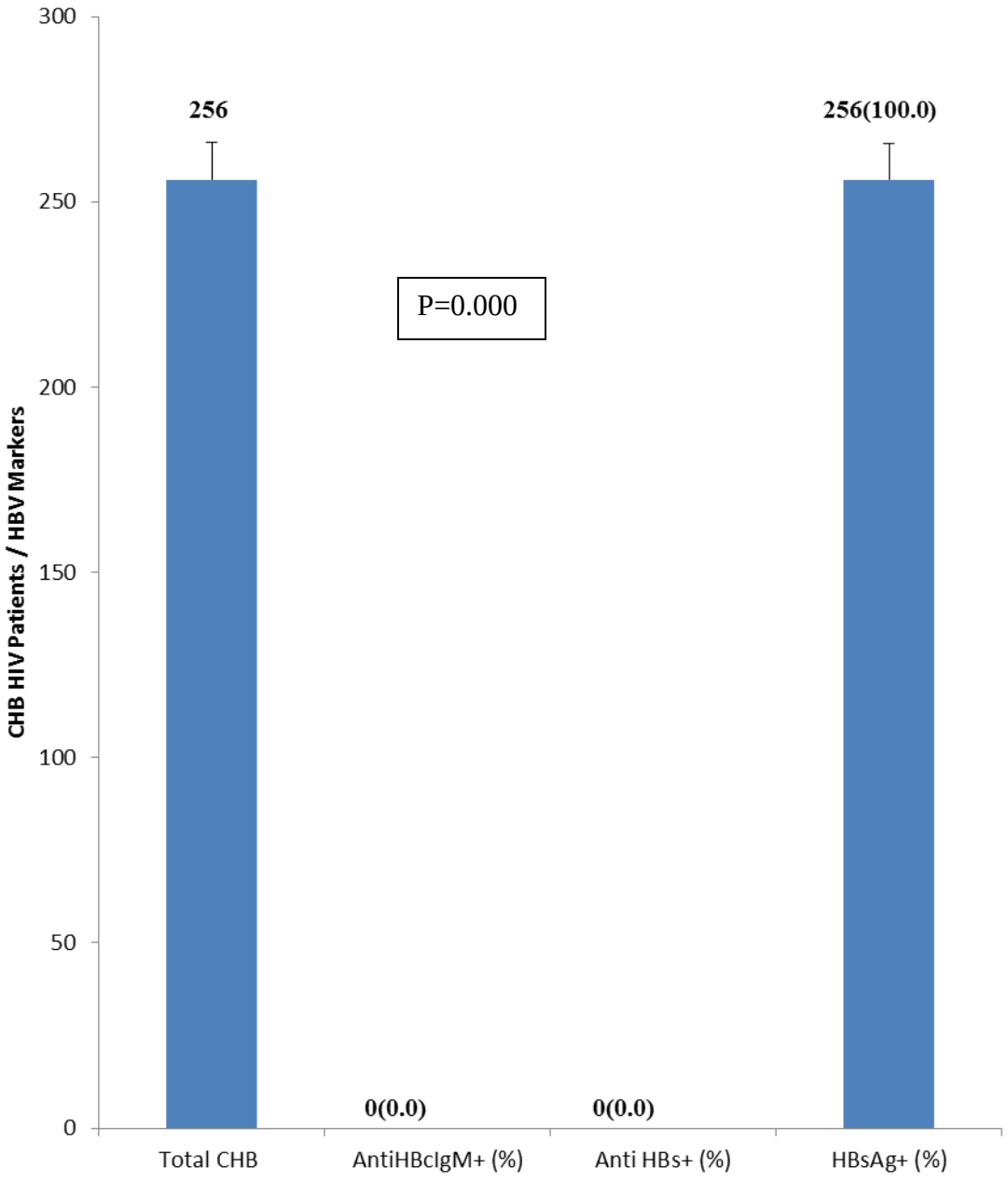


Figure 4.7: Seroprevalence of HBV Serological Markers in CHB HIV Patients

Key:

HBV: Hepatitis B Virus

HBsAg+: Hepatitis B Surface Antigen

Anti-HBs+: Hepatitis B Surface

Anti-HBcIgM: Hepatitis B Core Immunoglobulin M

CHB: Chronic Hepatitis B virus infection

HIV: Human Immunodeficiency Virus

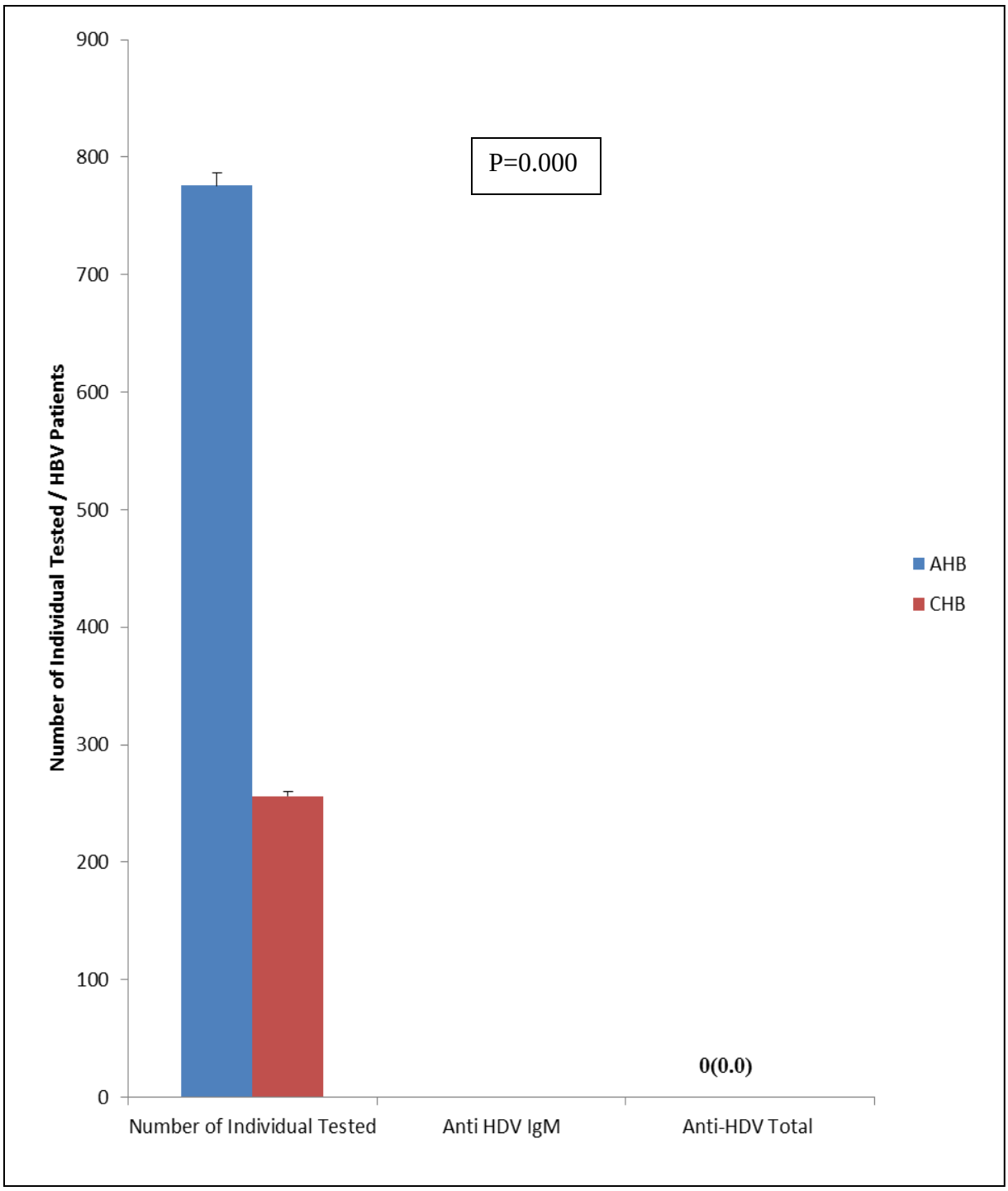


Figure 4.8: Seroprevalence of HDV Sero-Markers among HBV Patients

Key:

HBV: Hepatitis B Virus
HDV: Hepatitis Delta Virus
Anti-HDVIgM: Hepatitis D Virus Immunoglobulin M
CHB: Chronic Hepatitis B virus
AHB: Acute Hepatitis B Virus
HIV: Human Immunodeficiency Virus

Out of the 100 $HBSAg^-$ (Hepatitis B Surface antigen negative), 20 (20%) were positive for anti-$HBcT^+$ with p value of 0.147, while 5 (25%) were positive to HBV DNA with p value 0.163 (Fig. 4.9).

Out of the 256 HIV patients with $HBsAg^+$, the highest viral load range of $>2\times10^4$ - $>1.7\times10^8$ IU/mL where observed in 11.3% of the population, followed by 2001- 2×10^4 IU/mL seen in 24.2% and 20- 2×10^3 IU/mL in 41.1% and lowest viral load range of <20 IU/mL was reported in 23.4% (Fig. 4.10). P value showed statistical significance of 0.000.

The highest viral load ($>1\times10^7$ IU/mL) was observed in 5.1% of the Hepatitis C positive HIV patients, followed by 100001-1×10^6IU/mL detected in 26.3% of the HIV patients and 1001-1×10^5 IU/mL seen in 42.3%. While the lowest viral load range of <1000 IU/mL was observed in 26.3% of the patients (Fig 4.11). P value showed statistical significance of 0.000.

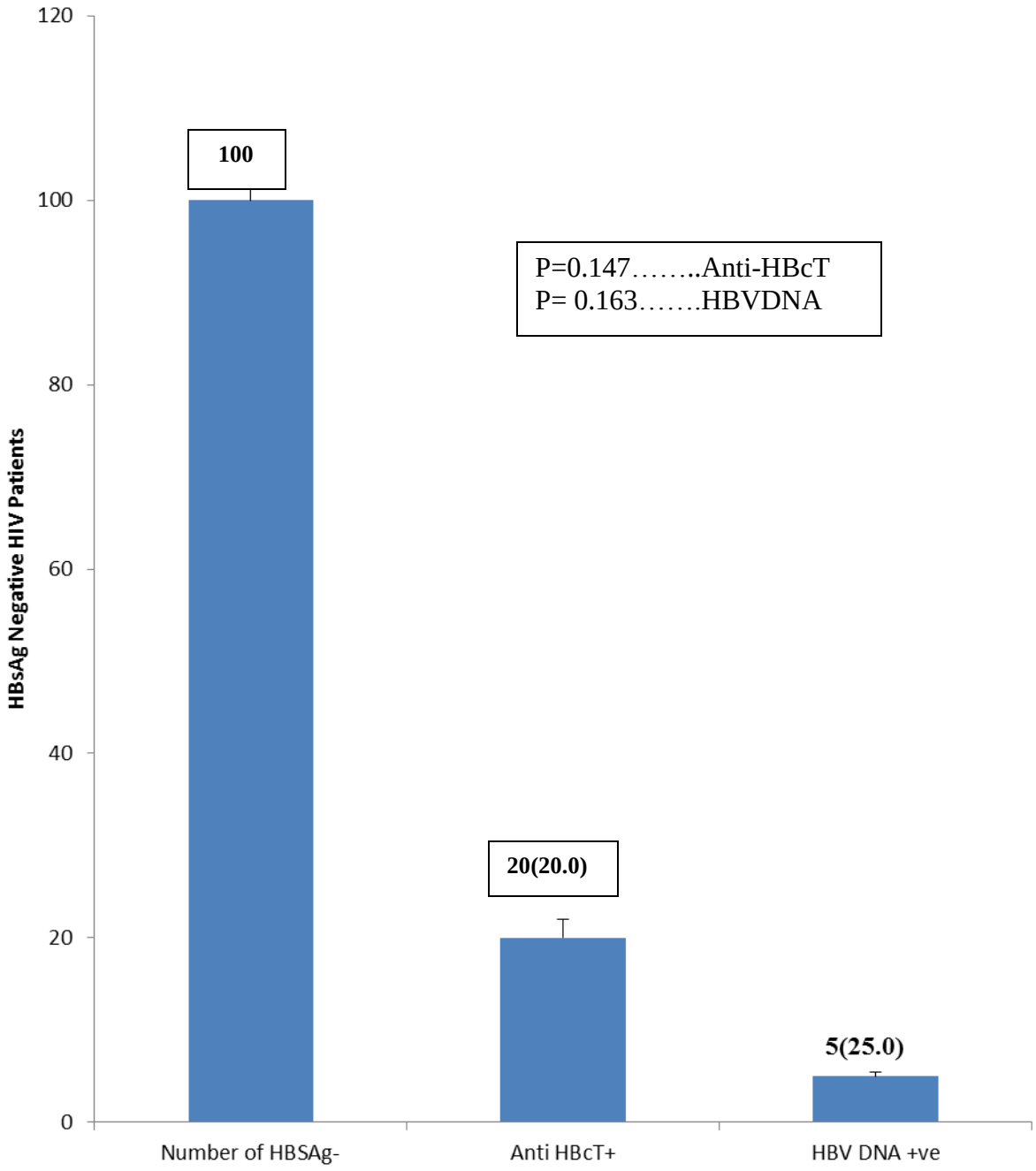


Figure 4.9: Seroprevalence of anti-HBcT and HBV DNA among HBsAg Negative HIV Patients

Key:

Anti HBcT: Hepatitis B Core Antibody Total
HBSAg- Hepatitis B Surface Antigen Negative
HBV DNA: Hepatitis B Virus DNA

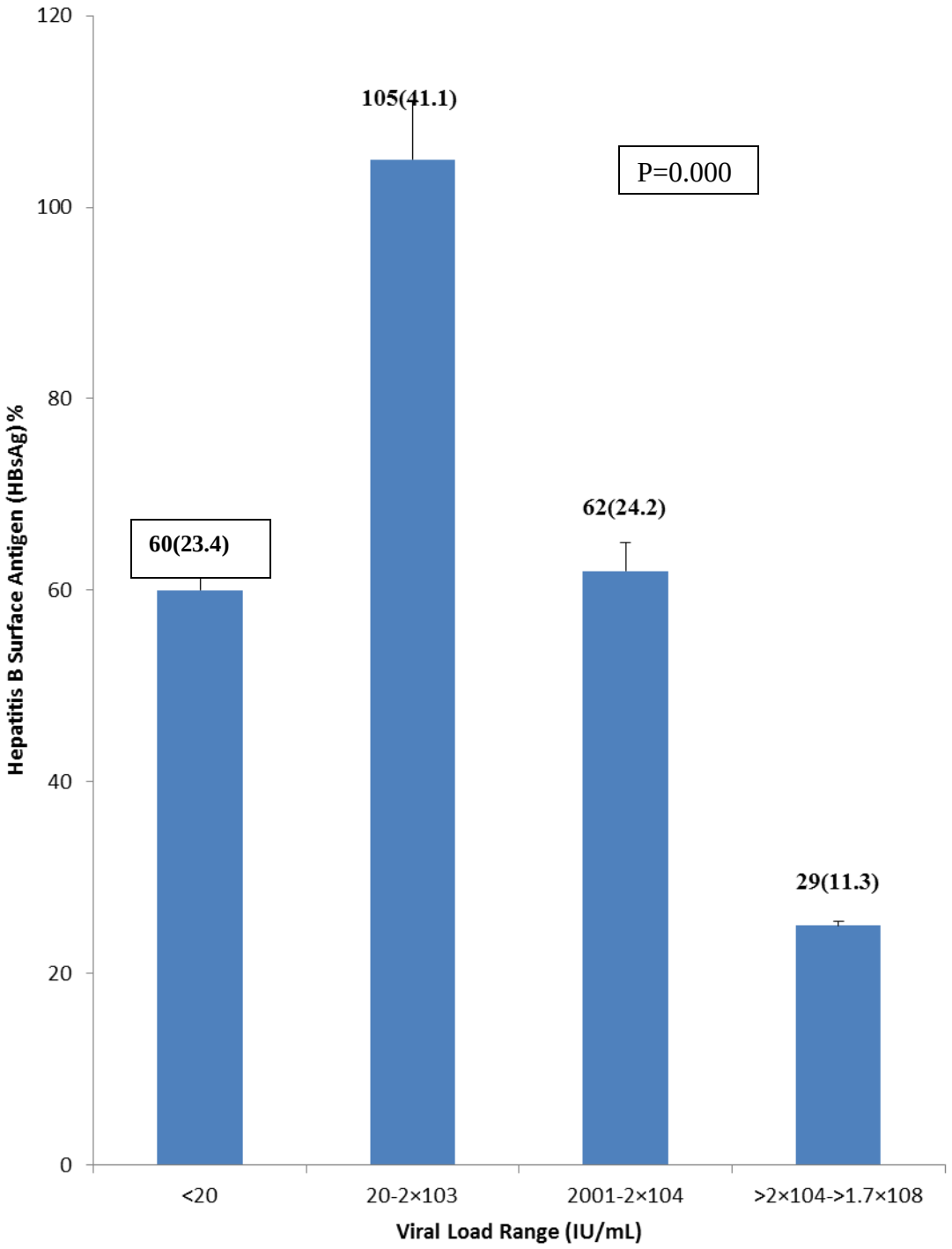


Figure 4.10: Profile of Hepatitis B Viral Load Results of Hepatitis B Surface Antigen (HBsAg) Positive

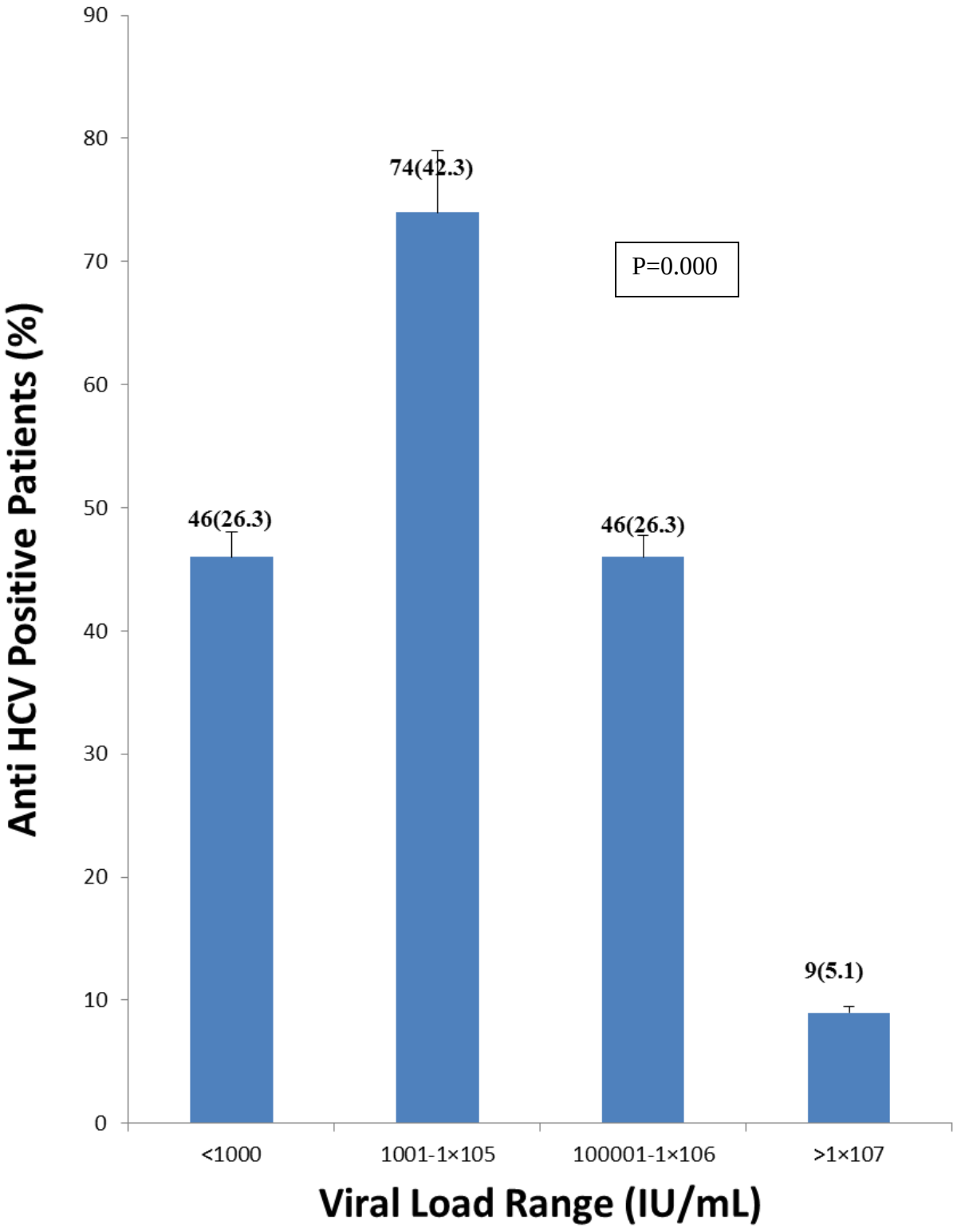


Figure 4.11: Profile of Hepatitis C Viral Load Results of anti HCV Positive Patients

Out of the 2500 positive HIV sampled, 5.7 % with tattooing/tribal mark were positive for HBsAg+ and 12.0 % without tattooing/tribal mark were positive for HBsAg+ with P-value of 0.000 (statistically significant). 3.1 % with body piercing/ear were positive for HBsAg+ and 21 % without body piercing/ear were positive for HBsAg+ with P-value of 0.002 (statistically significant). 5.0 % who have had blood transfusion were positive for HBsAg+ and 11.2 % without blood transfusion were positive for HBsAg+ with P-value of 0.000 (statistically significant). 16.7 % who have had surgical procedure were positive for HBsAg+ and 6.6% without surgical procedure were negative for HBsAg+ with P-value of 0.000. 12.5 % who have had dental extraction were positive for HBsAg+ and 9.8 % without dental extraction were positive for HBsAg+ with P-value of 0.000. 13.3 % who consume alcohol were positive for HBsAg+ and 5.6% who do not consume alcohol were positive for HBsAg+ with P-value of 0.000. 2.5 % who had circumcisions were positive for HBsAg+ and 24.0 % who do not had circumcisions were positive for HBsAg+ with P-value of 0.000. 10.1 % who had sexual activity were positive for HBsAg+ and 10.8 % who do not have sexual activity were positive for HBsAg+ with P-value of 0.002. 15.9 % who had intravenous injection were positive for HBsAg+ and 7.6 % who do not have intravenous injection were positive for HBsAg+ with P-value of 0.000. All the risk factors (tattooing/tribal mark, body piercing/ear, blood transfusion, surgical procedure, dental extraction, alcohol consumption, circumcision, sexual activity and intravenous injection) distribution amongst HIV patients with respect to HBV sero-status were all indicated in Table 4.3.

Table 4.3: Risk Factors Distribution amongst HIV Patients with respect to HBV Sero-Status

Associated Risk Factors	Total (2500)	$HBsAg^+$ (%)	P-value
Tattooing/Tribal mark			
Yes	700	40 (5.7)	0.000
No	1800	216 (12.0)	
Body Piercing/Ear			
Yes	1500	46 (3.1)	0.002
No	1000	210 (21)	
Blood Transfusion			
Yes	400	20 (5.0)	0.000
No	2100	236 (11.2)	
Surgical Procedure			
Yes	900	150 (16.7)	0.000
No	1600	106 (6.6)	
Dental Extraction			
Yes	400	50 (12.5)	0.000
No	2100	206 (9.8)	
Alcohol Consumption			
Yes	1500	200 (13.3)	0.000
No	1000	56 (5.6)	
Circumcision			
Yes	1600	40 (2.5)	0.000
No	900	216 (24.0)	
Sexual Activity			
Yes	2000	202 (10.1)	0.002
No	500	54 (10.8)	

Table 4.3: Risk Factors Distribution amongst HIV Patients with respect to HBV Sero-Status

Associated Risk Factors	Total (2500)	$HBsAg^+$ (%)	P-value)
Intravenous Injection			
Yes	800	127 (15.9)	0.000
No	1700	129 (7.6)	

Out of the 2500 positive HIV sampled, 7.1% with tattooing/tribal mark were positive for HCV^+ and 6.9% without tattooing/tribal mark were positive for HCV^+ with P-value of 0.000. 1.3% with body piercing/ear was positive for HCV^+ and 15.5% without body piercing/ear was negative for HCV^+ with P-value of 0.002. 1.2% who has had blood transfusion was positive for HCV^+ and 8.0 % without blood transfusion were positive for HCV^+ with P-value of 0.000. 4.4% who have had surgical procedure were positive for HCV^+ and 8.4% without surgical procedure were positive for HCV^+ with P-value of 0.000. 9.5% who have had dental extraction were positive for HCV^+ and 6.5% without dental extraction were positive for HCV^+ with P-value of 0.000. 0.7% who consumes alcohol was positive for HCV^+ and 16.5% who do not consume alcohol was positive for HCV^+ with P-value of 0.000. 2.5% who had circumcisions were positive for HCV^+ and 15.0% who do not had circumcisions were positive for HCV^+ with P-value of 0.000. 6.3% who had sexual activity were positive for HCV^+ and 9.6% who do not had sexual activity were positive for HCV^+ with P-value of 0.002. 17.8% who had intravenous injection were positive for HCV^+ and 1.9% who does not have intravenous injection were positive for HCV^+ with P-value of 0.001. All the risk factors (tattooing/tribal mark, body piercing/ear, blood transfusion, surgical procedure, dental extraction, alcohol consumption, circumcision, sexual activity and intravenous injection) distribution amongst HIV patients with respect to HCV sero-status were all indicated in Table 4.4 below.

Table 4.4: Risk Factors Distribution amongst HIV Patients with respect to HCV Sero-Status

Associated Risk Factors	Total (2500)	HCV^+ (%)	P-value
Tattooing/Tribal mark			
Yes	700	50(7.1)	0.000
No	1800	125(6.9)	
Body Piercing/Ear			
Yes	1500	20(1.3)	0.002
No	1000	155(15.5)	
Blood Transfusion			
Yes	400	05(1.2)	0.000
No	2100	170(8.0)	
Surgical Procedure			
Yes	900	40(4.4)	0.000
No	1600	135(8.4)	
Dental Extraction			
Yes	400	38(9.5)	0.000
No	2100	137(6.5)	
Alcohol Consumption			
Yes	1500	10(0.7)	0.000
No	1000	165(16.5)	
Circumcision			
Yes	1600	40(2.5)	0.000
No	900	135(15.0)	
Sexual Activity			
Yes	2000	127(6.3)	0.002
No	500	48(9.6)	

Table 4.4: Risk Factors Distribution amongst HIV Patients with respect to HCV Sero-Status

Associated Risk Factors	Total (2500)	HCV^{+} (%)	P-value
Intravenous Injection			
Yes	800	142(17.8)	0.001
No	1700	33(1.9)	

On the basis of socio–demographic parameters of HBV sero-status in HIV patients as indicated in Table 4.5, out of the 2500 HIV patients in this study, 6.3% educated were positive for $HBsAg^+$, 16.1 % uneducated were postive for $HBsAg^+$ with P-Value of 0.004 showing educational level. 11.0% of the Igbo ethnic group was positive for $HBsAg^+$, while 7.2 % of the other ethnic groups were positive for $HBsAg^+$ and ethnicity has a P- value of 0.001. 8.6 % of the civil servants were positive for $HBsAg^+$. Occupation has a P- value of 0.002, 10.4 % of the students were positive for $HBsAg^+$, 12.0 % of the artisans were positive for $HBsAg^+$ and 10.5 % of the unemployed were positive for $HBsAg^+$. Locality has a P- value of 0.001, 8.6 % of the urban population were positive for $HBsAg^+$ and 14.4 % of the rural population were positive for $HBsAg^+$. Marital status has a P- value of 0.000, 8.7 % of the single were positive for $HBsAg^+$, 8.1 % of the married were positive for $HBsAg^+$, 50.0 % of the divorced were positive for $HBsAg^+$ and 10.0 % of the widow/widower were positive for $HBsAg^+$.

Table 4.5: Socio–Demographic Characteristics of HBV Sero-status in HIV Patients

Socio–Demographic Characteristics	Total (2500)	$HBsAg^+$ (%)	P-Value
Educational Level			0.004
Educated	1500	95(6.3)	
Uneducated	1000	161(16.1)	
Ethnicity			0.001
Igbo	2000	220(11.0)	
Others	500	36(7.2)	
Occupation			0.002
Civil Servants	700	60(8.6)	
Students	900	94(10.4)	
Artisans	500	60(12.0)	
Unemployed	400	42(10.5)	
Locality			0.001
Urban	1800	155(8.6)	
Rural	700	101(14.4)	
Marital Status			0.000
Single	800	70(8.7)	
Married	1300	106(8.1)	
Divorced	100	50(50.0)	
Widow/Widower	300	30(10.0)	

On the basis on socio–demographic parameters of HCV sero-status in HIV patients as indicated in Table 4.6, out of the 2500 HIV patients in this study, educational level of the patients were P-value of 0.049, 6.7 % educated were positive for HCV^{+}, 7.5 % uneducated were positive for HCV^{+}. 7.25 % of the Igbo ethnic group was positive for $HCV^{+,}$ while 6.0 % of the other ethnic groups were positive for HCV^{+} and ethnicity with P- value of 0.001. Occupation had a P- value of 0.021, 5.7 % of the civil servants were positive for HCV^{+}, 6.1 % of the students were positive for HCV^{+}, 10.0 % of the artisans were positive for HCV^{+} and 7.5 % of the unemployed were positive for HCV^{+}. 8.1 % of the urban population were positive for HCV^{+} and 4.1 % of the rural population were positive for $HCV^{+,}$ locality with P-Value of 0.000. 9.0 % of the singles were positive for HCV^{+}, 3.2 % of the married were positive for HCV^{+}, 25.0 % of the divorced were positive for HCV^{+} and 12.3 % of the widow/widower were positive for HCV^{+}, marital status with P-Value of 0.000.

Table 4.6: Socio–Demographic Characteristics of HCV Sero-Status in HIV Patients

Socio–Demographic Characteristics	Total (2500)	HCV^+ (%)	P-Value
Educational Level			0.049
Educated	1500	100 (6.7)	
Uneducated	1000	75(7.5)	
Ethnicity			0.001
Igbo	2000	145 (7.25)	
Others	500	30 (6.0)	
Occupation			0.021
Civil Servants	700	40 (5.7)	
Students	900	55 (6.1)	
Artisans	500	50 (10.0)	
Unemployed	400	30 (7.5)	
Locality			0.000
Urban	1800	146 (8.1)	
Rural	700	29 (4.1)	
Marital Status			0.000
Single	800	72 (9.0)	
Married	1300	41 (3.2)	
Divorced	100	25 (25.0)	
Widow/Widower	300	37 (12.3)	

The heamatological parameter ($CD4^+$), biochemical parameters (ALT, AST and ALP) and weights of the HIV patients coinfected with HBV were ascertained as show in Table 4.7. Heamatological parameter (heamoglobin), biochemical parameter (ALT, AST and ALP) and weights of all the HIV patients positive for HBV infection did not show statistical significant variation ($P>0.05$), but heamatological parameter ($CD4^+$) was highly significant ($P<0.05$). There was increase in mean $CD4^+$ of HBV/HIV coinfected individuals from 146.22±2.73cells/µl to 149.89±2.17 cells/µl at 6 months, to 172.07±l.88cells/µl at 12 months and to 176.22±1.73cells/µl at 18 months. HBV/HIV coinfected patients were associated with increase in mean body weight from 57.00±11.31kg to 57.29±10.39kg at 6 months, to 57.71±8.94kg at 12 months and to 58.00±0.31kg at 18 months. There was associated increase in mean heamoglobin from 10.93±l.98 g/dl to 11.13±l.43g/dl at 6 months, to 11.19±1.61g/dl at 12 months and to 12.13±l.80g/dl at 18 months. There was an increase in mean ALT (from 28.86±10.9U/L to 31.29±11.42U/L), AST (from 25.29±9.93U/L to 27.00±8.91U/L) and mean ALP (from 63.43±23.95 U/L to 67.50±20.74U/L) levels in the first six months before decreasing at 12 months to 28.71±11.08 for ALT, to 26.64±7.29U/L for AST, to 65.57±18.27 U/L for ALP and subsequently increasing at 18 months to 29.86±11.8 for ALT, to 27.29±1.30U/L for AST, to 68.43±25.15U/L for ALP.

Table 4.7: Hematological and Biochemical Parameters among HBV/HIV Patients

Parameter		Baseline	Month-6	Month-I2	Month-18	Control	P-Value
$CD4^+$(cells/µl)	Hepatitis–Positive	146.22±2.73	149.89±2.17	172.07±l.88	176.22±1.73	217.28±4.56	0.000
Weight(kg)	Hepatitis –Positive	57.00±11.31	57.29±10.39	57.71±8.94	58.00±0.31	65.6±7.3	0.154
Haemoglobin(g/dl)	Hepatitis-Positive	10.93±l.98	11.13±l.43	11.19±1.61	12.13±l.80	13.7±0.91	0.823
ALT(U/L)	Hepatitis-Positive	28.86±10.9	31.29±11.42	28.71±11.08	29.86±11.8	24.89±8.14	0.079
AST(U/L)	Hepatitis-Positive	25.29±9.93	27.00±8.91	26.64±7.29	27.29±1.30	22.66±4.57	0.163
ALP(U/L)	Hepatitis-Positive	63.43±23.95	67.50±20.74	65.57±18.27	68.43±25.15	60.30±22.37	0.134

Values = mean ± standard deviation (SD)
ALT- Alanine aminotransferase
AST- Aspartate aminotransferase
$CD4^+$- Cluster of Differentiation
ALP - Alkaline phosphate

The heamatological parameter ($CD4^+$ and haemoglobin), biochemical parameter (ALT, AST and ALP) and weights of all the HIV patients receiving HAART therapy were ascertained as show in Table 4.8. All the heamatological parameter (heamoglobin), biochemical parameter (ALT and ALP) and weights of all the HIV patients positive for HCV infection did not show statistical significant variation ($P>0.05$), but $CD4^+$ and AST were statistically significant ($P<0.05$) as shown in Table 4.19. There was associated with increase in mean $CD4^+$ from 186.90 ±2.2cells/µl to 189.93±2.05 cells/µl at 6 months, to 192.27±1.78cells/µl at 12 months and to 192.10±12.0 cells/µl at 18 months. There was an increase in mean weight (from 67.67±7.77 to 72.33±2.89kg) in the first six months before decreasing at 12 months to 66.67±4.93 kg and subsequently decrease again at 18 months to 65.67±5.77 kg. There was an increase in mean heamoglobin (from 9.30±0.53g/dl to 10.00±0.7 g/dl) in the first six months before decreasing at 12 months to 9.73±0.64g/dl and subsequently increase at 18 months to 10.30±0.23g/dl. There was an increase in mean ALT (from 30.00±4.58U/L to 45.33±9.29U/L) in the first six months before decreasing at 12 months to 42.33±9.71U/L and subsequently increase slightly at 18 months to 43.00±4.28U/L. There was an increase in mean AST (from 28.33±6.03U/L to 44.67±6.66U/L) in the first six months before decreasing at 12 months to 40.33±6.35U/L and subsequently increase at 18 months to 45.29±2.93U/L. There was an increase in mean ALP (from 95.67±11.68U/L to 107.67±24.38U/L) in the first six months before decreasing at 12 months to 103.67±9.29U/L and subsequently increase at 18 months to 105.17±10.68U/L.

Table 4.8: Hematological and Biochemical Parameters among HCV/HIV Patients

Parameter		Baseline	Month-6	Month-I2	Month-18	Control	P-Value
$CD4^+$(cells/µl)	Hepatitis –Positive	186.90±2.2	189.93±2.05	192.27±1.78	192.10±12.0	217.28±4.56	0.000
Weight(kg)	Hepatitis –Positive	67.67±7.77	72.33±2.89	66.67±4.93	65.67±5.77	65.6±7.3	0.100
Haemoglobin (g/dl)	Hepatitis-Positive	9.30±0.53	10.00±0.7	9.73±0.64	10.30±0.23	13.7±0.91	0.908
ALT(U/L)	Hepatitis-Positive	30.00±4.58	45.33±9.29	42.33±9.71	43.00±4.28	24.89±8.14	0.281
AST(U/L)	Hepatitis-Positive	28.33±6.03	44.67±6.66	40.33±6.35	45.29±2.93	22.66±4.57	0.006
ALP(U/L)	Hepatitis-Positive	95.67±11.68	107.67±24.38	103.67±9.29	105.17±10.68	60.30±22.37	0.058

Values = mean ± standard deviation (SD)

$CD4^+$-Cluster of Differentiation
ALT- Alanine aminotransferase
AST- Aspartate aminotransferase
ALP- Alkaline phosphate

The heamatological parameter ($CD4^+$ and heamoglobin), biochemical parameters (ALT, AST and ALP) and weights of the HIV patients coinfected with HBV/HCV were ascertained as shown in Table 4.9. All the heamatological parameter ($CD4^+$ and heamoglobin), biochemical parameter (AST and ALP) and weights of all the HIV patients positive for HCV infection did not show statistical significant variation ($P>0.05$) except ALT which showed $P= 0.026$, hence all the heamatological parameters are not significant in HIV patients coinfected with HCV infection as shown in Table 4.20. This was associated with decrease in mean $CD4^+$ (from 152.69±2.9cells/µl to 150.24±2.12 cells/µl) in the first six months, increase at 12 months to 181.23±1.54cells/µl and also increase at 18 months to 189.04±1.4 cells/µl. There was a slight increase in mean weight (from 59.38±11.36 kg to 59.94±11.12kg) in the first six months, followed by a decrease at 12 months to 59.29±8.44kg and subsequently decrease again at 18 months to 58.45±2.2 kg. There was an increase in mean heamoglobin from 10.64±1.9g/dl to 10.93±1.38g/dl at 6 months, to 10.93±1.572g/dl at 12 months and a decrease to 9.00±1.0 g/dl at 18 months. There was an increase in mean ALT (from 29.06±9.97U/L to 33.76±12.14U/L) in the first six months before decreasing at 12 months to 31.12±0.83 U/L and subsequently increase slightly at 18 months to 33.23±0.1 U/L.There was an increase in mean AST (from 25.82±9.28 U/L to 30.12±10.87U/L) in the first six months before decreasing at 12 months to 29.06±8.78 U/L and subsequently increase at 18 months to 33.23±7.3 U/L. There was an increase in mean ALP (from 69.12±25.37U/L to 74.59±29.94U/L) in the first six months before decreasing at 12 months to 72.29±22.44U/L and subsequently increase at 18 months to 72.47±23.9U/L.

Table 4.9: Hematological and Biochemical Parameters among HIV/HBV/HCV Patients

Parameter		Baseline	Month-6	Month-I2	Month-18	Control	P-Value
$CD4^+$(cell/µl)	Hepatitis –Positive	152.69±2.9	150.24±2.12	181.23±1.54	189.04±1.4	217.28±4.56	0.319
Weight(kg)	Hepatitis –Positive	59.38±11.36	59.94±11.12	59.29±8.44	58.45±2.2	65.6±7.3	0.407
Haemoglobin (g/dl)	Hepatitis-Positive	10.64±1.9	10.93±1.38	10.93±1.572	9.00±1.0	13.7±0.91	0.964
ALT(U/L)	Hepatitis-Positive	29.06±9.97	33.76±12.14	31.12±0.83	33.23±0.1	24.89±8.14	0.026
AST(U/L)	Hepatitis-Positive	25.82±9.28	30.12±10.87	29.06±8.78	33.23±7.3	22.66±4.57	0.830
ALP(U/L)	Hepatitis-Positive	69.12±25.37	74.59±25.94	72.29±22.44	72.47±23.9	60.30±22.37	0.978

Values = mean ± standard deviation (SD)
$CD4^+$-Cluster of Differentiation
ALT-Alanine aminotransferase
AST-Aspartate aminotransferase
ALP-Alkaline phosphatase

The polymerase chain reaction (PCR) amplification of occult HBV DNA isolates is shown in Plate 4.1 below. The Lane M is the marker DNA Ladder which posse's molecular weight of 100 bp, Lane 9 serves as the positive control. Lane 3–7 HBV with molecular weight of 300 bp, Lane 8 serves as the negative control and Lane 1 and 2 have molecular weight of 298bp.

The polymerase chain reaction (PCR) amplification of HCV isolates is shown in Plate 4.2 below. The Lane M is the marker DNA Ladder which posses molecular weight of 100 bp, Lane 1 serves as the positive control. Lane 2, 4, 5, 6 and 7 are HCV with molecular weight of 250 bp and Lane 3 serve as the negative control.

HBV Singleplex PCR is shown in Plate 4.3 below. The Lane L is the marker DNA Ladder which posses molecular weight of 100 bp. It has 4 controls Lane 1 serves as the positive control, Lane 2 DNA Neg Control, Lane 6 weak positive and Lane 7 Negative Control and Lane 3 and 4 are positive samples with Ampilicon size of 116 bp.

Plate 4.4 is HBV Genotyping by Multiplex PCR, L is 100 bp ladder, Lane 1 is Positive control (N1 Reaction), Lane 3 is the positive sample TH21: (Genotype Type E) in N2 Reaction with Amplicon size of 666bp and Lane 4 is Negative control in N2 Reaction

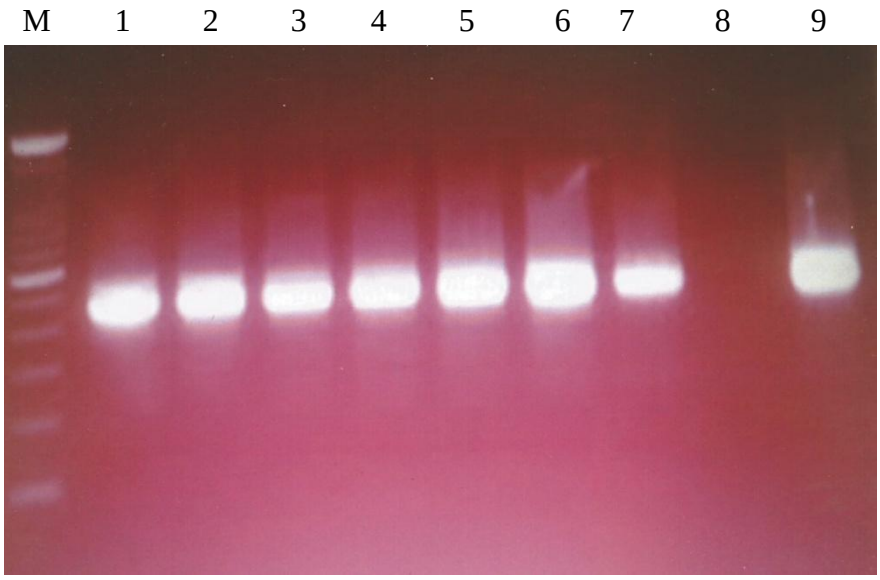


Plate 4.1: PCR Amplification of Occult Hepatitis B Virus

Lane M: Size marker DNA Ladder (100bp)

Lane 1 and 2: Specimens positive for Occult Hepatitis B Virus (298bp)

Lane 3, 4, 5, 6, and 7: Specimens positive for Occult Hepatitis B Virus (300bp PCR band).

Lane 9: positive control for Occult Hepatitis B Virus

Lane 8: Negative control.

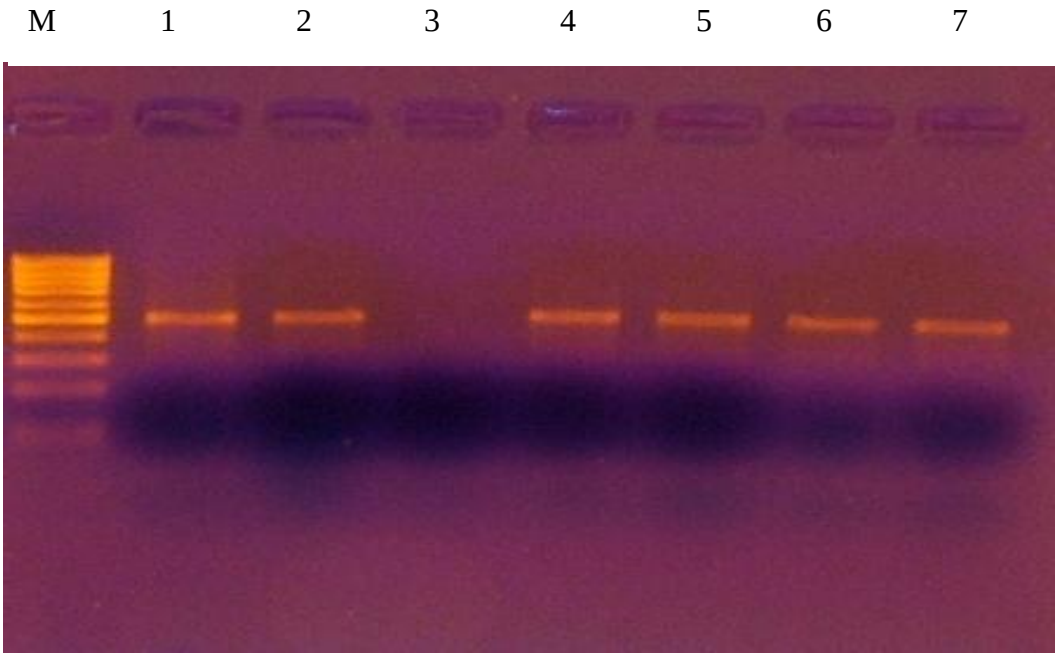


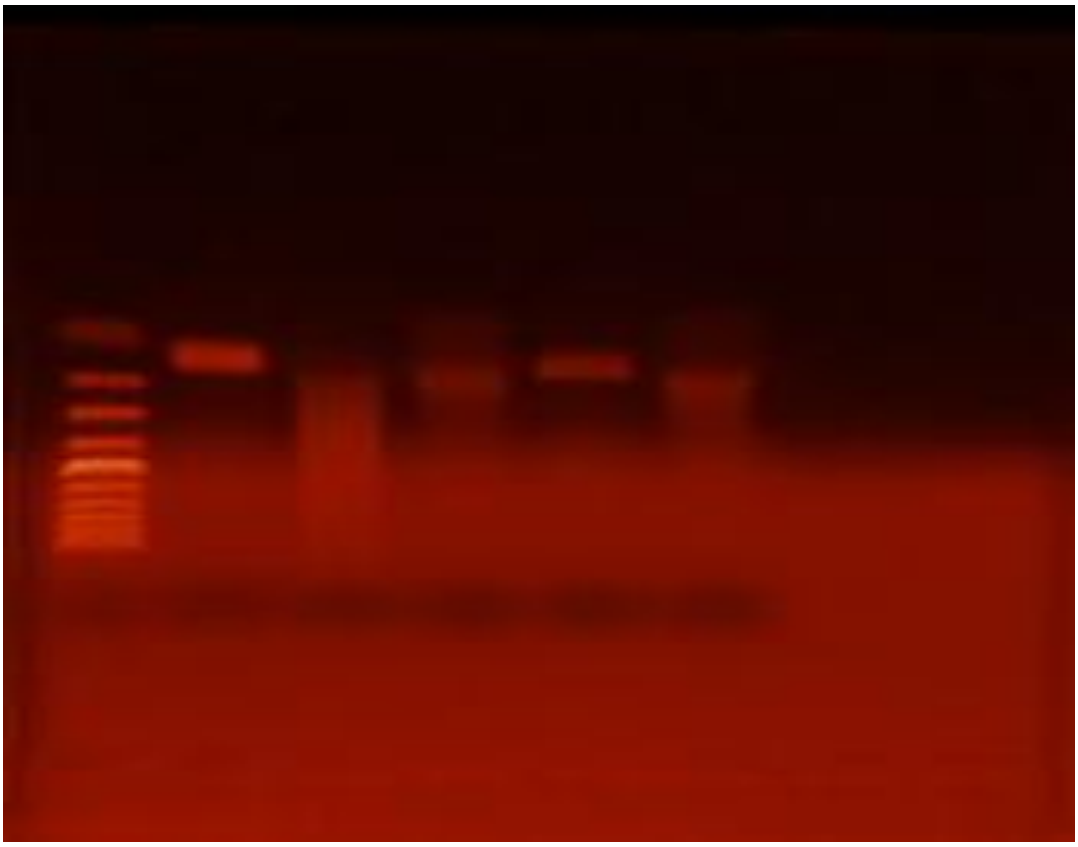
Plate 4.2: PCR Amplification of Hepatitis C Virus

Lane M: Size marker DNA Ladder (100bp)

Lane 1: positive control

Lane 2, 4, 5, 6 and 7: Specimens positive for Hepatitis C Virus (250bp PCR band).

Lane 3: Negative control.



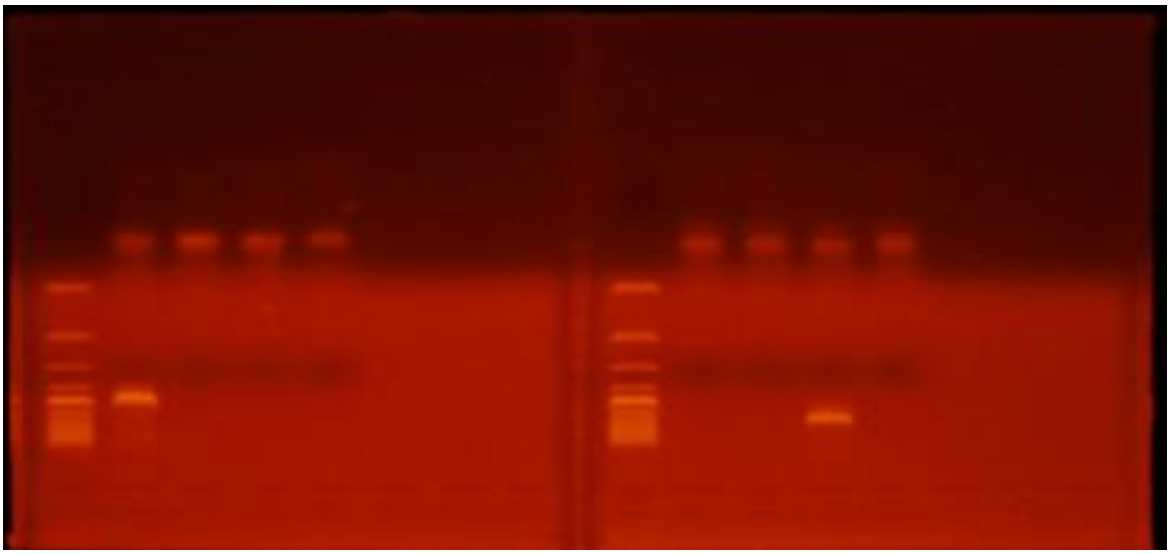
L 1 2 3 4 5 6

Plate 4.3: HBV Singleplex PCR

1- Positive Control
2- DNA Neg Control
3- TH201
4- TH21
5- Weak Positive
6- Negative

Amplicon size is 116 bp
L is 100 bp ladder

N1 N2



L 1 2 3 4 5 6 L 1 2 3 4

Plate 4.4: HBV Genotyping by Multiplex PCR

L is 100 bp ladder

1 Positive control (N1 Reaction)
3 TH21: (Genotype Type E) (N2 Reaction) Amplicon size is 666bp
4. Negative control (N2 Reaction)

Table 4.10 shows the DNA sequencing analysis of hepatitis B and C viruses with evidence attached in Appendix below. Samples TH21 and TH201 been hepatitis B viruses are Genotype E and shared consensus sequence of 99 % homology with accession number of 10407.

Samples A18, B9, TH144, and TH176 been hepatitis C viruses were all Genotype 2, they all have a consensus sequence of A18 (97 % homology), B9 (98 % homology), TH144 (96 % homology) and TH176 (96 % homology).

Table 4.10: Genotype and Homology with Consensus Sequence of Hepatitis B and C Viruses

Samples Tested (Type of hepatitis)	Genotype	Homology with Consensus Sequence (%)
TH21 (Hepatitis B)	E	99
TH201 (Hepatitis B)	E	99
A18 (Hepatitis C)	2	97
B9 (Hepatitis C)	2	98
TH144 (Hepatitis C)	2	96
TH176 (Hepatitis C)	2	96

CHAPTER FIVE

DISCUSSION

This study investigated the epidemiology of Hepatitis B and C virus infections amongst HIV patients on HAART in SouthEastern Nigeria. Generally, the transmission of the viruses were associated with risk factors such as tattoing/tribal mark, body piercing/ear, blood transfusion, surgical procedure, dental extraction, alcochol consumption, circumcision, sexual activity and intravenous injection amongst drug users. HIV, Hepatitis B and C viruses are prevalent infections in sub-Saharan Africa (Kamenya *et al*., 2017).

The study showed a prevalence rate of 10.2% of HBsAg in the study population within the SouthEast, hence is a seromarker for acute and chronic infections to Hepatitis B virus. This figure of 256 (10.2%) is classified as high endemicity of Hepatitis B virus infection (≥ 8%) (Lavanya *et al*., 2012). According to WHO (2009) classification for Hepatitis B virus endemicity, areas with 8.0% prevalence are endemic for Hepatitis B virus infection. Anambra, Enugu, Abia, Imo and Ebonyi States had prevalence rates of 11.4%, 12.4%, 9.0%, 10.0% and 8.4% respectively, hence SouthEast are endemic to Hepatitis B virus infection. Studies on HIV/HBV co-infection in Nigeria yielded prevalence ranging between 10% and 70% giving the widest variation in prevalence of HIV/HBV co-infection from studies emanating from any country all over the world (Owolabi *et al*., 2014). In line with results of this work, similar results have been observed with 10.4 % from Gombe (Mustapha *et al*., 2004), 7.9 % from Abuja (Tremeau-Bravard *et al*., 2012). The results of this work are greater than the report of Umutesi *et al*. (2017) who disclosed 4.3 % of HIV patients sampled were positive for Hepatitis B infection. In the same vein, 5.1 % HIV–HBV co-infection prevalence in some rural settings of Botswana were detected (Mandiwanaa and Tshitenge, 2017). Higher than the result of this study, Ashir *et*

al. (2009) revealed 19 % HBV-HIV co-infection in Maiduguri. The seroprevalence rate amongst various states are lower than the 12.5% earlier reported amongst asymptomatic students in Ahmadu Bello University, Zaria (Main Campus) (Aminu, *et al.*, 2013), 15.5% found among Medical students of Usman Danfodio University, Sokoto, (Alo *et al.*, 2013). In contrast, Ugwuja and Ugwu (2009) reported a lower seroprevalence of 4.1% among adolescents in Abakiliki, South Eastern, Nigeria and 4.7% among students in University of Uyo (Mboto and Edet, 2012). High prevalence of Hepatitis B virus infection in the studied area might be due to lack of vaccination among the general population which has enhanced the transmission of the virus and the difference in prevalence rates between the states might also be due to differences in social, economic and educational background. The implication of this finding is that most of the patients carrying Hepatitis B virus are asymptomatic and might serve as a source of transmission of the virus and may even become chronic carriers, which develop to chronic hepatic inflammation that can slowly progress to severe liver diseases such as cirrhosis and hepatocellular carcinomas.

Prevalence of HCV infection varied across different regions and populations (Messina *et al*., 2015). The seroprevalence of Hepatitis C virus out of 2,500 HIV patients sampled in this study, 175 (7.0 %) were positive for anti-HCV. Contrary to the result of this work, Tremeau-Bravard *et al.* (2012) reported 2.3 % of HCV in HIV patients in Abuja. Umutesi *et al*. (2017) revealed 4.6 % of HCV in HIV patients from public and private health facilities in Rwanda. It's however, slightly higher than 3.0% reported by Ejele *et al.* (2006) in the Niger Delta, Nigeria and 3.6% prevalence reported by Ugbebor *et al.* (2011) but less than 8.4% seropositivity documented for blood donors in Lagos (Ayolabi *et al.*, 2006). The figure 7.0% is much lower when compared to values obtained with studies in Enugu, Jos and Kaduna with 14.9%, 15.2%

and 11.9% respectively (Ebie and Pela, 2006). The prevalence of HCV infection is also comparable to what was reported elsewhere outside Nigeria. In this study the prevalence rate (7.0%) was found to be higher when compared to reports from SouthEast Asia (2.15%), America (1.17%) and Europe (1.03%) but lower when compared with Egypt (20%), (WHO, 2007). The highest seroprevalence rate was recorded in Anambra state (10.0 %) and Enugu state (10.0 %), followed by Abia state (6.0 %) and Ebonyi State (5.0 %), Imo state had the least (4.0 %). However, the prevalence of HCV for the general population is not clear. This is because of challenges such as barriers to screening, cost-related factors, and inadequate knowledge and awareness of hepatitis C (Averhoff *et al*., 2012). Co-infection of HCV with HIV accelerates the progression of hepatic fibrosis and results in a more aggressive course of liver disease (Wyles *et al*., 2015).

Co-infection of HIV with the hepatitis B virus (HBV) and the Hepatitis C viruses (HCV) were common event due to the similar routes of transmission (Bhaumik *et al*., 2015). Studying patterns of co-infection with HBV, HCV and HIV were of great importance, particularly in the context of controlling morbidity and mortality caused by liver disease (Daw *et al*., 2014). The knowledge on co-infections in a patient of HIV was vital, little were known about the prevalence HIV and Hepatitis co-infection among SouthEastern Nigerian states (Abia, Anambra, Enugu, Ebonyi and Imo). However, Tripathi *et al.* (2007) stated that the prevalence of co-infection may vary according to the geographical differences and the modes of transmission. Only 25 (1.0 %) HIV patients were recorded positive for HBV/HCV co-infection of the total 2,500 HIV patients sampled in this study. The result obtained in this work was found consistent with the studies conducted by Muriuki *et al*. (2013), who reported 1.0 % in Nairobi-Kenya and Otegbayo *et al*. (2012) who reported dual co-infection (HBV/HCV) of 0.16 % among HIV patients in Ibadan. In

the same vein, Padmapriyadarsin *et al.* (2006) reported 2.1 % prevalence of HBV and HCV co-infection among HIV patients in Chennai, India. Other low HBV/HCV co-infection among HIV patients have been reported in Libya 0.02 % (Daw *et al*., 2014), 0.15 % in Kenya (Kerubo *et al*., 2015), 0.1 % in Northern India (Tripathi *et al*., 2007), 1.1 % in Northern India (Ankur *et al*., 2012), 1.25 % in Iran (Amiri *et al*., 2016), 1.6 % in France (Larsen *et al*., 2008) and 0.7 % in Abuja (Tremeau-Bravard *et al*., 2012). The result also
reflected what was found earlier in Nigeria and in other West African countries (Kerubo *et al*., 2010; Mboto *et al*., 2010; Di'Bisceglie *et al*., 2010; Belay *et al*., 2010; Adewole *et al*., 2009; Otegbayo *et al*., 2008; Diop-Ndiaye *et al*., 2008; Firnhaber *et al*., 2008; Pirillo *et al*., 2007). Even though only 25 (1.0 %) patients showed a triple infection (HBV/BCV/HIV), they must be closely monitored for treatment. In contrast, higher prevalence of HBV-HCV among HIV patients (7.1% %) have been reported in China (Zhou *et al*., 2012), 3.9 % in Lagos (Balogun *et al*., 2012), 7.2 % in Keffi (Forbi *et al*., 2007) and 19 % in Nepal, India (Ionita *et al*., 2017).

In this study, age range of 31-40 years showed highest prevalence of $HBsAg^{+}$ (17.5%) (Table 4.2). The finding of this study is in line with Pennap *et al.* (2010), who reported high prevalence of 13.8% occurring among those within the ages of 1 – 40 years. However, there was no significant difference ($P>0.05$) established between the viral infection and age (Table 4.2). Kamenya *et al*. (2017) reported that HIV within the age range of 35-44 years showed the highest prevalence of HBV/HIV co-infection (22.7 %). Similar to the result of this study which was done at Keffi, highest HBV/HIV prevalence (53.85 %) was recorded between age ranges of 32-38 years (Gyar *et al*., 2014). Also according to the studies done by Okechukwu *et al*. (2014), Lar *et al*. (2013), and Sarkar *et al*. (2013), there results showed the age range of 36– 40 years, 36-40 years and 30-39 years, respectively. This study showed a decreasing prevalence of HBsAg

positivity with age. This could be attributed to risk factors such as ear piercing, dental extraction, intravenous injections and tattoing which people of younger age groups are exposed to than the older groups. Contrarily, Ishaku *et al*. (2013) reported the highest prevalence (33.3 %) of HBV/HIV co-infection in patients within age range of 51-60.

The presence of anti-HBs is generally interpreted as indicating recovery and immunity from Hepatitis B virus infection. Only 34 % of 2500 HIV infected participants had protective Anti-HBs titers. Age range between 31-40 years (52.5 %) showed the highest Anti-HBs^{+}, this was a clear indication that age range between 31-40 years indicated the highest recovery and immunity from HBV infection, followed by patients by age range between 21-30 years (40.0%). The lowest responsiveness to HBV vaccine among the HIV patients between age range of >70 years (8.0 %) observed in this study could also be explained by a faster decline of vaccine titer after the initial response. The results were consistent with the study of Haban *et al*. (2017), who reported 29 % prevalence of protective anti-HBs response among HIV patients in Morocco. This result is also in accordance with previous studies that have reported similar low vaccine responsiveness, in patients with HIV/AIDS (Pippi *et al*., 2008). The result of this work is higher than the work of Freitas *et al*. (2014) who reported the prevalence rate of Anti-HBs (16.7 %) among HIV patients sampled in Brazil. Despite immunization against Hepatitis B not being routinely offered for all HIV infected individuals to prevent primary HBV infection, the findings emphasizes the need to ensure early vaccination as a means of primary prevention against HBV infection.

A strong positive reaction to anti-HBcIgM during acute Hepatitis is indicative of an acute HBV infection (Mandell *et al*., 2010). Anti-HBcIgM (20.8 %) was observed in the overall sampled population. 31-40 years age range showed the highest anti-HBcIgM (35.0 %), Martins

et al. (2014) analyzed for the presence of anti-HBcIgM in HIV patients, but recorded no result, an indication of no acute HBV infection. 31-40 years revealed the highest anti-HBcIgM, a clear indication of highest acute HBV infection associated with this age range.

Anti-HCV^{+} (7.0 %) were observed in the overall sampled population. In this study, patients aged between 31-40 years (17.5 %) had the highest HCV antibody (anti-HCV^{+}) seroprevalence rate. Similarly, Chandra *et al*. (2013) reported 8.3 % of anti-HCV^{+} in HIV infected patients. Higher prevalence of anti-HCV among HIV patients in Ughelli, Delta State was determined to be 15.0 % (Newton *et al*., 2015). This was contrary to observations of Sule *et al.* (2009) who reported that high prevalence was found in patients aged 50 years and above, but agrees with the findings of Ejele *et al.* (2006) and Ayolabi *et al*. (2006) who reported highest prevalence of HCV antibodies in the age group 30 – 39 years; the supposedly sexually-active group. In this study age bracket of 18 – 49 years of which age 31– 40 years is a subset had the highest anti–HCV antibody seropositivity. However, there was no significant association ($P > 0.05$) between the age of the patients and prevalence rate of HCV antibodies. Presence of anti-HCV^{+} is an indication of past or present infection with the HCV virus. The difference in prevalence could be attributed to differences in social behaviour of the HIV patients involved in this study and population size of the individual countries.

On the basis of the sex parameter in the study population, males showed higher sero-markers ($HBsAg^{+}$, anti-HBs^{+} and anti-HBcIgM) with the corresponding seroprevalence rate of 11.8 %, 35.7% and 22.1 % respectively. While, females showed the lowest seroprevalence rate of $HBsAg^{+}$ (8.2 %), anti-HBs^{+} (31.8 %) and anti-HBcIgM (19.1 %) as shown in Fig 4.4. In the same vein, Umutesi *et al*. (2017) revealed higher prevalence of $HBsAg^{+}$ in male (5.4 %) than in female (3.7 %), Nada and Atwa (2013) showed 2.3 % in male and 2.1 % in female. High

prevalence of HBsAg in males was reported by other studies. This was 52.9% in Georgia, 51.5 % in the USA, 93 % in Jordan and 91 % in Kuwait (Ameen *et al*., 2005 and Wang *et al*., 2003), 75 % in Ibadan (Balogun *et al*., 2012) and 95.2 % in Philippine (Rodenas *et al.*, 2006). In disagreement, Luma *et al*. (2017) reported higher prevalence of $HBsAg^+$ in female (13.9 %) than in male (8.8 %). The higher sero-markers ($HBsAg^+$, anti-HBs^+ and anti-HBcIgM) obtained in male than in female in this present study might be attributed to some risk behaviors of males, such as outside socialization, multiple sex relationships etc. and may also be due to fewer females were screened compared to males. The prevalence of Hepatitis B virus infection with respect to gender was found to be high in males (11.8%) than females (8.2%), with respect to $HBsAg^+$. However, there was no significant difference ($P \geq 0.05$) between the sexes.

These findings were in contrast with Okonko *et al*. (2010), who reported that there was significant difference ($P \leq 0.05$) between males and females with respect to hepatitis B infection. But it is consistent with the finding of Pennap *et al.* (2010), who reported that, no significant association ($P \geq 0.05$) between gender and viral infection, eventhough the prevalence was higher (11.8%) among the males. This study is also in agreement with earlier findings of Agbede *et al.* (2007), Lawal *et al.* (2009), Fasola *et al.* (2009) Sule *et al.* (2010), Opaleye *et al.* (2010) and Mabayoje *et al.* (2010), who reported that no significant difference ($P \geq 0.05$) was observed among sexes. There was no obvious explanation for the difference in gender as a risk factor for this viral infection, although, Bwogi *et al.* (2009), reported a lower prevalence of HBV in men than in women and suggested that due to circumcision in male that serve as protective cover. In line with Pennap *et al.* (2010), this was not the case in this study even though it was in anarea that male circumcision is mandatory. The result of this study is not surprising, since most of the males in the study area have multiple sex partners which could serve as asource for the infection.

The presence of anti-hepatitis C virus antibody (anti-HCV Ab) indicates previous exposure to hepatitis C virus. Female (8.2 %) showed the higher seroprevalence rate of anti-HCV^+ than male (6.1 %) as shown in Fig 4.5. In the same vein, 64.1 % were recorded in men and 35.9 % in women (Tizzot *et al*., 2014). Higher seroprevalence was also recorded in female (7.1 %) than in male (6.7 %) in Benin City as observed by Ojide *et al*. (2015). However, Ramarokoto *et al.* (2008) in their study on the seroprevalence of hepatitis C in urban areas of Madagascar reported that the prevalence did not differ significantly according to gender. The reason for this is not obvious, but may be due to the epidemiological differences in the different study populations and variations in methodology.

Acute hepatitis B virus (HBV) is a common cause of acute icteric hepatitis in adults (Shiffman, 2014). HIV and acute hepatitis B coinfection is extremely rare (Bansal *et al*., 2010). Serologic tests for hepatitis B surface antigen (HBsAg) and hepatitis B core antibody immunoglobulin M (anti-HBcIgM) are required for the diagnosis of acute hepatitis B virus (HBV) (CDC, 2015). Out of the total 776 Acute Hepatitis B (AHB) patients obtained from this study, 520 (67.0 %) HIV patients revealed the presence of anti-HBc IgM^+, followed by $HBsAg^+$ obtained in 256 (33.0 %) HIV patients and no HIV patients were detected with anti-HBs^+ (Fig 4.6). The results of anti-HBc IgM among AHB patients group in the present study is lower than the result obtained in China, where all the 35 HIV patients positive for AHB revealed the presence of anti-HBc IgM (Fayyadh and Ma, 2017). This difference may due to early stage of acute infection among patients in present study. In consistence with the report of this study, Fayyadh and Ma (2017) revealed that no anti-HBs^+ was recorded in all the AHB HIV positive patients. Chen *et al*. (2004) revealed that the likelihood of spontaneous HBsAg seroconversion during acute HBV infection varies according to patient age and immune competence and also

that up to 97% of healthy adults with acute HBV will clear the infection. People who test positive for the hepatitis B virus for more than six months (after their first blood test result) are diagnosed as having a chronic infection. This means their immune system was not able to get rid of the hepatitis B virus and it still remains in their blood and liver (HBF, 2016). Chronic hepatitis B virus (HBV) infection is an increasing cause of morbidity and mortality in human immunodeficiency virus (HIV)-infected individuals (Bautista-Amorocho *et al*., 2014). Chronic hepatitis B (CHB) is associated with cirrhosis and hepatocellular carcinoma (HCC) in patients suffering from congenital and/or acquired immunodeficiency (Askari *et al*., 2014).

A total of 256 HIV patients which were positive for Chronic Hepatitis B (CHB) virus were all positive for $HBsAg^{+}$ with 100% seroprevalence rate. Other serological markers (anti HBs^{+} and antiHBc IgM^{+}) were not detected in the CHB patients. Contrary to result of this work, Rockstroh (2006) reported 9.48 % of cases suffered from CHB among 232 HIV- infected patients in Germany and 45 % of 383 HIV positive patients had detectable CHB in France (Bloquel *et al*., 2014). Very high prevalence rate of CHB among HIV patients obtained in this study indicates that the immune system of all the HIV patients sampled in this study were not able to get rid of the hepatitis B virus and it still remains in their blood and liver.

Anti-HDV IgM and anti-HDV are serological parameters used to ascertain Hepatitis D Virus (HDV) infection. IgM anti-HDV is detectable during the 'window' phase of the infection (Patel and Goswami, 2016). HDV infections occur epidemically or endemically in countries where hepatitis B is endemic. A decline in the prevalence of hepatitis D infection has been noted worldwide (Kailash *et al*., 2005). HDV infection occurs only in association with HBV infection.

Out of the 776 Acute Hepatitis B (AHB) virus infected patients, none (0 %) showed the presence of the serological markers (anti-HDV IgM and anti-HDV). In the same vein, the

serological markers (anti-HDV IgM and anti-HDV) were also not detected in Chronic Hepatitis B (CHB) patients (Fig 4.8). The results of this work are in line with the results obtained by Patel and Goswami (2016) who stated that all 150 patients positive for HBV were negative for HDV IgM. The absence of these serological markers (anti-HDV IgM and anti-HDV) is an indication of absence of Hepatitis D virus infection in all samples analyzed in this study, with p value of 0.000, highly significant($P \leq 0.05$).

Viral load, also known as viral titre, is a numerical expression of the quantity of virus in a given volume. It is often expressed as viral particles, or infectious particles per mL depending on the type of assay. A higher viral burden, titre, or viral load often correlates with the severity of an active viral infection *(*Adrian *et al*., 2010*).* Viral load (VL) determination using polymerase chain reaction techniques is a useful tool in decision-making (Iregbu and Nwajiobi-Princewill, 2016). Out of the 256 HIV patients with $HBsAg^{+}$, the highest viral load range of $>2\times10^{4}$ ->1.7×10^{8} IU/mL where observed in 11.3 % of the population, followed by 2001 - 2×10^{4} seen in 24.2 % and 20-2×10^{3} IU/mL in 41.1 % and lowest viral load range of <20 IU/mL was reported in 23.4 % of the population. In consistency with result of this study, Iregbu and Nwajiobi-Princewill (2016) reported the highest load range of > 2×10^{4} ->1.7×10^{8} IU/mL within 76.1 % of the sampled population. And HBV viral load greater than 20,000 international units per milliliter (IU/mL) of blood indicates that the virus is active and has the greatest potential to cause damage to the liver and viral loads less than 2,000 IU/mL generally do not require treatment (HBF, 2016). Hence 35.5% (11.3 % + 24.2 %) of the population are at risk of experiencing liver damage due to the greater level of hepatitis B viral load observed. A baseline high HBV-DNA level > 10 000 copies/mL was associated with a significant increased risk of hepato-cellular carcinoma, HCC (Chen *et al*., 2006) and with progression towards cirrhosis (Iloeje *et al*. 2007). Low viral load

(<20 IU/mL and 20 - 2×10^3 IU/mL) obtained in study among 64.5 % (23.4 % + 41.1 %) of the sample population, Shao *et al*. (2007) stated that patients with low HBV-DNA levels, between 300 and 10^4 copies/mL, have a very low risk of progression to cirrhosis and HCC. Our findings extend the utility of HBV-DNA measurements for predicting cirrhosis or HCC risk to include HBV-infected persons among HIV positive individuals from Nigeria and Africa.

High viral load of Hepatitis C is usually > 800, 000 IU/L, or and low viral load of Hepatitis C is usually <800,000 IU/L. Knowing the viral load before starting treatment is useful because patients with "high" viral loads can have a difficult time getting the virus to become completely undetectable on treatment (VHF, 2016). The highest viral load (>1×10^7 IU/mL) was observed in 5.1 % of the Hepatitis C positive HIV patients, followed by 100001-1×10^6 IU/mL detected in 26.3 % of the HIV patients and 1001-1×10^5 IU/mL seen in42.3%. While the lowest viral load range of <1000 was observed in 26.3 % of the patients (Fig 4.11). VHF (2016) stated that patients with "low" viral loads have a better chance of getting their virus to become completely undetectable on treatment. For every 10-fold increase in baseline HCV viral load, the relative risk for clinical progression to AIDS (Mohsen *et al*., 2002).

Statistically significant variations ($P\leq0.05$) were observed in HIV patients on HAART that were positive for $HBsAg^+$ with all the risk factors; tattooing/tribal mark, body piercing/ear, blood transfusion, surgical procedure, dental extraction, alcohol consumption, circumcision, sexual activity and intravenous injection as indicated in Table 4.3. In consistency with report of this study, Ayele and Gebre-Selassie (2013) stated that alcohol consumption, dental extraction, body piercing and tattooing on body showed significant variation ($P\leq0.05$) in HIV patients positive for $HBsAg^+$. The result of this study indicates that HIV patients with the above risk factors are more likely to have infection with HBV infection than their counter parts that are negative. The result

is in disagreement with the report of Erena and Tefera (2014), who stated that risk factors such as history of blood transfusion, unsafe injection, tooth extraction, history of surgery, catheterization, abortion, tattooing and having a history of family liver disease did not show statistically significant association with HBV infection. In the same vein, Weldemhret *et al*. (2016) report that HIV/HBV infection did not show significant variation with respect to surgical, multiple sexual partners and history abortion. Ayele and Gebre-Selassie (2013) reported no significant variation ($P \leq 0.05$) with blood transfusion, surgical procedure, abortion, circumcision, ear piercing HIV patients' positive for HBV infection. Likewise, Kamenya *et al.* (2017) revealed no statistical significant variation with alcohol intake, history of blood transfusion and history of tattooing in HIV patients positive for HBV infection. The finding of this study is not surprising for the fact that the instruments used for such procedures are usually not washed and passed through naked flame before and after each procedure for sterilization. This might have been serving as a good means of HBV transmission.

The significant risk factors for HCV found among the HIV patients sampled in this work are: tattooing/tribal mark, body piercing/ear, blood transfusion, surgical procedure, dental extraction, alcohol consumption, circumcision, sexual activity and intravenous injection as shown in Table 4.4. Similarly, Luma *et al*. (2016), Eze *et al*. (2014), Freitas *et al*. (2014) and Khin *et al*. (2010) reported that tattoo is a significant risk factor in HIV/HCV co-infection. Still in line with the study of Inyama *et al*. (2005), Anaedobe *et al*. (2016), Umumararungu *et al*. (2017), they reported that blood transfusion was found as a significant risk factor in HIV/HCV co-infection. These studies do not contradict each other, but sustain the fact that the prevalence and risk factors for each region depends on the predominant socio-cultural activity and life style found among them. Body piercing/ear, blood transfusion, surgical procedure, alcohol

consumption, circumcision, sexual activity and intravenous injection showed statistical significance ($P \leq 0.05$) as also stated in Table 4.4. Contrary, Freitas *et al*. (2014), Pineda *et al*. (2005) and D'Almeida *et al*. (2017) revealed that blood transfusion is not a risk factor in HCV infection in HIV patients. The findings of this study shows that the risk factors are major route of HCV transmission in the population studied.

All the socio–demographic parameters (educational level, ethnicity, occupation, locality and marital status) were statistically significant ($P \leq 0.05$) associated with HBV infection. Out of the 2500 HIV patients in this study, 16.1 % uneducated were positive for $HBsAg^{+}$, while 6.3 % educated were positive for $HBsAg^{+}$. This report is also in line with the study of Weldemhret *et al.* (2016) and Umare *et al*. (2016). In the same vein, Ionita *et al*. (2017) also reported that HBV/HIV coinfected patients with no formal education revealed the highest number (35.7 %) of HBV/HIV coinfection in India. Contrary to this report, Marcelin *et al*. (2017) reported highest HBV/HIV coinfection among high schools in Cameroon. The distribution of HBV infections according to educational status was analyzed and the result was shown in Table 4.5. High prevalence occurred among uneducated (16.1%), followed by those who are educated (6.3%). This finding does not agree with Eke *et al.* (2011) who reported that, high prevalence was obtained among those who are educated (9.5%), followed by those without education (9.1%). However, significant relationship ($P<0.05$) was observed between educational attainment and the prevalence of Hepatitis B infection obtained. Igbo ethnic group recorded the highest (11.0 %) and other recorded (7.2 %) as obtained in this study, this is attributed to the majority of the population in SouthEastern Nigeria are Igbo ethnic group. P value was statistically significant($P<0.05$) Occupation is a known predisposing factor for HBsAg infection (Bunyamin *et al*., 2009). The distribution of Hepatitis B virus infection based on occupation

(Table 4.5) showed that Artisans had the higher prevalence of 60(12.0%), followed by unemployed 42 (10.5%), students 94(10.4%), and civil servants 60 (8.6%). The finding of this study is not surprising since most artisans and unemployed in the study area had no or little knowledge of formal education. Therefore, awareness of the preventive measures of this disease is low among such group which might have led to high prevalence rate of the infection among such groups, although significant difference ($P<0.05$) was observed between occupations and prevalence of the virus. Contrarily, Agaba *et al*. (2014) also reported highest prevalence rate HBV/HIV infection among civil servants. In line withthis study, Adefemi *et al*. (2015) reported highest prevalence rate among unemployed (38.4 %), followed by civil servants (36.80 %). High prevalence noticed among civil servants in this study may be attributed to the high exposure rate of civil servants to the community. Similarly, Marceline *et al*. (2017) revealed that daily labourers (10.56 %) showed the highest with HBV/HIV infection. The urban dwellers showed the highest prevalence rate (8.6 %) of HBV/HIV co-infection. In line with this present work, Yohanes *et al.* (2016) reported that urban (92.2 %) showed the highest, while rural showed the lowest (7.8 %) with regard to HBV/HIV co-infection. In the same vein, study from Eastern Sudan had shown higher prevalence of HBsAg among HIV patients from urban area than the rural counterparts (Abdallah *et al*., 2011). This difference might be due to the varied numbers of urban and rural study participants as compared to our study. Marital status is also considered as socio-demographic characteristics for Hepatitis B infection. There was high prevalence of HBV among divorced (50.0%) than widow/widower (10.0%), singles (8.7%) and married individuals (13.3%). This finding is in line with Okonko *et al.* (2010), who reported a prevalence of 8.0% among the singles with highly significant difference ($P < 0.05$). The high prevalence in this group (single) in comparison with the married individuals might be due to the fact that, they are

single (Unmarried, unattached) and thus free to indulge in more sexual activity. This finding is not in agreement with Eke *et al.* (2011) who reported that no statistical significant (P> 0.05) relationship was observed between the HBV infection and marital status.

The differences in socio-dermographic parameters may be attributed to sociocultural and economic differences.

It was observed that all the socio–demographic parameters (educational level, ethnicity, occupation, locality and marital status) were statistical significant (P≤0.05) associated with HCV infection (Table 4.6). On the basis of educational level, educated HIV patients showed the highest (6.7 %) of HCV infection than uneducated HIV patients. The result is in consistent with the work of Torre *et al*. (2007) and Zhang *et al*. (2014). Igbo ethnic group of (7.25 %) were the highest ethnic group of HIV/HCV infection. On the basis of occupation, the results showed students (6.1 %) were at the highest risk of HIV/HCV coinfection. The urban (8.1 %) dwellers are at the highest risk of HCV/HIV coinfection. The majority of the HIV/HCV coinfection participants are married (9.0 %). Contrary to the report of this work, Wu *et al*. (2017), reported highest prevalence of HCV/HIV coinfection among unmarried patients in China.

These patients were receiving HAART at the time of sample collection. The limitation of this study lies in the fact that, we did not know the drug combination administered to HIV patients and the duration of drug administration at the time of sample collection. Nevertheless, our finding compares favorably with the reports that HIV disease impacts adversely on haematological profile of a patient due to the enormous assault of the virus on haemopoietic cells/system (Spivak, 2002 and Klein *et al*., 2005). This occurs in addition to hepatotoxicity and nephrotoxicity of anti- retroviral drugs (ARD) administered to AIDS patients (Spivak, 2002 and Klein *et al*., 2005). Hematological and biochemical abnormalities are among the most frequent

clinicopathological manifestations of HIV patients on HAART. Hence the development and assessment of indigenous antiretroviral drugs with minimal abnormalities becomes a necessity (Ibeh *et al*., 2013).

Haematological parameter (haemoglobin), biochemical parameter (ALT, AST and ALP) and weights of all the HIV patients positive for HBV infection did not show statistical significant variation ($P \geq 0.05$), as shown in Table 4.7. This was associated with increase in mean $CD4^+$ of HBV/HIV coinfected individuals from 146.22±2.73 cells/µl to 149.89±2.17 cells/µl at 6 months, to 172.07±l.88 cells/µl at 12 months and to 176.22±1.73cells/µl at 18 months. Thus, HBV/HIV-coinfected patients might benefit from an earlier introduction of HAART (Núñez *et al*., 2006). This finding compared favorably with a report that $CD4^+$ count is lower in the HBV/HCV co-infected patients compared with the HIV mono-infected control subjects (Obienu and Nwokediuko, 2011). These authors speculated that the resultant effect of HBV/HIV co-infection could be due to an additive or synergistic effect of the two infections.

The initiation of HAART in HBV/HIV coinfected patients was associated with increase in mean heamoglobin from 10.93±l.98 g/dl to 11.13±l.43 g/dl at 6 months, to 11.19±1.61 g/dl at 12 months and to 12.13±l.80 g/dl at 18 months. The findings from our study are in agreement with the report of Shaukat *et al.* (2017) documenting increasing heamoglobin from HBV/HIV coinfected patients receiving HAART. In the same vein, Kiragga (2010) demonstrated that the majority of HBV/HIV patients initiating HAART experienced an improvement in hemoglobin. There was an increase in mean ALT (from 28.86±10.9 U/L to 31.29±11.42U/L), AST (from 25.29±9.93 U/L to 27.00±8.91 U/L) and mean ALP (from 63.43±23.95 U/L to 67.50±20.74 U/L) levels in the first six months before decreasing at 12 months to 28.71±11.08 for ALT, to 26.64±7.29 U/L for AST, to 65.57±18.27 U/L for ALP and subsequently increasing at 18 months

to 29.86±11.8 for ALT, to 27.29±1.30 U/L for AST, to 68.43±25.15 U/L for ALP as shown in Table 4.7. The transaminases are enzymes known to be associated with hepatocytes which are poured into circulation when the liver cells are attacked by the virus or the antiretroviral agent (Ngala *et al*., 2015). The results not withstanding, indicate an association of hepatocellular injury with HAART treatment in participants with retroviral infection. The findings concur with those obtained by Ngala *et al*. (2015) among Ghanaian HAART-experienced individuals for at least 1-6 months and Bello *et al.* (2014) among HIV/AIDS patients on HAART for 2-8 years in the Nigerian population.

All the heamatological parameter (haemoglobin), biochemical parameter (ALT, AST and ALP) and weights of all the HIV patients positive for HCV infection receiving HAART did not show statistical significant variation ($P \geq 0.05$) as shown in Table 4.8. The initiation of HAART in HCV/HIV conifected patients was associated with increase in mean $CD4^+$ from 186.90±2.2 cells/µl to 189.93±2.05 cells/µl at 6 months, to 192.27±1.78 cells/µl at 12 months and to 192.10±12.0 cells/µl at 18 months. As evidenced by $CD4^+$ count in our study, the degree of immune suppression may increase in HIV co-infection with HCV in agreement with International Association of Providers of AIDS Care (IAPAC, 2013), Otegbayo *et al*. (2008) in the South Western Nigeria and Idoko *et al*. (2009) in North Central Nigeria. There was an increase in mean weight (from 67.67±7.77 to 72.33±2.89 kg) in the first six months before decreasing at 12 months to 66.67±4.93 kg and subsequently decrease again at 18 months to 65.67±5.77 kg. There was an increase in mean heamoglobin (from 9.30±0.53 g/dl to 10.00±0.7 g/dl) in the first six months before decreasing at 12 months to 9.73±0.64 g/dl and subsequently increase at 18 months to 10.30±0.23g/dl. In the same vein, Kiragga (2010) revealed that HAART initiation led to increased hemoglobin levels for the majority of patients but 3.5% developed

early severe anemia within 12 months of HAART initiation. Sloand (2005) and Opie (2012) revealed that although there are multiple of possible causes of decrease in heamoglobin in HCV/HIV coinfections, but it is commonly attributed to bone marrow failure, peripheral destruction and opportunistic infections and HAART therapy. Increase in mean ALT (from 30.00±4.58 U/Lto 45.33±9.29 U/L) in the first six months was observed in HCV/HIV, followed by decrease at 12 months to 42.33±9.71 U/L and subsequently increase slightly at 18 months to 43.00±4.28 U/L. This result supports other work as documented by Yemanebrhane *et al*. (2017), Olawumi *et al*. (2014) and Abera *et al*. (2014) who reported a high mean level of ALT among HIV patients co-infected either with HBV or HCV. Moreover this study strongly supported the study conducted in Northwest Ethiopia which reported a raised level of liver enzyme in coinfected HIV patients even though it is not statistically significant (Wondimeneh *et al*., 2013). There was an increase in mean AST (from 28.33±6.03 U/Lto 44.67±6.66 U/L) in the first six months before decreasing at 12 months to 40.33±6.35 U/L and subsequently increase at 18 months to 45.29±2.93 U/L. There was an increase in mean ALP (from 95.67±11.68 U/Lto 107.67±24.38 U/L) in the first six months, followed by decrease at 12 months to 103.67±9.29 U/L and subsequently increase at 18 months to 105.17±10.68 U/L. In this study increment in the liver enzymes ALT and AST within the first six months, a decrease at the 12 months and subsequently increase at the 18 months was observed among HIV patients co-infected with HCV. This might be as a result of HCVs' nature to cause chronic viral hepatitis that can lead to cirrhosis and hepatocellular carcinoma**.** In addition these enzymes level further increment at month 6 and month 18 in case of HCV-HIV co-infection could be attributed to viral hepatitis infection as well as patient's condition like having chronic alcoholism or due to drug induced hepatotoxicity.

All the heamatological parameter ($CD4^+$ and heamoglobin), biochemical parameter (AST and ALP) and weights of all the HIV patients positive for HBV/HCV coinfection receiving HAART did not show statistical significant variation ($P \geq 0.05$), hence all the heamatological parameters are not significant in HIV patients coinfected with HCV infection receiving HAART as shown in Table 4.9. The initiation of HAART in HBV/HCV/HIV conifected patients was associated with decrease in mean $CD4^+$ from 152.69±2.9 cells/µl to 150.24±2.12 cells/µl at 6 months, followed by an increase to 181.23±1.54cells/µl at 12 months and to 189.04±1.4 cells/µl at 18 months. This report is in line with the work of Petoumenos and Ringland (2005); Van-Griensven *et al*. (2014). The mean $CD4^+$ in HBV/HCV in HIV patients in this study is lower than mean $CD4^+$ in HIV/HCV coinfected patients. Re *et al*. (2014) revealed that HIV/HBV/HCV infected patients had higher rates of hepatic decompensation than HIV/HCV-infected patients. A clear indication that the risk of decompensation was increased among triply infected patients who are receiving HAART. Higher mean $CD4^+$ obtained at month 18 is a clear indication that HIV/HBV/HCV triply infected patients should start and continue HAART, regardless of $CD4^+$ count. A cohort study from the United States found that liver related deaths occurred more frequently among patients with HIV/HBV/HCV infection than among those with either HIV/HCV or HIV infection alone (Bonacini *et al*., 2004). Similarly, a cross-sectional study from Spain showed that HIV/HBV/HCV-infected patients had a higher prevalence of cirrhosis than HIV/HBV or HIV/HCV-infected patients (Arribas *et al*., 2005). Another cohort study from Spain observed that HIV/HBV/HCV-infected patients had higher rates of cirrhosis and hepatic decompensation than HIV/HBV-infected patients (Martin-Carbonero *et al*., 2011). There was a slight increase in mean weight (from 59.38±11.36 kg to 59.94±11.12 kg) in the first six months, followed by a decrease at 12 months to 59.29±8.44 kg

and subsequently decrease again at 18 months to 58.45±2.2 kg. In the same vein, Yemanebrhane *et al*. (2017) reported mean body mass index (BMI) of 0.72 ± 0.61 kg in HIV patients coinfected with HBV/HCV receiving HAART and mean BMI of 0.95 ± 4.70 kg in HIV patients coinfected with HBV. Mijiti *et al*. (2016) reported that patients co-infected with HIV/HBV/HCV appeared to have lower BMI compared to HBV/HIV-only, HCV/HIV-only and patients with mono HIV infection. The initiation of HAART was associated with increase in mean heamoglobin from 10.64±1.9 g/dl to 10.93±1.38 g/dl at 6 months, to 10.93±1.572 g/dl at 12 months and a decrease to 9.00±1.0 g/dl at 18 months. This is consistent with the report of Curkendall *et al*. (2007), who reported lowest mean heamoglobin in triple infected (HIV/HBC/HCV) and also slight increment of mean heamoglobin at 6 months and 12 months, subsequently decrease at 18 months. The increment in mean heamoglobin values at 6 months and 12 months is associated with treatment with HAART and the decrease at 18 months might be attributed to the higher doses of the HAART in the triple infected individuals. In this study, the higher mean value of ALT, AST and ALP were observed in triple infection (HIV/HBV/HCV) compare to HIV/HBV and HIV/HCV infected individuals. This observation made might be due to viral hepatitis infection as well as patient's condition like having chronic alcoholism or due to drug induced hepatotoxicity.

Polymerase chain reaction (PCR) is a technique used in molecular biology to amplify a single copy or a few copies of a segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence (Bartlett and Stirling, 2003). Hepatitis B virus (HBV) DNA quantitation is used extensively for monitoring of antiviral treatment of HBV infection (Lole and Arankalle, 2006). The polymerase chain reaction (PCR) amplification of Occult HBV isolates is shown in Plate 4.1 above. In this study HBV DNA was detected in HBsAg-negative serum using nested PCR, which is in line with previous studies that

demonstrated the same result in peripheral blood mononuclear cells, serum, and liver samples. The polymerase chain reaction (PCR) amplification of occult HBV DNA isolates has molecular weight of 298bp. which agreed with the work done by Nna *et al*. (2014). As indicated in plate 4.2, the polymerase chain reaction (PCR) amplification of HCV isolates in this study had molecular weights of 250 bp which also agrees with the work done by Banaz and Gaza, 2014.

HBV genotype E was the most prevalent genotype, genotype E is thought to be the most recent genotype originating in Africa. It is exclusively found in Africa or in African descendants living worldwide (Bekondi *et al.,* 2007, Kramvis & Kew 2007, Palumbo *et al*., 2007, Sitnik *et al*., 2007). Within Africa, it has a higher prevalence in Western African countries, including Senegal, Cote d'Ivoire, Ghana, Nigeria and Namibia (Hübschen *et al.* 2008; Kramvis & Kew 2007; Vray *et al.* 2006; Kramvis *et al.* 2005; Suzuki *et al.* 2003). In our study, most of the HBV isolates belonged to genotype E, which supports the idea that this genotype is the most prevalent genotype in West Africa (Bekondi *et al*. 2007, Kramvis & Kew 2007).

The current study revealed that genotypes 2 were found to be the major causes of the disease and this was in accordance with the work done by Umar *et al.,* 2017 were genotype 2 of hepatitis C virus was detected in infected individuals in Northwest Nigeria.

5.1 Conclusion

This study indicates that HBV and HCV infections are common among HIV positive patients in our environment and rapid detection of these co-infections may attract better management to avoid complications such as liver cirrhosis and hepatocellular carcinoma. Triple infected (HIV/HBV/HCV) patients are more likely to have abnormal liver enzyme test result than dual-infected (HIV/HBV or HIV/HCV).

Regardless of tattooing/tribal mark and dental extraction being uncommon practice in the South Eastern Nigeria, this study provides evidence for tattooing/tribal mark and dental extraction as significant risk factors. Hence there is a need to create public awareness to address the risk associated with this practice.

Finally, our study highlights the risks that South Eastern Nigeria (Abia, Anambra, Ebonyi, Enugu and Imo) may face and provides data that would be useful for designing measures to limit HBV, HCV and HIV infections and coinfections.

5.2 Recommendations

Based on our research findings, a national management and active surveillance program for HIV and hepatitis co-infections is essential in South Eastern Nigeria, as an important step to reduce the morbidity and mortality rates of these affections. The new guidelines shall incorporate and consider viral hepatitis as serious as HIV infection. These will assist in making significant impact in reducing the burden of this disease.

Prompt diagnosis of HCV and HBV co-infection in HIV patients has both individual and public health benefits. Proper counseling of all co-infected patients with HIV/ HBV or HIV/HCV on reducing the risk of transmitting HBV or HCV to close household members, sex partners and children with close physical contact through dried blood, open cuts, and shared toothbrushes, needles or razors is a necessity. Those who shared injection drug equipment with the patient should be screened for HBV and vaccinated against these viruses if they are not actively infected. As with HIV prevention, condom use with sex and avoidance of shared needles and other equipment for injection drug use are recommended measures for reducing the risk of HBV transmission. Counseling should include advising the patients against intake of hepatotoxins

which include alcohol and high doses of acetaminophen. Early antiretroviral therapy is especially recommended for co-infected patients.

Finally, haematological and biochemical parameters could serve as pointers for early detection of liver disease and renal function in HIV patients. The development of novel therapeutic approaches to impede co-infection of HIV and hepatotropic viruses is encouraged

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APPENDIX I

Structured Questionnaire

I am a post graduate student in Department of Microbiology and Brewing Nnamdi Azikiwe Univ ersity, Awka undertaking a research work on the "Prevalence and Trends of Hepatitis B andC Vi rus Epidemic Amongst HIV Patients in South Eastern Nigeria: A Systematic Review and Meta-Analysis". The research will require somedata concerning you and this structured questionnaire is aimed at providing the neededdata. Sir/Madam, your cooperation is highly needed in providing the correct data and will be treatedwith utmost confidentiality. Thanks.

S/No: 1H21.
Name of Hospital: Amaku Teaching Hospital.
1. Age: 40 years.
2. Sex (~~for children only~~): Male [ ]; Female [✓]
3. Marital status: married [ ]; single [ ]; widow [ ]; Divorced [✓]
4. Educational status: none [ ]; primary [ ]; secondary [✓]; tertiary [ ] others [ ]
5. Place of residence: rural [ ]; urban [✓]
6. Are you on antiretroviral drugs? Yes [✓]; No [ ]
7. Number of sex partners: Many
8. Occupation: civil servant [ ]; trader [✓]; housewife [ ]; student [ ]; farmer [ ]
9. Blood transfusion/ History of receiving blood: Yes [ ]; No [✓]
10. History of jaundice: Yes [✓]; No [ ]
11. **Risk Behaviors:**

a. History of tattooing: Yes [ ]; No [✓]

b. History of intravenous drug use: Yes [✓]; No [ ]

c. History of regular alcohol consumption: Yes [✓]; No [ ]

d. History of STDs: Yes [✓]; No [ ]

e. History of extramarital sexual relations without a condom: Yes [✓]; No [ ]

f. History of sexual contact before marriage: Yes [✓]; No [ ]

g. Surgical Procedure: Yes [✓]; No [ ]

Thanks for your response.

Sign _____

APPENDIX II

Structured Questionnaire

I am a post graduate student in Department of Microbiology and Brewing Nnamdi Azikiwe Univ ersity, Awka undertaking a research work on the "Prevalence and Trends of Hepatitis B andC Vi rus Epidemic Amongst HIV Patients in South Eastern Nigeria: A Systematic Review and Meta-Analysis". The research will require somedata concerning you and this structured questionnaire is aimed at providing the neededdata. Sir/Madam, your cooperation is highly needed in providing the correct data and will be treatedwith utmost confidentiality. Thanks.

S/No: B9
Name of Hospital: Federal Teaching Hospital Abakaliki
1. Age: 27.
2. Sex (~~for children only~~): Male [✓]; Female [ ]
3. Marital status: married [ ]; single [✓]; widow [ ]; Divorced [ ]
4. Educational status: none [ ]; primary [ ]; secondary [ ]; tertiary [✓] others [ ]
5. Place of residence: rural [ ]; urban [✓]
6. Are you on antiretroviral drugs? Yes [✓]; No [ ]
7. Number of sex partners: More than 2.
8. Occupation: civil servant [ ]; trader [ ]; housewife [ ]; student [✓]; farmer [ ]
9. Blood transfusion/ History of receiving blood: Yes [✓]; No [ ]
10. History of jaundice: Yes [ ]; No [✓]
11. **Risk Behaviors:**

a. History of tattooing: Yes [ ]; No [✓]

b. History of intravenous drug use: Yes [✓]; No [ ]

c. History of regular alcohol consumption: Yes [✓]; No [ ]

d. History of STDs: Yes [✓]; No [ ]

e. History of extramarital sexual relations without a condom: Yes [✓]; No [ ]

f. History of sexual contact before marriage: Yes [✓]; No [ ]

g. Surgical Procedure: Yes [ ]; No [✓]

Thanks for your response.

Sign

Appendix III

SAFETY MOLECULAR PATHOLOGY LABORATORY
Plot 44 Rangers Avenue, Independence Layout, Enugu
Enugu State
Nigeria

DNA Sequencing Service

Background	HBV DNA genotyping by DNA sequencing involves two key steps: Nested PCR to amplify a 2.7 kb region of HBV DNA and sequencing using a Big Dye Terminator chemistry in a Capillary Electrophoresis Genetic Analyzer, ABI 3130. Sequenced data are aligned and search in Genomic Database (NCBI).
Target	**HBV DNA Genotype**
Targeted amplicon	**P region, 2.7 kb size**
Primers for Ist round	5'-**GAGTATTTGGTGTCTTTTGGAGTGTGGATT**-3' (P-F1(nt2287-2316), sense 5'- **GAAGTATGCCTCAAGGTCGGTCGTT**-3' (P-R1(nt1710-1686), antisense
2nd Round primers	5'- **CACCAAATGCCCCTATCTTATCAACACT**-3' (P-F2(nt2339-2366), sense 5'- **GGCGTTCACGGTGGTCTCCAT**-3' (P-R2(nt1629-1609): antisense
Primer synthesis	All primers used were synthesized by Eurofins, Germany. Lyophilized primers were reconstituted in 1x TE buffer from IDT Europe
Sequencing Method	Big Dye Terminator chemistry (Thermofisher, UK) was used. Genomic DNA were first amplified using the Ist round primers, following by second PCR using the second-round primers and PCR product of first round (Nested PCR). The sequencing primer, 5'-**CACCAAATGCCCCTATCTTATCAACACT-3'** was used at a final concentration of 0.1 uM. Sequencing was done in Genetic Analyzer AB3130 (Applied Biosystems).
Sequencing Data Analysis	Sequencing data was aligned in the SeqMan Pro software of DNA Star. Aligned data were searched in BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for genotype identification using the HBV taxid (Accession No) 10407 as consensus sequence.
Samples tested	TH21 and TH201
Results	TH21: Genotype E TH201: Genotype E
Homology	Both samples shared 99 % homology

Thank you

Dr. E Nna

Safety Molecular Path Lab Services Ltd
Head Office: Plot 44 Rangers Avenue,
Independence Layout, Enugu
Faculty of Health Sciences & Technology
University of Nigeria,Enugu Campus
Enugu, 400001, Enugu State, Nigeria

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Tel: +234 8129806856 (Customer Service)
Email:info@safetybiomedical.org
Website:www.safetybiomedical.org
Registration No: RC-854975
Page 11

CEO: Dr. E Nna
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enna@safetybiomedical.org
Admin: Ogo Obi
ogobi@safetybiomedical.org
Tel: +234 8036315162

Appendix IV

SAFETY MOLECULAR PATHOLOGY LABORATORY
Plot 44 Rangers Avenue, Independence Layout, Enugu
Enugu State
Nigeria

DNA Sequencing Service

Background	HCV RNA genotyping by DNA sequencing involves three key steps: Reverse transcription of HCV RNA into cDNA, Nested PCR to amplify a 266 bp 5' UTR (Untranslated Region) and sequencing using a Big Dye Terminator chemistry in a Capillary Electrophoresis Genetic Analyzer, ABI 3130. Sequenced data are aligned using DNAStar and searched in Genomic Database (NCBI).
Target	**5' UTR HCV RNA**
Targeted amplicon	**5' UTR, 266 bp**
Primers for Ist round	5'-CCCCTGTGAGGAACTWCTGTCTTCACGC -3' (Forward) 5'-AGGTTTAGGATTTGTGCTCAT-3' (Reverse
2nd Round primers	5'-GAAAGCGYCTAGCCATGGCGTTAG-3' (Forward) 5'-ACGGTCTACGAGACCTCCCGGGGC-3' (Reverse)
Primer synthesis	All primers used were synthesized by Eurofins, Germany. Lyophilized primers were reconstituted in 1x TE buffer from IDT Europe
Sequencing Method	Big Dye Terminator chemistry (Thermofisher, UK) was used. HCV RNA was extracted using Zymo viral RNA kit, cDNA was made using Thermofisher First Strand DNA synthesis. HCV targets were first amplified using the Ist round primers, following by second PCR using the second-round primers and PCR product of first round (Nested PCR) in Eppendorf Nexus Gradient Master Cycler. The sequencing primer, 5'-GAAAGCGYCTAGCCATGGCGTTAG -3' was used at a final concentration of 0.1 uM. Sequencing was done in Genetic Analyzer AB3130 (Applied Biosystems).
Sequencing Data Analysis	Sequencing data was aligned in the SeqMan Pro software of DNA Star. MegAlign was used searching BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for genotype identification using the HBV taxid (Accession No) 11103 as consensus sequence.
Samples tested	A18, B9, TH144, TH176
Results: Sample No, Genotype and Homology	A18: Type 2 (97 % homology with Consensus sequence) B9: Type 2 (98 % homology with Consensus sequence) TH144: Type 2 (96 % homology with Consensus sequence) TH176: Type 2 (96 % homology with Consensus sequence)

Thank you

Dr. E Nna

Safety Molecular Path Lab Services Ltd
Head Office: Plot 44 Rangers Avenue,
Independence Layout, Enugu
Faculty of Health Sciences & Technology
University of Nigeria,Enugu Campus
Enugu, 400001, Enugu State, Nigeria

Tel: +234 8037435616 (Direct to the Lab)
Tel: +234 8129806856 (Customer Service)
Email: info@safetybiiomedical.org
Website: www.safetybiiomedical.org
Registration No: RC-854975

CEO: Dr. E Nna
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enna@safetybiiomedical.org
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ogobi@safetybiiomedical.org
Tel: +234 8036315162

Appendix V

FEDERAL TEACHING HOSPITAL, ABAKALIKI

P.M.B. 102, ABAKALIKI, EBONYI STATE, NIGERIA website.www.fetha.ng Email:info@fetha.ng

Dr. ONWE EMEKA OGAH
MB, BS, FWACP
Chief Medical Director

CHIEF C.C. OGBU JP, KSM
B.Sc (IIONS), MBA, M.Sc
FCAL, FNIMN, FHAN, MNIN
Chief Medical Director

Our Ref: FETHA/ADMIST/17/VOL.II/190

Date: *11th January, 2016*

Mr. Okoli Chukwudum Somadina
Department of Applied Microbiology
Nnamdi Azikiwe University, Awka.

ETHICAL CLEARANCE

Following your application and subsequent interview, you are hereby informed that Ethical Clearance Management Committee of Federal teaching Hospital Abakaliki has approved Ph.D Project Research Thesis "Prevalence and Trends of Hepatitis B and C Virus Epidemic Amongst HIV Patients in South Eastern Nigeria: A systematic Review and Meta Analysis" by Mr. Okoli Chukwudum Somadina with the Registration Number 2013487011F, Department of Applied Microbiology and Brewing. Faculty of Bioscience, Nnamdi Azikiwe University, Awka, to be carried out in out establishment.

The management believed that you are going to carryout this research according to rules and regulation guiding Hospital Ethical.

Yours faithfully

Chief C.C. Ogbu
Director Administration

Appendix VI

ESUT TEACHING HOSPITAL PARKLANE

P.M.B. 1030 ENUGU
parklancehospitalenugu@yahoo.com

DR. G.E. NJEZE, BM. Bch (Nig), FMCS, FWACS,FICS, FACS
CHIEF MEDICAL DIRECTOR

DR. W.O. OKENWA, MBBS (NIG), FWACS
CHAIRMAN MEDICAL ADVISORY COMMITTEE

PROF. H.E. ONAH, MBBS, FMDGG, FWACS, MPA, M.PHARM
CHAIRMAN
ESUT TEACHING HOSPITAL PARKLANE
MANAGEMENT BOARD

MR. ONODU I. N., B.Sc(pol. Sc.), M.Sc Int. Relation
Ag. DIRECTOR OF ADMINISTRATION

Our Ref: 00906 *Date: 12TH February, 2016*

Sequel to your application for ethical clearance on "Prevalence and Trends of Hepatitis B and C Virus Epidemic Amongst HIV Patients in South Eastern Nigeria: A systematic Review and Meta Analysis". I am directed to inform you that your letter was received and has been approved as requested. I urge you to maintain confidentiality in your findings.

We look forward to your cooperation.

Yours faithfully,

DR. W.O. OKENWA

Appendix VII

FEDERAL MEDICAL CENTRE UMUAHIA
P.M.B. 7001, UMUAHIA, ABIA STATE, NIGERIA
email:fmcumuahia@fmcumuahia.com
fmcqeh@yahoo.com
website:www.fmcumuahia.com

Dr. Wakil Chibok, B.sc, M.sc, MBA, Phd.
Chairman, Management Board
Dr. Chuku Abali, MBBS, FWACS, FICS, Dip HSM, Cert HRM, OPTH MICRO SURG. FCIPSMN.
Medical Director

Ekpemu Rowland, B.Sc, ACAI, MCIPM, AHAN.
Head of Administration/Secretary to the Board
Dr. Chukwuonye I. I. MBBS, FMCP
Chairman Medical Advisory Commttee.

OUR REF: FMC/QEH/S.16/44

DATE: 26th January, 2016

Mr. Okoli Chukwudum Somadina
Department of Applied Microbiology
Nnamdi Azikiwe University, Awka.

ETHICAL CLEARANCE

With reference to your letter dated 2nd December, 2015, I am directed to inform you that approval has been granted to you to carry on with your research work on "Prevalence and Trends of Hepatitis B and C Virus Epidemic Amongst HIV Patients in South Eastern Nigeria: A systematic Review and Meta Analysis".

We look forward to your cooperation.

Yours faithfully

Dr. Chukwuonye I.I
CMAC

Serving Beyond Your Expectation

Appendix VIII

FEDERAL MEDICAL CENTRE

P.M.B. 1010, Orlu Road Owerri, Imo State, Nigeria

Medical Director/CEO
Dr. A.C. Uwakwem
MBBS, FWACS, FICS, FICA, IMAAO
Chief Medical Director

Chairman Medical Advisory Committee
Dr. E.C. Osuagwu
MBBS, FWACS

Head of Administration Service
Mrs. Nnenna Onyegula
B.Sc, MPA, FHAH, MCIPM,

e-mail:tmcowerri@yahoo.com

Our Ref:FMC/OW/P/195/30

Date: 3rd February, 2016

RE: APPROVAL FOR A RESEARCH WORK

I am directed to Inform Okoli Chukwudum Somadina that the Ethical Committee have approved that he the above mentioned student to carry out a research work on "Prevalence and Trends of Hepatitis B and C Virus Epidemic Amongst HIV Patients in South Eastern Nigeria: A systematic Review and Meta Analysis".

In view of the above, the approval is in line with the ethical clearance regulations.

Yours faithfully,

Dr. E.C. Osuagwu

Appendix IX

CHUKWUEMEKA ODUMEGWU OJUKWU UNIVERSITYTEACHING HOSPITAL, AMAKU AWKA

(Formerly Anambra State University Teaching Hospital)

P.M.B, 5022 AWKA, ANAMBRA STATE, NIGIERA

HRH Igwe Chukwuemeka Ilouno
MBBS, DA, DBA, PGDE, MPH, FWACS, FMCOG, FICS, FAGP, FBSC, FNISM, LLB
Chairman, COOUTH Management Board
Chairman Medical Advisory Committee

Dr. L.C. Ikeako MBBS, FWACS, FMCOG, FICS
Chief Medical Director

Dr. Ifeanyi Ezeobi MBBS, FWACS
(CMAC)

Dr. Ejiofor O.S. MBBS, FWACP (paed)
Deputy- CMAC

Our Ref: COOUTH/AA/VOL.020

Date: 10th February, 2016

ETHICAL CLEARANCE

TOPIC: Prevalence and Trends of Hepatitis B and C Virus Epidemic Amongst HIV Patients in South Eastern Nigeria: A systematic Review and Meta Analysis.

BY: Okoli Chukwudum Somadina

FOR: Award of P.hD., in department of Applied Micro-biology and Brewing, Faculty of Biosciences. Nnamdi Azikiwe University Awka.

The above named research topic has been reviewed and approved by the Ethical Committee of COOUTH Amaku, Awka.

Yours faithfully,

DR. EJIOFOR O.S. (FWACP)
Chairman Ethical Committee

STATISTICAL ANALYSIS

Table 1: Oneway Analysis of Variance (ANOVA) for Seroprevalence of HBV Markers amongst HIV Patients in South East, Nigeria

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
NUMBEROF INDIVIDUALS TESTED	**Between Groups**	**.000**	**4**	**.000**	**.000**	**1.000**
	Within Groups	**600000.000**	**10**	**60000.000**		
	Total	**600000.000**	**14**			
HBSAG	**Between Groups**	**824.400**	**4**	**206.100**	**18.736**	**.000**
	Within Groups	**110.000**	**10**	**11.000**		
	Total	**934.400**	**14**			
ANTIHBS	**Between Groups**	**81666.667**	**4**	**20416.667**	**3.237**	**.060**
	Within Groups	**63066.667**	**10**	**6306.667**		
	Total	**144733.333**	**14**			
ANTIHBCIGM	**Between Groups**	**15960.000**	**4**	**3990.000**	**1.750**	**.215**
	Within Groups	**22800.000**	**10**	**2280.000**		
	Total	**38760.000**	**14**			

Table 2: Oneway Analysis of Variance (ANOVA) for Seroprevalence of HCV Markers amongst HIV patients in South East, Nigeria

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
NUMBER OF INDIVIDUALS TESTED	Between Groups	.000	4	.000	.000	1.000
	Within Groups	600000.000	10	60000.000		
	Total	600000.000	14			
HEPATITIS C ANTI HCV	Between Groups	2400.000	4	600.000	1.875	.191
	Within Groups	3200.000	10	320.000		
	Total	5600.000	14			

Table 3: Oneway Analysis of Variance (ANOVA) for Seroprevalence of HBV/HCV Co-infection amongst HIV Patients in South East, Nigeria

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
NUMBER OF INDIVIDUALS TESTED	Between Groups	.000	4	.000	.000	1.000
	Within Groups	600000.000	10	60000.000		
	Total	600000.000	14			
HBV/HCV CO-INFECTION (%)	Between Groups	300.000	4	75.000	3.151	.064
	Within Groups	238.000	10	23.800		

	Total	538.000	14			

Table 4: Oneway Analysis of Variance (ANOVA) for Distribution of HBVSero-markers amongst HIV patients by Age in South East, Nigeria

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
NUMBERTESTED	Between Groups	378229.167	7	54032.738	.574	.767
	Within Groups	1507266.667	16	94204.167		
	Total	1885495.833	23			
HBSAG	Between Groups	13554.000	7	1936.286	1.202	.356
	Within Groups	25770.000	16	1610.625		
	Total	39324.000	23			
ANTIHBS	Between Groups	151162.500	7	21594.643	5.182	.003
	Within Groups	66678.000	16	4167.375		
	Total	217840.500	23			
ANTIHBCLGM	Between Groups	52980.292	7	7568.613	25.664	.000
	Within Groups	4718.667	16	294.917		
	Total	57698.958	23			

Table 5: Oneway Analysis of Variance (ANOVA) for Distribution of HCVsero-markers amongst HIV patients by age in South East, Nigeria

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
NUMBERTESTED	Between Groups	378229.167	7	54032.738	.574	.767
	Within Groups	1507266.667	16	94204.167		
	Total	1885495.833	23			
ANTIHCV	Between Groups	10790.625	7	1541.518	.854	.561
	Within Groups	28864.000	16	1804.000		
	Total	39654.625	23			

Table 6: Oneway Analysis of Variance (ANOVA) for Distribution of HBVsero-markers amongst HIV patients by Sex in South East, Nigeria

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
NUMBEROFINDIVIDUALTESTED	Between Groups	135000.000	1	135000.000	5.400	.081
	Within Groups	100000.000	4	25000.000		
	Total	235000.000	5			
HBSAG	Between Groups	8664.000	1	8664.000	1.856	.245

	Total	27336.000	5			
ANTIHBS	Between Groups	33750.000	1	33750.000	.128	.739
	Within Groups	1054200.000	4	263550.000		
	Total	1087950.000	5			
ANTIHBCLGM	Between Groups	15000.000	1	15000.000	60.000	.001
	Within Groups	1000.000	4	250.000		
	Total	16000.000	5			

Table 7: Oneway Analysis of Variance (ANOVA) for Distribution of HCVsero-markers amongst HIV patients by Sex in South East, Nigeria

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
NUMBER TESTED	Between Groups	135000.000	1	135000.000	5.400	.081
	Within Groups	100000.000	4	25000.000		
	Total	235000.000	5			
ANTIHCV	Between Groups	37.500	1	37.500	.176	.696
	Within Groups	850.000	4	212.500		
	Total	887.500	5			

TABLE 8: T-TEST FOR SEROPREVALENCE OF HBV SEROLOGICAL MARKERS IN AHB HIV PATIENTS

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
TOTAL AHB PATIENTS	1344.071	2	.000	776.00000	773.5159	778.4841
ANTIHBCIGM	45.033	2	.000	520.00000	470.3172	569.6828
HBSAG	147.802	2	.000	256.00000	248.5476	263.4524

TABLE 9: T-TEST FOR SEROPREVALENCE OF HBV SEROLOGICAL MARKERS IN CHB HIV PATIENTS

One-Sample Test

	Test Value = 0				
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference

TABLE 10: ONEWAY ANALYSIS OF VARIANCE (ANOVA) FOR SEROPREVALENCE OF HDV AND HCV MARKERS AMONG HBV PATIENTS

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
UMBER TESTED	Between Groups	405600.000	1	405600.000	20280.000	.000
	Within Groups	80.000	4	20.000		
	Total	405680.000	5			
NTIHDVIGM	Between Groups	.000	1	.000	.	.
	Within Groups	.000	4	.000		
	Total	.000	5			
NTIHDVTOTAL	Between Groups	.000	1	.000	.	.
	Within Groups	.000	4	.000		
	Total	.000	5			

Table 11: T-TEST FOR SEROPREVALENCE OF ANTI-HBCT AND HBV DNA AMONG HBSAG NEGATIVE HIV PATIENTS

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
NUMBER OF HBSAG	3.780	2	.063	100.00000	-13.8375	213.8375
ANTIHBCT POSITIVE	2.309	2	.147	20.00000	-17.2621	57.2621
HBV DNA POSITIVE	2.165	2	.163	5.00000	-4.9366	14.9366

TABLE 12: ONEWAY ANALYSIS OF VARIANCE (ANOVA) FOR PROFILE OF HEPATITIS B VIRAL LOAD RESULTS OF HEPATITIS B SURFACE ANTIGEN (HBSAG) POSITIVE

ANOVA

NUMBER

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8778.000	3	2926.000	100.034	.000
Within Groups	234.000	8	29.250		
Total	9012.000	11			

TABLE 12: ONEWAY ANALYSIS OF VARIANCE (ANOVA) FOR PROFILE OF HEPATITIS B VIRAL LOAD RESULTS OF HEPATITIS B SURFACE ANTIGEN (HBSAG) POSITIVE

ANOVA

NUMBER

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8778.000	3	2926.000	100.034	.000
Within Groups	234.000	8	29.250		
Total	9012.000	11			

TABLE 13: ONEWAY ANALYSIS OF VARIANCE (ANOVA) FOR PROFILE OF HEPATITIS C VIRAL LOAD RESULTS OF ANTI HCV POSITIVE PATIENTS

ANOVA

NUMBER

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8778.000	3	2926.000	100.034	.000
Within Groups	234.000	8	29.250		
Total	9012.000	11			

Table 14: Oneway Analysis of Variance (ANOVA) for Risk factors distribution amongst HIV patients with respect to HBV sero-status

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
TATTOOING TRIBAL MARK	Between Groups	3758744.000	3	1252914.667	240.922	.000
	Within Groups	20802.000	4	5200.500		
	Total	3779546.000	7			
BODY PIERCING EAR	Between Groups	2794664.000	3	931554.667	37.261	.002
	Within Groups	100002.000	4	25000.500		
	Total	2894666.000	7			
BLOOD TRANSFUSION	Between Groups	5454424.000	3	1818141.333	359.992	.000
	Within Groups	20202.000	4	5050.500		
	Total	5474626.000	7			
SURGICAL PROCEDURE	Between Groups	3009704.000	3	1003234.667	200.627	.000
	Within Groups	20002.000	4	5000.500		
	Total	3029706.000	7			
DENTAL EXTRACTION	Between Groups	5432104.000	3	1810701.333	358.519	.000
	Within Groups	20202.000	4	5050.500		
	Total	5452306.000	7			
ALCOHOL	Between Groups	2788504.000	3	929501.333	92.950	.000

Table 15: Oneway Analysis of Variance (ANOVA) for Continue: Risk factors distribution amongst HIV patients with respect to HCV Sero-status

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
TATTOOING TRIBAL MARK	Between Groups	3906100.000	3	1302033.333	245.066	.000
	Within Groups	21252.000	4	5313.000		
	Total	3927352.000	7			
BODY PIERCIN GEAR	Between Groups	2971037.500	3	990345.833	39.535	.002
	Within Groups	100200.000	4	25050.000		
	Total	3071237.500	7			
BLOOD TRANSFUSION	Between Groups	5620037.500	3	1873345.833	374.594	.000
	Within Groups	20004.000	4	5001.000		
	Total	5640041.500	7			
SURGICAL PROCEDURE	Between Groups	3201837.500	3	1067279.167	210.821	.000
	Within Groups	20250.000	4	5062.500		
	Total	3222087.500	7			
DENTAL EXTRACTION	Between Groups	5602613.500	3	1867537.833	373.433	.000
	Within Groups	20004.000	4	5001.000		
	Total	5622617.500	7			
ALCOHOL CONSUMPTION	Between Groups	2976837.500	3	992279.167	198.060	.000
	Within Groups	20040.000	4	5010.000		
	Total	2996877.500	7			
CIRCUMCISION	Between Groups	3201837.500	3	1067279.167	104.125	.000
	Within Groups	41000.000	4	10250.000		
	Total	3242837.500	7			
SEXUALACTIVITY	Between Groups	4959053.500	3	1653017.833	41.292	.002
	Within Groups	160130.000	4	40032.500		
	Total	5119183.500	7			
INTRAVENOUS INJECTION	Between Groups	3524693.500	3	1174897.833	46.987	.001
	Within Groups	100020.000	4	25005.000		
	Total	3624713.500	7			

ıble 16: Oneway Analysis of Variance (ANOVA) for Socio–demographic characteristics of HBV Sero-status in HIV Patients

ıble 16: Oneway Analysis of Variance (ANOVA) for Socio–demographic characteristics of HBV Sero-status in HIV Patients

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
EDUCATION LEVEL	Between Groups	3109901.500	3	1036633.833	25.852	.004
	Within Groups	160394.000	4	40098.500		
	Total	3270295.500	7			
ETHNICITY	Between Groups	4801624.000	3	1600541.333	63.848	.001
	Within Groups	100272.000	4	25068.000		
	Total	4901896.000	7			
OCCUPATION	Between Groups	1556716.000	7	222388.000	9.565	.002
	Within Groups	186002.000	8	23250.250		
	Total	1742718.000	15			
LOCALITY	Between Groups	3730684.000	3	1243561.333	49.717	.001
	Within Groups	100052.000	4	25013.000		
	Total	3830736.000	7			
MARITAL STATUS	Between Groups	3000188.000	7	428598.286	32.045	.000
	Within Groups	107000.000	8	13375.000		
	Total	3107188.000	15			

Table 17: Oneway Analysis of Variance (ANOVA) for Socio–demographic characteristics of HCV Sero-status in HIV Patients

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
EDUCATIONLEVEL	Between Groups	2953437.500	3	984479.167	6.642	.049
	Within Groups	592850.000	4	148212.500		
	Total	3546287.500	7			
ETHNICITY	Between Groups	4966037.500	3	1655345.833	65.688	.001
	Within Groups	100800.000	4	25200.000		
	Total	5066837.500	7			
OCCUPATION	Between Groups	1647143.750	7	235306.250	4.796	.021

LOCALITY	Between Groups	3926501.500	3	1308833.833	130.825	.000
	Within Groups	40018.000	4	10004.500		
	Total	3966519.500	7			
MARITALSTATUS	Between Groups	3088225.438	7	441175.063	86.474	.000
	Within Groups	40814.500	8	5101.813		
	Total	3129039.938	15			

Table 18: Oneway Analysis of Variance (ANOVA) for Hematological and biochemical parameters among HBV/HIV patients

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
CDFOURHEPATITISPOSITIVE	Between Groups	34475.658	4	8618.914	439.741	.000
	Within Groups	98.000	5	19.600		
	Total	34573.658	9			
WEIGHTHEPATITISPOSITIVE	Between Groups	102.892	4	25.723	2.679	.154
	Within Groups	48.000	5	9.600		
	Total	150.892	9			
HAEMOGLOBIN HEPATITIS	Between Groups	10.591	4	2.648	.368	.823
	Within Groups	36.000	5	7.200		
	Total	46.591	9			
ALT HEPATITIS	Between Groups	45.186	4	11.297	4.034	.079
	Within Groups	14.000	5	2.800		
	Total	59.186	9			
AST HEPATITIS	Between Groups	28.965	4	7.241	2.586	.163
	Within Groups	14.000	5	2.800		
	Total	42.965	9			
ALPHEPATITIS	Between Groups	84.635	4	21.159	2.939	.134
	Within Groups	36.000	5	7.200		
	Total	120.635	9			

Table 19: Oneway Analysis of Variance (ANOVA) for Hematological and Biochemical parameters among HCV/HIV patients

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
CDFOURHEPATITISPO	Between Groups	1218.292	4	304.573	50.762	.000

LOCALITY	Between Groups	3926501.500	3	1308833.833	130.825	.000
	Within Groups	40018.000	4	10004.500		
	Total	3966519.500	7			
MARITALSTATUS	Between Groups	3088225.438	7	441175.063	86.474	.000
	Within Groups	40814.500	8	5101.813		
	Total	3129039.938	15			

Table 18: Oneway Analysis of Variance (ANOVA) for Hematological and biochemical parameters among HBV/HIV patients

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
CDFOURHEPATITISPOSITIVE	Between Groups	34475.658	4	8618.914	439.741	.000
	Within Groups	98.000	5	19.600		
	Total	34573.658	9			
WEIGHTHEPATITISPOSITIVE	Between Groups	102.892	4	25.723	2.679	.154
	Within Groups	48.000	5	9.600		
	Total	150.892	9			
HAEMOGLOBIN HEPATITIS	Between Groups	10.591	4	2.648	.368	.823
	Within Groups	36.000	5	7.200		
	Total	46.591	9			
ALT HEPATITIS	Between Groups	45.186	4	11.297	4.034	.079
	Within Groups	14.000	5	2.800		
	Total	59.186	9			
AST HEPATITIS	Between Groups	28.965	4	7.241	2.586	.163
	Within Groups	14.000	5	2.800		
	Total	42.965	9			
ALPHEPATITIS	Between Groups	84.635	4	21.159	2.939	.134
	Within Groups	36.000	5	7.200		
	Total	120.635	9			

Table 19: Oneway Analysis of Variance (ANOVA) for Hematological and Biochemical parameters among HCV/HIV patients

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
CDFOURHEPATITISPO	Between Groups	1218.292	4	304.573	50.762	.000

CDFOURHEPATITISPOSITIVE	Between Groups	1218.292	4	304.573	50.762	.000
	Within Groups	30.000	5	6.000		
	Total	1248.292	9			
WEIGHTHEPATITISPOSITIVE	Between Groups	61.934	4	15.483	3.519	.100
	Within Groups	22.000	5	4.400		
	Total	83.934	9			
HAEMOGLOBINHEPATITIS	Between Groups	25.013	4	6.253	.233	.908
	Within Groups	134.000	5	26.800		
	Total	159.013	9			
ALTHEPATITIS	Between Groups	658.779	4	164.695	1.723	.281
	Within Groups	478.000	5	95.600		
	Total	1136.779	9			
ASTHEPATITIS	Between Groups	833.357	4	208.339	14.077	.006
	Within Groups	74.000	5	14.800		
	Total	907.357	9			
ALPHEPATITIS	Between Groups	3084.791	4	771.198	4.784	.058
	Within Groups	806.000	5	161.200		
	Total	3890.791	9			