CHAPTER 1
1.0 INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Hepatitis is derived from the Greek word 'hepatos' meaning the liver and 'itis' meaning inflammation. Hepatitis is an inflammation of the liver. The condition can be self-limiting or can progress to fibrosis (scarring), cirrhosis or liver cancer (Kudesia and Wright, 2009). Hepatitis viruses are the most common cause of hepatitis in the world but other infections, toxic substances (e.g. alcohol, certain drugs), and autoimmune diseases can also cause hepatitis (WHO, 2015). Viral hepatitis is a necroinflammatory liver disease of variable severity. It is a systemic infection caused by various hepatitis viruses that primarily infect the hepatocyte cells of the liver (Levinson, 2014). These viruses are taxonomically unrelated but are called hepatitis viruses because they cause similar disease symptoms as a result of infection of the liver (Acheson, 2011). The major types of hepatitis viruses are designated types A, B, C, D, E and G (Burtis *et al*., 2006). Most liver damage as a result of viral hepatitis is caused by hepatitis B and C viruses (Papadakis *et al.*, 2013). Hepatitis B Virus (HBV) particularly is the major cause of inflammation of the liver and tends to be more serious than other hepatitis viruses. It is also the most common cause of acute hepatitis and most common chronic viral infection worldwide (Burtis *et al.*, 2006; Lennette *et al.*, 2008; Kudsia and Wright, 2009). Hepatitis B Virus is the 9th leading cause of death worldwide. It causes Cirrhosis, liver failure and Hepatocellular Carcinoma (HCC) (Levinson, 2014; WHO, 2015).

Hepatitis B Virus (HBV) belongs to the Hepadnaviridae family. It is a double stranded circular DNA virus composed of an outer envelope containing hepatitis B surface antigen (HBsAg) and an inner nucleocapsid consisting of hepatitis B envelope antigen (HBeAg) and hepatitis B core antigen (HBcAg) (Beck and Nassal, 2007). The viral core also contains double stranded DNA genome and DNA polymerase (Bruss, 2007). Hepatitis B virus (HBV) is transmissible by the parenteral route and may be found in blood, blood product, saliva and other body fluids such as semen and vaginal secretions (Acheson, 2011; Papadakis *et al.*, 2013). Once in the bloodstream, the virus travels to the liver where it

replicates in hepatocytes. Hepatitis B virus induces wide spectrum of clinical forms, ranging from a healthy carrier state, acute hepatitis B infection, and chronic hepatitis infection, which could lead to cirrhosis and hepatocellular carcinoma (HCC) (Lok, 2000; WHO, 2009a; Salawu *et al.*, 2011; Samal *et al.*, 2012).

Hepatitis B virus (HBV) infection is a major public health problem worldwide because of the manner it affects human's life. The endemicity of HBV infection in Nigeria has been variously described (Forbi *et al*., 2008; Adoga *et al.*, 2009). Liver disease due to HBV is an enormous global health problem. One third of the world population (about 2 billion people) has been infected with HBV, and over 400 million people have chronic infection. Seventy five percent of all chronic carriers live in Asia and the Western Pacific region (Kumar *et al*., 2010). The global prevalence of chronic hepatitis B virus infection varies widely from high (>8%) in Africa, Asia and Western Pacific region to intermediate (2% to 7%) in southern and eastern Europe to low (<2%) in Western Europe, North America and Australia (Hou *et al*., 2005; Kumar *et al.*, 2010). Every year there are over four million acute clinical cases of HBV infection (WHO, 2009a). The risk of death from HBV-related liver cancer or cirrhosis is approximately 25% for persons who acquired chronic infection at childhood (Weinbaum *et al.*, 2008; WHO, 2009a). Moreover, 8% to 10% of people in the general population in the developing world become chronically infected and most acquire infection with HBV at childhood (WHO, 2009a). Although Hepatitis B vaccination is highly effective in preventing infection with HBV and consequent acute and chronic liver disease (Weinbaum *et al.*, 2008], this infection is still a major problem in Nigeria as reported by various scientists and scholars (Otegbayo *et al.*, 2003 Adoga *et al.*, 2009).

Hepatitis B virus (HBV) serologic markers are antigen and antibodies of HBV that serve as markers of infection, often used to determine the stages of HBV infection. The presence of Hepatitis B virus serologic markers such as HBsAg, HBeAg, Anti-HBc, Anti-HBs and Anti-HBe in individuals signifies infection or exposure to HBV (Acheson, 2011). Utilizing these markers, studies have revealed that HBV is cleared after the resolution of this acute infection; however, in approximately 5% to 10% of adults and in 80–90% of neonates a chronic carrier state still may persist (WHO, 2015). Hepatitis B surface antigen (HBsAg) is the viral marker that indicates infection (acute or chronic) with HBV. The concurrent

presence of HBsAg and HBs antibody are observed in both acute and chronic hepatitis B infection (Levinson, 2014). Chronic hepatitis B is clinically defined as the frequent detection of HBsAg for at least six months after acute infection (Torbenson and Thomas, 2002). In addition, chronically infected patients potentially have high levels of the hepatitis B envelope antigen (HBeAg) in their serum (Papadakis *et al.*, 2013). Chronic HBV carriers are clinically defined by the presence of HBsAg and anti-HBc, as HBV produce the core antigen (HBcAg) during an active replication phase (Allain, 2017). Altogether, the active chronic state is associated with high levels of HBV-DNA, liver inflammation, elevated liver enzymes and the highest risk of cirrhosis and hepato cellular carcinoma (HCC) (Allain, 2017).

Screening for transfusion transmissible infections (TTIs) is an established procedure in Blood Banks globally before accepting an individual to donate blood for clinical use. Most important of these TTIs screened for include hepatitis viruses (including Hepatitis B and C), HIV, and syphilis (WHO, 2009a; Acheson, 2011). Hepatitis B virus (HBV) infection screening is particularly important because it is a major public health problem affecting over 350 million people worldwide (Asim *et al.,* 2010). Studies have shown that the carrier rate of hepatitis B virus (HBsAg marker) in blood donors in Nigeria appears to be between 5% and 17% depending on the geographical location (Baba *et al.*, 2010; Okwesili *et al.*, 2014). The prevalence of HBV infection in Nigeria was estimated to be 2.4-18.4% of the population (Olokoba *et al.*, 2009; Ndako *et al.*, 2011). The risk of transfusion-transmitted HBV infection has been reduced by screening all blood donations for HBV surface antigen (HBsAg) since 1970 as most blood banks in resource limited economies screen for hepatitis B virus infection mainly by screening for the hepatitis B surface antigen (HBsAg) and bled donors based on its negativity (Badur and Akgun, 2001). Although this serologic method reduces transfusion transmissible HBV infections, some HBsAg-negative blood samples can still induce post transfusion hepatitis in recipients (Allain, 2004a; Asim *et al.*, 2010). Advances in the genomic amplification of viral DNA have, however, shown that it is possible to carry hepatitis B virus and still be negative on screening for HBsAg (Seo *et al.*, 2015). This is known as occult hepatitis B virus infection (Allain, 2004a).

Occult hepatitis B virus infection (OBI) is characterized by the presence of HBV DNA in serum and/or in the liver of patients negative for hepatitis B surface antigen (Urbani *et al.*, 2010). Occult hepatitis B virus infection (OBI) has been described for decades, and Nucleic Acid Testing (NAT) for HBV-DNA detection has confirmed the existence of the OBI, which is defined as the presence of HBV-DNA in the absence of detectable HBsAg with or without anti-HBV antibodies (Torbenson and Thomas, 2002). This phenomenon is becoming increasingly recognized in several clinical settings worldwide (Hudu, *et al.*, 2016). Occult HBV infection may impact in several different clinical contexts including the risk of HBV transmission with transfusion or transplantation, and endogenous viral reactivation (Seo *et al.*, 2015). In most cases, occult HBV infection is related to low level HBV infection with sub-detectable levels of HBsAg and not infection with HBV variants that cannot express S protein or produce S proteins with aberrant epitopes which are not detected by conventional serological assays (Lok, 2004). An important observation in subjects with occult HBV infection is the low HBV DNA levels in the serum and in the liver cells. The frequency of detection of HBV DNA is higher in liver tissue than in serum (Cacciola *et al.*, 2000; Lok, 2004). Because of low amounts of HBV DNA, sensitive Polymerase Chain Reaction (PCR) is increasingly used for accurate diagnosis (Allain, 2017).

Screening for occult hepatitis B infection among blood donors is important to reduce the risk of HBV transmission through blood transfusion. The gold standard test for detection of occult HBV infection is the amplification of HBV DNA (Allain, 2017). Studies on a large set of blood donors using NAT confirmed this phenomenon of OBI and formed the basis of mandatory NAT for transfused blood units in many developed countries (Panhotra, *et al*., 2005; Prati, *et al*., 2006). Such a testing regimen has not been incorporated into the testing algorithms of many laboratories in developing countries including Nigeria. The frequency of post transfusion HBV infection is apparently due to the fact that HBsAg is in circulation at very low and undetectable level for screening assays. Nonetheless, anti-HBc (antibody to hepatitis B core antigen) antibody screening tests are able to eliminate some of these donor units (Hudu *et al.*, 2016) is not done. The prevalence of OBI in blood donors has been confirmed from different geographic areas and ranges from less than 1% to 16% depending on the endemicity of HBV infection (Candotti *et al*., 2008; Huang *et al*., 2012). In a

general Korean adult population, the prevalence of OBI was 0.7% (Song *et al.*, 2009) and the prevalence of OBI was 18% and 8% in resolved HBV infection group and in HBV seronegative individuals (negative for HBsAg, anti-HBs and anti-HBc), respectively (Minuk, *et al.*, 2005). However, the prevalence of OBI was 64% in liver transplanted patients, 62% in HCC patients, 27% in hemodialysis patients and up to 45% in HCV and HIV infected patients (Raimondo, *et al.*, 2007; Samal *et al*., 2012).

Studies on OBI prevalence in Nigeria is unclear, with one study that utilized a smaller sample size (n = 28) reported no prevalence of OBI in healthy subjects (Ola *et al*., 2009). Recent study on OBI by Nna *et al.,* (2014) at Abakaliki, south eastern Nigeria observed that 8% of blood donors with HBsAg negative were positive with anti-HBc and HBV-DNA by nested PCR. In another study by Oluyinka *et al*., (2015), they recorded a 17% prevalence of OBI among blood donors in south western Nigeria and genotype E is the most prevalent. However, they did not test for anti-HBc IgM and its involvement in OBI. Recently, Olotu *et al.*, (2016) reported 5.4% prevalence of OBI among blood donors at Ile Ife, Western Nigeria that were anti-HBc positive. However, they did not determine the anti-HBc IgM status as well as OBI prevalence among blood donors that tested negative to all HBV serologic markers and donors that tested positive for anti-HBs marker.

Studies have shown that Alanine Aminotransferase (ALT) is markedly elevated in acute HBV infection moderately increased in chronic infection and remain normal in past and occult HBV infection (Gitlin, 1997; WHO, 2009a). In another study at Elele, Rivers States, Nigeria, Ajugwo *et al*., (2015) found that there is no significant difference in hematological indices (PCV, HGB, WBC Total and ESR) between symptomatic and asymptomatic patients infected with Hepatitis B Virus. Lavanya *et al.*, (2012) posited that the prevalence of HBV markers among blood donors are as follow; HBsAg 3.5%, Anti-HBc total 10.9%, anti-HBc IgM 5.7% and anti-HBs 3%. They found that this prevalence are instigated with risk factors like alcoholism, smoking, tattooing, ear piercing, visiting barber's shop and family history of jaundice. However, they found that there is no statistical significant difference in HBV infection among the various blood groups studied.

1.2 STATEMENT OF RESEARCH PROBLEM

The use of unscreened or inadequately screened blood for transfusion keeps the patient at risk of acquiring many transfusion transmissible infections (TTIs) like hepatitis viruses (HBV, HCV), Human Immuno-deficiency Viruses (HIV), syphilis, malaria, to mention but a few (WHO, 2009b). In Nigeria, which is highly endemic for HBV infection (Siriena *et al.*, 2002; Jombo *et al.*, 2005; Adoga *et al.*, 2010), current blood banking practices do not include laboratory testing/ procedures that would identify occult hepatitis B virus infection (OBI) and prevent transfusion of blood or blood products from apparently healthy donors with OBI to recipients (Salawu *et al.*, 2010). Most blood banks in Nigeria including Nnamdi Azikiwe University Teaching Hospital and University of Abuja Teaching Hospital use only hepatitis B surface antigen (HBsAg) marker, rapid test device to screen blood donors for HBV infection before donation based on its negativity. Enzyme linked Immunosorbent assay (ELISA) for HBsAg which has been adjuged to be more sensitive than rapid test kit is not used in screening blood donors (Erhabor *et al*., 2014). In addition, serological markers of HBV infection such as HBsAb, HBcAb, HBeAg, HBeAb and HBcAb IgM are not included in the screening tests of blood donors. Studies have shown that the presence of HBV serologic markers in individual signifies exposure or infection by HBV (Servoss and Friedman, 2006; Salawu *et al.*, 2011; Levinson, 2014). This means some potential blood units containing HBV are being transfused to patients unknowingly by screening blood units for hepatitis B suface antigen marker (HBsAg) only, using rapid test kit. The implications of transfusion of infected blood units could lead to increase in prevalence of overt infection (Liu, *et al.*, 2006a). All these could translate to increase of patients who may be at risk of HBV infection from occult blood donors with its attendant health consequence such as cirrhosis and hepatocellular carcinoma.

Other factors such as blood donations during the window period of transfusion transmissible infections, emergence of newer transmissible pathogens, and prevalence of asymptomatic carriers pose a serious challenge to blood transfusion safety. The blood donors who are apparently healthy and HBsAg, HIV and HCV negative by rapid test kit devices may be infected with hepatitis B virus and other transfusion transmissible viral infections such as HIV and HCV as these viral agents have a common route of

transmission. Hence the need to use more sensitive and specific diagnostic tests such as ELISA and molecular methods for screening of blood donors cannot be overemphasized.

1.3 JUSTIFICATION FOR THE STUDY

Occult Hepatitis B virus infection among blood donors presents a global health risk not only to the infected blood donors but also to the blood and organ recipients (Olotu *et al.*, 2016). Knowledge of the prevalence of occult HBV infection, viral markers and genotype among blood donor population is important as it may help to formulate new policy for blood donor screening that could mitigate transmission of transfusion infectious agents in Nigeria (Oluyinka *et al.*, 2015). Hence, screening blood donors for HBV using Hepatitis B markers and Nucleic Acid Amplification Testing (NAT) can give a reliable prevalence of the HBV infection in a population and provide an avenue for preventing transmission of the virus to blood and organ recipients (Allain, 2017). The implications of transfusion of infected blood units to recipients will not only result to increase in prevalence of transfusion transmissible infections (TTIs) in the population, it could as well lead to serious infections such as HIV/AIDS and viral hepatitis with resultant cirrhosis, hepatocellular carcinoma and even death of infected persons (Seo *et al.*, 2015).

Occult Hepatitis B virus infection (OBI) through blood transfusion is thought to be a major route of transmission in low resource areas. In spite of this, blood donor screening for hepatitis B infection using only HBsAg marker is in practice in Nigeria including Nnamdi Azikiwe University Teaching Hospital, Nnewi and University of Abuja Teaching, Hospital, Abuja. Nucleic Acid Testing for HIV, HCV/HBV and anti HBc testing which is obtainable in some developed countries among blood donors is not practiced in many Nigerian hospitals' blood banks. One significant importance of OBI is the risk of transmission of HBV from individuals with OBI to recipients. This could occur if blood or blood components, stem cells or solid organs are transfused or transplanted following negative HBsAg results in donors with OBI. Such infections could manifest overtly becoming HBsAg positive with possibly fatal consequences, the risk rising with immune suppressed recipients (Levicnik-Stezinar *et al.*, 2008; Hollinger and Sood, 2010). Nevertheless there is limited data on the prevalence of OBI in blood donors in Nigeria as only HBsAg screening

is still relied upon for screening of blood donors (Salawu and Murainah, 2006; Salawu *et al.*, 2010).

Few studies on OBI among blood donors in Nigeria were done in South west Nigeria (Oluyinka *et al.*, 2015; Olotu *et al.*, 2016). This is the first study on occult Hepatitis B viral infection among blood donors in the study population. There is need for adequate screening of blood donors with inclusion of testing regimens to determine the tests suitable for screening blood donors that can detect occult hepatitis B virus infection. In addition, the need for testing the HBV serologic markers to determine the pattern that could be used as surrogate marker for detection of occult HBV infection cannot be overemphasized. It is against this background that this study was embarked upon to investigate the prevalence, viral markers, viral load and genotype of occult HBV infection as well as assess transfusion of transmissible viral infections among blood donors in these two teaching hospitals. This will enable us make evidence-based recommendations for effective screening of blood donors in Nigeria.

1.4 RESEARCH QUESTIONS

To solve this health problem and ensure that blood units for transfusion are safe, the following research questions are pertinent;

- What is the prevalence of occult HBV Infection among blood donors in Nnamdi Azikiwe University Teaching Hospital, Nnewi and University of Abuja Teaching Hospital, Gwagwalada, Abuja?
- Which HBV genotype is most prevalent in the study population?
- Is the positivity of HBV serologic markers in HBsAg negative blood donor an indication of occult HBV infection?
- Is ABO blood group associated with occult HBV infection? Which ABO blood group is most frequently infected among blood donors with occult HBV Infection?
- Which of the test methods (Rapid test device, ELISA or PCR) is most effective (sensitive/specific) in detection of transfusion transmissible viral (HBV, HCV and HIV) infectious agents?
- How safe is the blood units for transfusion in these two Teaching Hospitals with respect to other Transfusion Transmissible Infectious agents such as HIV and HCV?

1.5 RESEARCH HYPOTHESES/ASSUMPTIONS

Hypothesis 1

Null (H0): Blood donors' samples that tested negative for HBV serologic markers (Seronegative) are not negative for HBV DNA.

Alternate (HA): Blood donors' samples that tested negative for HBV serologic markers (Seronegative) are negative for HBV DNA.

Hypothesis 2

Null (H0): Blood donor sample that tested positive for anti-HBc marker is not positive for HBV DNA.

Alternate (HA): Blood donor sample that tested positive for anti-HBc marker is positive for HBV DNA.

Hypothesis 3

Null (H0): Blood donors' samples that tested positive by HBsAg ELISA technique are not positive for HBV DNA.

Alternate (HA): Blood donors' samples that tested positive by HBsAg ELISA technique are positive for HBV DNA.

Hypothesis 4

Null (H0): Blood donors' samples that tested positive for HBsAb are not positive for HBV DNA.

Alternate (HA): Blood donors' samples that tested positive for HBsAb are positive for HBV DNA.

1.6 AIM AND SPECIFIC OBJECTIVES OF THE STUDY

1.6.1 Aim:

The main purpose of the research is to evaluate the status of occult hepatitis B virus infection and other transfusion transmissible viral infections among blood donors at

University of Abuja Teaching Hospital, Gwagwalada and Nnamdi Azikiwe University Teaching Hospital, Nnewi.

1.6.2 Specific Objectives

1. To determine the prevalence of occult HBV infection among blood donors that tested negative and positive for HBV serologic markers.
2. To determine HBV serologic markers pattern among blood donors with occult HBV infection.
3. To quantify the viral load of blood donors with occult and overt HBV infection.
4. To determine the prevalence of other transfusion transmissible viral (HBV, HCV and HIV) infections and coinfections among blood donor participants.
5. To sequence HBV gene of blood donors with occult and overt HBV infection to determine the HBV genotype.
6. To determine Alanine Aminotransferase (ALT) and Alpha-Feto Protein (AFP) levels of blood donors with occult and overt HBV infection.

1.7 SIGNIFICANCE OF THE STUDY

This study is relevant as it will aid in the following;

- Assessment of HBV status among blood donors using serological markers and HBV DNA in order to detect occult HBV infection.
- To detect occult Hepatitis B viral infection among blood donors which will go a long way in preventing post transfusion of hepatitis B virus to blood recipients.
- Define and identify the most prevalent HBV genotype among blood donors with occult and overt HBV infection.
- Identify and create public awareness on risk factors of hepatitis B virus infection thereby leading to prevention and control of hepatitis B Virus infection in our community.
- Make relevant recommendations based on its finding that will minimize the transmission of HBV via blood transfusion or organ transplantation.
- Be beneficial to blood donors identified with HBV infection including occult HBV infection and other transfusion transmissible viral infections. This will enable them

to commence early treatment thereby preventing associated liver problems such as cirrhosis and Hepatocellular carcinoma.

- Definitely add a new knowledge that could spur further studies on HBV infection leading to improvement of health of mankind.

1.8 SCOPE OF THE STUDY

The study is targeted to determine the transfusion safety of blood units by blood donors certified fit for donation and transfusion to their recipients by detecting occult HBV infection and other transfusion transmissible viral infections such as HIV and HCV infection. Nucleic Acid Testing (NAT) and Enzyme Linked Immuno-Sorbent Assay were used to compare with rapid test devices most often used in blood banks in Nigeria. By using these techniques, the study was able to detect transfusion transmissible viral infections (HIV, HBV and HCV) which the rapid test kits missed to identify. The study did not screen for syphilis using Treponema Pallidum Haemagglutination Assay (TPHA) because of its low prevalence in our community. In addition the study did not attempt to detect other transfusion transmissible infections such as Malaria, Cytomegalovirus (CMV) infection, Epistein bar virus infection and Trypanosomiasis. The study did not involve all blood donors all over Nigeria but considered some blood donors attending University of Abuja Teaching Hospital, Abuja and Nnamdi Azikiwe University Teaching Hospital, Nnewi between 1st June, 2016 and 30th October, 2016. The study is limited to detection of occult hepatitis B virus infection using only blood specimens from blood donors in the studied population. Liver biopsies were not assayed for HBV DNA because of difficulty in obtaining specimens as the procedure is invasive.

1.9 DEFINITION OF KEY TERMS

- **Occult hepatitis B Virus Infection (OBI)**: is defined as the detection of HBV DNA in the serum or liver tissue of patients who tested negative for HBsAg marker. It is classified as seronegative and seropositive OBI.
- **Seronegative OBI**: Seronegative OBI are individuals that tested negative to all five HBV serologic markers (HBsAg, HBsAb, HBeAg, HBeAb and HBcAb) but positive for HBV DNA.

- **Seropositive OBI**: Seropositive OBI is an individual that tested negative for HBsAg but positive for one or more of HBV serologic markers and also HBV DNA.
- **Competitive Immunoassay**: A competitive immunoassay enables detection of an antigen (target) with use of a single capture antibody attached to the surface of a microsphere and a competitive, labeled antigen reversibly bound to the antibody. An analyte of interest in the sample competes with the labeled antigen causing a decrease in detected signal. A reverse version of the assay is also possible in which the analyte in the sample competes away the labeled antibody in solution rather than on the surface of the microsphere.
- **Cirrhosis**: Liver disease characterized by diffuse interlacing bands of fibrous tissues dividing the hepatic parenchyma into micronodular or macronodular areas. A chronic disease of the liver marked by degeneration of cells, inflammation and fibrous thickening of tissue. It is typically a result of alcoholism or viral hepatitis.
- **Hepatocellular Carcinoma (HCC)**: Hepatocellular Carcinoma (HCC), also called malignant hepatoma, is the most common type of liver cancer. Most cases of HCC are as a result of either a viral hepatitis infection (hepatitis B or C), metabolic toxins such as alcohol or aflatoxin, conditions like hemochromatosis and alpha 1-antitrypsin deficiency or NASH (Non-Alcoholic Steato-Hepatitis).
- **Polymerase Chain Reaction (PCR)**: PCR can be defined as amplification of a piece of DNA by in-vitro enzymatic replication. It entails extraction of nucleic acid (DNA/RNA), amplification i.e. replication of DNA or gene and identification of amplified extracts. The major components of PCR include; DNA template, Primers (forward and reverse), enzyme and buffering solutions.
- **Primers**: These are short oligonucleotide (DNA fragments) with defined sequence, between 18-30 base pairs that will flank the target sequence of DNA during the amplification phase of PCR process. A good primer for PCR reaction should have a volume range of 0.lul- 1.0ul. The G+C content must be 50/50.
- **Multiplex PCR:** In multiplex PCR, two or more primer pairs are included in one reaction tube and two or more DNA templates are targeted simultaneously. This is a relatively simple molecular way to detect few different microorganisms in one PCR

reaction. In multiplex PCR, the primer pairs should be specific to the target gene and the PCR products should be in different sizes.

- **Nucleic Acid Testing:** Molecular technique that involves amplification of genetic material of an organism with ultimate aim of its detection. It is highly sensitive and specific method of detection and identification of microorganisms.
- **Gene:** A gene is the basic physical and functional unit of heredity that can be transferred from parents to offspring. A segment of DNA, occupying a specific place on a chromosome, which is the basic unit of heredity. Genes act by directing the synthesis of proteins which are the main components of cells and are catalysts of all cellular processes. Physical traits such as texture of person's hairs or color are determined by gene. Genes undergo mutation when their DNA sequences change.
- **Genome:** Gene component of an organism or individual.
- **Genotype:** The genetic makeup of an organism or group of organisms with reference to a single set of traits. It is sum total of genes transmitted from parents to offspring. Genotype is of the three factors that determine specific characteristics (phenotype) of that cell or organism, the other two include inherited epigenetic factor and non-inherited environmental factor.
- **Window Period:** The window period for a test designed to detect a specific disease (particularly infectious disease) is the time between first infection and when the test can reliably detect that infection. In antibody-based testing, the window period is dependent on the time taken for seroconversion**.** During the window period (or equivalence zone) of hepatitis B, both serological markers HBsAg (Hepatitis B surface antigen) and Anti-HBs (antibody against HBsAg) are negative (which is because, although Anti-HBs are present, they are actively bound to the HBsAg). In other words, the window period is the time interval between the disappearance of the HBsAg and the appearance of Anti-HBs (in terms of detecting them in the serum). Other serological markers, IgM (antibody) against HBc can be positive at this point.
- **Phylogenetic Analysis:** Phylogenetics is the study of evolutionary relationship. Phylogenetic analysis is the means of inferring or estimating these relationships. The evolutionary history inferred from phylogenetic analysis is usually depicted as

branching, treelike diagrams that represent an estimated pedigree of the inherited relationships among molecules ("gene tree"), organism or both.

- **Gene Sequencing:** Genome sequencing or DNA sequencing is the process of determining the precise order of nucleotides within DNA molecules. It includes any method or technology that is used to determine the order of the four bases- adenine, guanine, cytosine and thymine in a strand of DNA.
- **Electrophoresis:** The separation of proteins in a complex mixture by subjecting them to electrical potential.
- **Enzyme Linked Immuno-Sorbent Assay (ELISA):** An assay for quantitating either antibody or antigen by use of an enzyme-linked antibody and substrate that forms a colored reaction product.
- **Neutralization:** Blockage of the activity of an organism or a toxin by antibody.
- **Reverse Transcriptase:** An enzyme that reversely transcribes RNA to DNA. It is found in retroviruses such as HIV
- **Reverse Transcriptase PCR:** RT- PCR is the technique of synthesis of cDNA from RNA by reverse transcription firstly, which is followed with amplification of a specific cDNA by PCR.
- **Real Time PCR:** A simple, quantitative assay for any amplifiable DNA sequence. This method is based on using fluorescent labeled probes to detect, confirm, and quantify the PCR products as they are generated in real time.
- **Nested PCR:** Genomic template DNA is amplified with two sets of primers. The first PCR set produces a larger PCR product than that in the second PCR set. The second PCR set uses the first PCR product as template DNA to amplify an internal region of DNA during the second (nested/semi-nested) amplification stage. The primers in the second PCR set can be different to the first set or one of the primers can be the same as the first set. This method can be used to increase the sensitivity of detection or to identify the first set PCR products when the primers in the second PCR reaction are species-specific.
- **Cytotoxic Cell:** Generally a CD8+ class 1 MHC- restricted T cell, which differentiates into CTL following interaction with altered self-cells (e.g. tumor cells, virus-infected cells).

- **Vaccination:** Intentional administration of a harmless or less harmful form of a pathogen (vaccine) to induce a specific immune response that protect against exposure to the same pathogen.
- **Cross Sectional Study:** A study design where expose and outcome are measured at the same time.
- **Descriptive Study:** Study designed to describe the distribution of variables in a population without regard to causal or other hypotheses.
- **BLAST:** This acronym stands for Basic Local Aligment Search Tool. It is one of sequence comparison/alignment algorithms used to determine whether observed similarities in sequence among proteins that perform similar metabolic functions are indicative of progressive changes in a common ancestral protein. It is used to identify unknown proteins and genes by searching for sequence homologs of known function. BLAST algorithms emphasize the regions of local alignment to detect relationships among sequences with only isolated regions of similarity.
- **YMDD:** This is a mutation involving Tyrosine-methionine-aspartate-aspartate in the polymerase gene of Hepatitis B virus.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 EVOLUTION, GROWTH AND CHALLENGES OF NATIONAL BLOOD TRANSFUSION SERVICE (NBTS) IN NIGERIA

Blood transfusion service (BTS) is an integral and indispensable part of the healthcare system. The priority objective of BTS is to ensure safety, adequacy, accessibility, and efficiency of blood supply at all levels (Islam, 2009). Transfusion of blood and blood products help save millions of lives every year. It can help patients suffering from life-threatening conditions live longer and with higher quality of life and supports complex medical and surgical procedures (Erhabor and Adias, 2011). It also has an essential, life-saving role in maternal and prenatal care. Access to safe and sufficient blood and blood products can help reduce rates of death and disability due to severe bleeding during delivery and after childbirth (WHO, 2015).

National Blood Transfusion Service (NBTS) is the organization with statutory national responsibility for the provision of blood for transfusion, and liaison with clinical services. The NBTS coordinates all activities concerned with blood donor recruitment and the collection, testing, processing, storage and distribution of blood and blood products, the clinical use of blood and surveillance of adverse transfusion events (WHO, 2010). Activities are carried out within a network of national/regional/ provincial blood centers and hospital blood banks. National Blood Transfusion Service (NBTS) has been empowered all over the world to perform this crucial service of provision of safe and adequate blood units in their respective countries (WHO, 2010). Efforts to establish a National Blood Transfusion Service in Nigeria date back to the early 60s after the Independence in October, 1960. These efforts were geared towards adequate provision and equitable distribution of safe donor blood throughout the country in order to eliminate the hazards associated with centralized, hospital-based and unregulated blood transfusion services (Erhabor and Adias, 2011). According to data from the Safe Blood for Africa initiative, it is estimated that about 1.5 million units of blood are needed on a yearly basis to take care of the estimated 170 million Nigerians (Erhabor and Adias, 2011). However, a

National Baseline Data Survey on blood transfusion in August 2005 indicated that only about half a million units of blood were collected from private and public sources in the previous one year with paid donors accounting for more than 90% of the blood donated (WHO, 2010).

The World Health Organization estimates that blood donation by 1% of a country population are the minimum required to meet a nation's most basic blood need. Nigeria with a country population of about 170 Million, it is expected that the donated blood requirement in a year be about 1.7 million units (WHO, 2010). But studies have shown that less than 1% of the population in many countries of the world, most especially developing countries, donates blood (CDC, 2008; WHO, 2010). In many countries, less than 25% of their blood supplies were from voluntary unpaid blood donor which is adjudged the safest blood source (Koistinen, 1992; CDC, 2008). Nigeria, as a member of World Health Organization (WHO) has made little progress with voluntary donor enrolment. Only about 5% of donor blood used in Nigeria comes from voluntary donors; family replacements and paid donors are still the major sources of donor blood procurement (Erhabor and Adias, 2011). The WHO and the International Federation of Red Cross and Red Crescent Societies have developed a global framework for action to achieve 100% voluntary blood donation around the world. It was put together after a series of collaborative activities were organized jointly by both bodies and stakeholders from around the world. The framework which was issued in 2010 would be considered for review from time to time depending on need and encompasses 20 strategies. These include advocating for 100% voluntary blood donation; establishing a national voluntary blood donor programme; strengthening collaboration and partnerships; understanding the mind set of blood donors; identifying target blood donor populations; developing communication strategies for donor education and community; active involvement; building partnerships with the media, mobilizing community partners and creation of viable networks (WHO, 2010). Other strategies include;

- Maximizing the impact of World Blood Donor Day and national blood donor events globally.
- Educating, motivating and recruiting new blood donors.
- Mobilizing the youthful populace as a new generation of voluntary blood donors.

- Converting eligible family/replacement donors to voluntary blood donors.
- Recalling infrequent, inactive and temporarily deferred blood donors.
- Retaining suitable voluntary blood donors; recognizing blood donors' contribution to the society and making it convenient for donors to give blood.

It also has strategies such as reaching out to donors through mobile donor sessions; assessing donors' suitability to donate blood; providing blood donor counselling and also making blood donation a safe and pleasant experience (WHO, 2005). But despite the efforts of these global bodies in sensitizing people on the need to voluntarily donate blood; many Nigerians still squirm at the thought of having their arms pierced with needles to extract the life giving liquid (Olaiya *et al.*, 2004). In some cities across the country, the solution for many is to source for blood in the unregulated laboratories that has emerged in recent years with its attendant health consequence (Erhabor and Adias, 2011).

Most countries in sub-Saharan Africa including Nigeria are not meeting with the best practices advocated by World Health Organization, and have resorted to diverse ways to meet the demand of blood supplies. This has led to proliferation of many unregulated blood transfusion services. The proliferation of unregulated blood services and profiteering has its attendant implications on the spread of infections that are transmissible through blood transfusion (Jayaraman *et al.*, 2010). Safe blood donors are the cornerstone of a safe and adequate supply of blood and blood products. The safest blood donors are voluntary, unpaid donors (WHO, 2010). Family/replacement and paid donations (as largely practiced in Nigeria) is associated with a higher risk of transfusion transmissible infections (TTI's) including: HIV, Hepatitis B, Hepatitis C and Syphilis (Erhabor and Adias, 2011). The statistics of people already infected with HIV and other blood borne infectious agents in Nigeria is alarming and transmission of infection through unsafe blood transfusion accounts for the second largest source of HIV infection among infected individuals (Ejele *et al.*, 2005). The above facts underscored the urgent need for a system that will effectively address the issue of blood transfusion safety in Nigeria. Blood saves lives if the blood itself is safe. To ensure that blood is safe, stringent measures should be put in place to screen blood before they are transfused into patients. These challenges led to the emergence of the National Blood

Transfusion Service (NBTS). The setback is further worsened by inadequate number of voluntary donors and the lack of appropriate legislation to guide actions as it concerns blood donation both in the public and private segments of the society. This has led to unsavory tactics employed by many hospitals in sourcing for the precious fluid, called blood. At present, blood transfusion services in Nigeria are based on the executive policies spearheaded by the Federal Ministry of Health and many have said this does not tally with global best practices (Erhabor and Adias, 2011). Experts advocate that with an enabling law and stringent mechanism to monitor sharp practices, the system would be better enhanced to serve its intended purpose. A study on the challenge of access to safe blood in Nigeria explained the importance of an efficient blood transfusion system and adds that lack of reliable testing mechanism, inadequate data on screened blood and lack of basic and advanced laboratory technologies/ services remain major challenges to blood transfusion services in Nigeria (Erhabor and Adias, 2011). In summary, blood transfusions worldwide and particularly in Nigeria is currently facing interesting challenges among which are transfusion transmissible infections (WHO, 2009a).

Transfusion transmissible infections, such as HIV, hepatitis B virus (HBV), hepatitis C virus (HCV), syphilis, and malaria have provoked a greatly heightened emphasis on safety with inescapable implications for the complexity and cost of providing a transfusion service (Fasola *et al.*, 2008). One of the biggest challenges to blood safety particularly in Nigeria and Sub-Saharan Africa in general is accessing safe and adequate quantities of blood and blood products (Tagny *et al.*, 2008). Communities in Africa face several enduring challenges: chronic blood shortages, high prevalence of transfusion transmissible infections (TTIs), lack of national blood transfusion services, recruitment and retention of voluntary non-remunerated donors, family replacement and commercial blood donation, and inadequate use of pharmacologic and non-pharmacologic alternatives to allogenic blood. Addressing these challenges should be a central priority for most blood transfusion services, particularly in Sub-Saharan African countries, to ensure the uninterrupted supply of safe blood and blood products (Erhabor and Adias, 2011). It is advocated that commercially remunerated donation of blood be discouraged. Political will and open-mindedness to innovative ways to improve supply

and safety of blood are essential to promote more evidence-based approaches to blood transfusion practice in Sub-Saharan Africa (Eahabor and Adias, 2011). We have a significant challenge to reduce unnecessary demand through world-class management of this precious product and the application of the best available evidenced-based medical practices. Strategies that reduce the use of allogenic blood, such as the correction of perioperative anemia, pharmacological and nonpharmacological measures to reduce blood loss, preoperative autologous blood donation, and perioperative red blood cell salvage, should be implemented to enable the optimal use of our limited blood supply in patients in whom these approaches are contraindicated (WHO, 2010).

2.2 TRANSFUSION TRANSMISSIBLE INFECTIONS (TTIs) AND THEIR CAUSATIVE AGENTS

2.2.1 Transfusion Transmissible Infections

Transfusion transmissible infections can be simply defined as infections acquired through transfusion of blood and blood products from donors to recipients (WHO, 2009a). The use of unscreened or inadequately screened blood for transfusion keeps the patient at risk of acquiring many transfusion transmissible infections (TTI) like Hepatitis viruses (HBV, HCV), Human Immuno-deficiency Viruses (HIV), syphilis, malaria, to mention but a few (WHO, 2009b). Screening for transfusion transmissible infections (TTIs) to exclude blood donations at risk of transmitting infection from donors to recipients is a critical part of the process of ensuring that transfusion is as safe as possible. Effective screening for evidence of the presence of the most common and dangerous TTIs can reduce the risk of transmission to very low levels (Dodd, 2007). Blood transfusion services should therefore establish efficient systems to ensure that all donated blood is correctly screened for specific TTIs and that only non-reactive blood and blood components are released for clinical use. Screening of donated blood for TTIs represents one element of strategies for blood safety and availability. The first line of defense in providing a safe blood supply and minimizing the risk of transfusion transmissible infections is to collect blood from well-selected, voluntary non-remunerated blood donors from low-risk populations, particularly those who donate regularly (Allain *et. al.*,2008). The prevalence of TTIs in voluntary non-remunerated blood donors is generally much lower than among family/replacement and

paid donors (Matee *et al*., 2006; Panda and Kar, 2008; WHO, 2008). Each country should establish voluntary blood donor programmes which provide donor information and education and develop stringent national criteria for blood donor selection and deferral to exclude prospective donors at the risk of TTIs (WHO, 2008). A lower prevalence of TTIs in the donor population also reduces the discard of donated blood and hence results in improved efficiency and use of resources. The microbial agents of importance to blood transfusion services are those that are transmissible by blood transfusion and can cause morbidity and mortality in recipients. In order to be transmissible by blood, the infectious agent or infection usually has the following characteristics:

- Presence in the blood for long periods, sometimes in high titres.
- Stability in blood stored at 4°C or lower.
- Asymptomatic phase or only mild symptoms in the blood donor, hence not identifiable during the blood donor selection process (Contreras, 2005).

As large volumes of blood or blood components are given to patients during transfusion therapy, even a blood unit with a low viral load may cause infection in the recipient (WHO, 2010). It is imperative that blood transfusion services have effective screening systems to detect, segregate and remove reactive blood donations and all components derived from these donations from the quarantined useable stock. Only non-reactive blood and blood components should be released for clinical or manufacturing use. The various markers of infection appear at different times after infection. Each TTI has one or more window periods, ranging from a few days to months, depending on the infectious agent, the screening marker used and the screening technology employed. During this period, the particular screening marker is not yet detectable in a recently infected individual, even though the individual may be infectious. Nucleic acid, as part of the native infectious agent itself, is the first detectable target to appear, followed within a few days by antigen, and subsequently by antibody as the immune response develops (Levinson, 2014). One or a combination of markers of infection can be used to detect a particular infection during the screening process (WHO, 2015). Various assay systems developed for blood screening detect:

- Antibodies that indicate an immune response to the infectious agent.
- Antigens that are produced by the infectious agent and indicate the presence of that agent.
- Nucleic acid (RNA/DNA) of the infectious agent.

In non-endemic countries, where the blood donor population includes travelers to or migrants from endemic areas, alternative strategies may be required, based on selective blood donor deferral and/or screening tests, if suitable assays are available. Similarly, some infections, such as human cytomegalovirus (CMV), present a risk to certain recipient groups only (Levinson, 2014). In this situation, the selective screening of donations for these specific recipients is normally adopted. To minimize the risk of the transmission of infection through the route of transfusion, the general recommendations by (WHO, 2009a) include:

1. All whole blood and apheresis donations should be screened for evidence of the presence of infection prior to the release of blood and blood components for clinical or manufacturing use.
2. Screening of all blood donations should be mandatory for the following infections and using the following markers:

- HIV-1 and HIV-2: screening for either a combination of HIV antigen-antibody or HIV antibodies.
- Hepatitis B: screening for hepatitis B surface antigen (HBsAg).
- Hepatitis C: screening for either a combination of HCV antigen antibody or HCV antibodies.
- Syphilis (*Treponema pallidum*): screening for specific treponemal antibodies.

3. Screening of donations for other infections, such as that causing malaria disease, should be based on local epidemiological evidence.

4. Screening should be performed using highly sensitive and specific assays that have been specifically evaluated and validated for blood screening.

5. Quality-assured screening of all donations using serology should be in place before additional technologies such as nucleic acid testing are considered.

6. Only blood and blood components from donations that are nonreactive in all screening tests for all markers should be released for clinical or manufacturing use.

7. All screened reactive units should be clearly marked, removed from the quarantined stock and stored separately and securely until they are disposed of safely or kept for quality assurance or research purposes, in accordance with national policies.

Screening for the following four infections that are transmissible by transfusion is recommended as mandatory for the provision of a safe blood supply in all countries (WHO, 2015). These infections can cause chronic disease with possible serious consequences and present the greatest infection risk to recipients of transfusion:

- Hepatitis B virus (HBV)
- Hepatitis C virus (HCV)
- Human immunodeficiency virus (HIV)
- Treponema pallidum (Syphilis).

Importantly, the risks of infection can be virtually eliminated if the screening of blood donations is performed in a quality-focused way. All efforts should be made to implement universal screening for these four infections by countries in which it is not currently fully in place. All blood donations should be screened for at least one suitable serological marker for each of these four infections. Screening for additional markers for these infections and for other transfusion transmissible infectious agents could then be considered, depending on the residual risk, logistics and level of resources available (Laperche, 2008).

2.2.2 Hepatitis B virus (HBV)

Hepatitis B virus (HBV) is a member of the hepadnavirus group and is an enveloped DNA virus. HBV is transmissible by the parenteral route and may be found in blood and other body fluids. Once in the bloodstream, the virus travels to the liver where it replicates in

hepatocytes. Hepatitis B Virus is endemic globally and hyper-endemic in parts of the world. It is difficult to determine the total number of cases of transfusion transmissible HBV globally (WHO, 2009a). Transmissibility of HBV depends on many factors among which the viral load level in the blood is important (Levinson, 2014). While HBV is present in the bloodstream, the levels of the virus itself are variable. In recently infected individuals, viral DNA is normally present, although not always at high levels. Chronically infected individuals may either be infectious (viral DNA present) or non-infectious (viral DNA absent) and viremia would generally be expected to be very low or absent entirely (WHO, 2015).

Screening for hepatitis B surface antigen (HBsAg) indicates infection with HBV, but does not in itself distinguish between recent and chronic infections. The distinction between acute and chronic infection is not relevant to blood screening. All HBsAg positive donations should be considered to be at high risk of transmitting HBV and should not be released for transfusion. Additionally, some studies indicate that even when HBsAg is negative, some individuals may have low levels of detectable viral DNA which will be transmitted by blood and may cause infection in the recipient (Gerlich *et al.*, 2007; Satake *et al.*, 2007). The use of unscreened HBV-infected blood and blood products will result in the transmission of HBV in the vast majority of cases. In general, the earlier in life that HBV is acquired, the more likely the individual is to develop chronic infection which then has a higher probability of progressing to cirrhosis and hepatocellular carcinoma. The serology of HBV is complex. A number of different serological markers develop during the course of infection, including hepatitis B surface antigen (HBsAg) and hepatitis B core antibody (anti-HBc). In addition, HBV DNA can be detected in the majority of cases, although in HBsAg negative phases of infection the DNA levels are generally relatively low and the viraemia may be transient. The methods used to identify the presence of HBV employ the following screening targets:

- Serological markers:
— Hepatitis B surface antigen
— Hepatitis B core antibody, in some situations
- Viral nucleic acid: HBV DNA.

Hepatitis B surface antigen

Hepatitis B surface antigen is the prime marker used in blood screening programmes. It normally appears within three weeks after the first appearance of HBV DNA and levels rise rapidly (Gerlich *et al.*, 2007). It can thus be detected easily by most of the highly sensitive HBsAg assays available. The presence of HBsAg may indicate current or chronic infection and thus potential infectivity. Most blood transfusion services screen donated blood for HBsAg using sensitive immunoassays. Particle agglutination assays are still available and used in some countries, although they are less sensitive than immunoassays or even simple/rapid assays (Levinson, 2014).

Hepatitis B Core Antibody

Antibody to hepatitis B core antigen is produced later in acute infection, after the appearance of HBsAg, and marks the start of the immune response to HBV infection. In general, anti-HBc persists for life, irrespective of whether the infection resolves or progresses to chronicity. In the vast majority of cases of hepatitis B, the detection of anti-HBc has limited value as HBsAg is already present. In some cases, however, during the resolution of the infection, HBsAg may decline to below detectable levels. Although anti-HBs usually then appears relatively rapidly, there may be a short period of time prior to its appearance when anti-HBc is the only detectable circulating serological marker of infection, even though the individual may still have low viraemia and would thus be potentially infectious. If anti-HBc screening is introduced for routine use, it would be necessary to distinguish between individuals who are anti-HBc reactive because of previous, resolved, natural HBV infection, and are thus non-infectious, from those who have unresolved HBV infection and are thus potentially infectious. In a population with a high prevalence of infection, the number of blood donors with evidence of natural, resolved infection is likely to be significant, resulting in the potentially unnecessary discard of many blood donations. As the presence of anti-HBs is protective, anti-HBs testing of all anti-HBc reactive donations would therefore be required to distinguish between infectious and non-infectious individuals. In general, a level of anti-HBs at 100 mIU/mL is usually accepted as the minimum protective level in the context of blood screening (Papadakis *et al.*, 2013).

Donations that are HBsAg negative, anti-HBc reactive with anti-HBs levels of 100 mIU/mL or more are generally considered to be safe and acceptable for release for clinical or manufacturing use. Another important consideration is that anti-HBc assays often demonstrate a high level of non-specificity. This, together with the problems associated with the confirmation of anti-HBc reactivity, often results in a situation where anti-HBc reactivity is identified in the absence of any other markers of HBV infection and where the majority of this reactivity is actually non-specific and does not reflect HBV infection. Thus, although anti-HBc screening may have advantages in some situations (Urbani *et al.*, 2010), the problems associated with the performance of anti-HBc assays and the complexity of dealing with HBV immune individuals may outweigh any potential benefits (WHO, 2009a).

Alanine Aminotransferase (ALT)

Testing for raised liver alanine aminotransferase (ALT) levels was originally introduced in some countries prior to the identification of hepatitis C and the introduction of HCV screening in an attempt to reduce the incidence of what was then called post-transfusion non-A, non-B hepatitis (WHO,2009a). Alanine Aminotransferase is an enzyme found predominantly in the liver. It circulates naturally at low levels in the bloodstream, but is released in high quantities as a result of liver damage; this is often, but not exclusively, due to viral infection (Burtis *et al.*, 2006). Alanine Aminotransferase is a non-specific marker of infection. With the advent of HCV screening, screening for raised ALT levels provides no identifiable benefit in terms of improving blood safety (Busch *et al.*, 1995; Contreras *et al.*, 2005).

Hepatitis B Virus DNA

The detection of HBV DNA further reduces the risk of HBV transmission through the transfusion of infected blood donated during the acute window period: i.e. when the results of HBsAg assays are negative, but HBV DNA is positive. Low levels of HBV DNA have also been detected in the blood of individuals after the resolution of acute HBV infection and the disappearance of HBsAg or in so called chronic occult HBV infection. To minimize the risk of HBV infection through the route of transfusion, it is recommended (WHO, 2015) that:

1. Screening should be performed using a highly sensitive and specific HBsAg immunoassay (EIA/CLIA).
2. Screening using a highly sensitive and specific HBsAg rapid assay or particle agglutination assay may be performed in laboratories with small throughput, in remote areas or in emergency situations.
3. Screening for anti-HBc is not recommended as a routine. Countries should determine the need for anti-HBc screening based on the prevalence and incidence of HBV infection.
4. Screening for ALT is not recommended.

2.2.3 Hepatitis C Virus (HCV)

Hepatitis C Virus (HCV) is a member of the flavivirus group and is an enveloped RNA virus (Levinson, 2014). It is transmissible by the parenteral route and may be found in blood and other body fluids. Once in the bloodstream, the virus travels to the liver where it replicates in hepatocytes, resulting in a similar picture to that seen with HBV infection. Seroreversion has been seen in numbers of individuals who have resolved their infections. The loss of circulating antibody may leave no readily detectable evidence of previous infection. HCV is endemic in many parts of the world, although in some regions its incidence and prevalence may be low (Buseri *et al.*, 2009). Several genotypes are identified and are associated with different geographical distributions and some differences in antigenicity and clinical features, including response to treatment with interferon alpha (IFN-α). While HCV is present in the bloodstream, the levels of the virus itself are variable. In recently infected individuals, virus is normally present.

However, only around 70% of chronically infected individuals are viraemic and the length of time that viraemia persists is not fully understood. Nonetheless, it is expected that most HCV infected donations would contain virus and thus be infectious. Screening for both HCV antigen and antibody does not in itself distinguish between recent and chronic infection. The distinction is, however, not relevant to the screening of blood for transfusion and all HCV antigen-antibody reactive donations should be considered to be at high risk of transmission of HCV and should not be used for clinical or manufacturing use.

The diagnostic methods used to identify the presence of HCV employ the following screening targets:

- Serological markers:
— HCV antibody
— HCV antigen
- Viral nucleic acid: HCV RNA.

HCV antibody and antigen

HCV antibody becomes detectable approximately 30 to 60 days after infection. Viral antigen normally appears between 0 and 20 days after viral RNA first appears. Antibody is generated and can be detected between 10 and 40 days after antigen is first detected. The serology of HCV is still not fully understood. Serological screening has been highly effective in significantly reducing the transmission of HCV through the route of transfusion. Until recently, anti-HCV has been the prime serological marker for blood screening programmes. However, HCV antigen can be detected in the peripheral blood earlier than antibody in the course of early infection. HCV antigen assays, both antigen only and combined antigen-antibody, have been commercially available for a number of years. These have been introduced in some countries to improve the overall effectiveness of serological HCV screening.

Hepatitis C Virus RNA

Viral RNA is normally detectable within a few weeks of infection and persists for 6–8 weeks prior to antibody seroconversion. The detection of HCV RNA may further reduce the risk of HCV transmission through the transfusion of infected blood donated during the window period of antigen and antibody assays: i.e. when the results of HCV antigen-antibody assays are negative, but HCV RNA is positive. However, any benefit is dependent upon HCV incidence and the actual number of donations that may be collected in the window period (Laperche *et al*., 2005). To minimize the risk of HCV infection through the route of transfusion, it is recommended that:

1. Screening should be performed using a highly sensitive and specific HCV antibody immunoassay or a combination HCV antigen-antibody immunoassay (EIA/CLIA).

The assay should be capable of detecting genotypes specific to the country or region.

2. Screening using a highly sensitive and specific HCV antibody rapid assay may be performed in laboratories with small throughput, in remote areas or emergency situations.

2.2.4 Human Immunodeficiency Virus (HIV)

The human immunodeficiency virus (HIV) is a retrovirus, an enveloped RNA virus, which is transmissible by the parenteral route. It is found in blood and other body fluids. Once in the bloodstream, the virus primarily infects and replicates in lymphocytes. The viral nucleic acid persists by integrating into the host cell DNA. A number of different groups and subtypes (clades) have been identified with some significant antigenic differences; HIV-1 and HIV-2 are the two major distinct virus types and there is significant cross-reactivity between them. HIV-1 is now endemic in many parts of the world, although its incidence and prevalence is low in some regions. HIV-1 group M is responsible for more than 99% of the infections worldwide, whereas the prevalence of HIV-2 is mainly restricted to countries in West Africa and India. Additionally, a few infections with HIV group O and group N have been observed in Africa. The appearance of antibody marks the onset and persistence of infection, but not immunity. As HIV can be present in the bloodstream in high concentrations and is stable at the temperatures at which blood and individual blood components are stored, the virus may be present in any donated blood from an HIV-infected individual. Infectivity estimates for the transfusion of infected blood products are much higher (around 95%) than for other modes of HIV transmission owing to the much larger viral dose per exposure than for other routes (Baggaley *et al.*, 2006). The screening methods used to identify the presence of HIV employ the following screening targets:

- Serological markers:

- anti-HIV-1, including group O, plus anti-HIV-2
- HIV p24 antigen (p24 Ag)

- Viral nucleic acid: HIV RNA.

The assay should be capable of detecting subtypes specific to the country or region. Screening donations for both antibody and antigen will identify the vast majority of donations from infected donors (Laperche *et al*., 2000). This entails the use of Anti-HIV-1 plus anti HIV-2 and p24 antigen. All screening strategies should employ, at minimum, the detection of antibody because the identification of specific antibody is still the most reliable screening method. They should preferably also employ the detection of antigen. Antibody may be detected approximately three weeks after infection and approximately six days after antigen is first detected (Kleinman *et al.,* 1997). Human Immunodeficiency Virus p24 antigen may appear from 3 to 26 10 days after viral RNA (Fiebig *et al.,* 2003), and its detection can further reduce the serological window period by 3 to 7 days before antibody detection. Screening for anti-HIV has been the basis for blood screening since the mid-1980s and HIV serology is therefore well understood. Although there is cross-reactivity between the main virus types (HIV-1 and HIV-2), it is not sufficient to rely on an HIV-1 specific assay to detect all cases of HIV-2. Since the early 1990s, anti-HIV assays have included specific antigens for both HIV-1 and HIV-2. However, the use of antibody-only assays has been superseded by the use of combination HIV antigen and antibody assays (combined HIV p24 Ag and anti-HIV-1 + anti-HIV-2), wherever possible. These provide an enhanced level of sensitivity in early infection over antibody-only assays by reducing the serological window period (Laperche, 2008).

HIV RNA

Viral RNA can be detected approximately 7 to 11 days after infection: i.e. when the results of HIV antigen-antibody assays are negative, but HIV RNA detection is positive. The detection of HIV RNA can reduce the risk of HIV being transmitted through the transfusion of infected blood donated during the serological window period of antigen and antibody assays. To minimize the risk of HIV infection through the route of transfusion the recommendations are as follow:

1. Screening should be performed using a highly sensitive and specific anti-HIV-1 + anti-HIV-2 immunoassay or HIV combination antigen antibody immunoassay (EIA/CLIA). The assay should be capable of detecting subtypes specific to the country or region.

2. Screening using a highly sensitive and specific anti-HIV-1 + anti-HIV-2 rapid assay may be performed in laboratories with small throughput, in remote areas or emergency situations.

2.2.5 Syphilis

Syphilis is caused by the bacterium *Treponema pallidum*. It is transmissible by the parenteral route and may be found in blood and other body fluids. Once in the bloodstream, the bacteria spread throughout the body. A primary lesion, chancre, usually occurs about three weeks after exposure, although the duration may be shorter in cases of transfusion-transmitted infection where the organism enters the bloodstream directly (Levinson, 2014). Syphilis is endemic in many parts of the world. While *T. pallidum* may be found in the bloodstream, levels are variable, even in acute primary syphilis, and the bacteremia is often short-lived. In addition, the treponemes are relatively fragile, in particular being heat-sensitive; storage below +20°C for more than 72 hours results in irreparable damage to the organism such that it is no longer infectious. Thus, although clearly potentially infectious, the risk of transmission through the transfusion of blood and blood components stored below +20°C is very low (WHO, 2010). Blood components stored at higher temperatures (above +20°C), such as platelet concentrates, or those not stored at lower temperatures for any length of time, such as blood collected and used within 48 hours, present a significantly higher risk of transmitting syphilis. Thus, although the risk of transmission of syphilis from unscreened donations is variable, the screening test is nonetheless considered essential. This is because most blood transfusion services provide some blood components that are either stored above +20°C or are not stored below +20°C for sufficient time to kill any organisms present. The screening methods used to identify the presence of syphilis employ the following screening targets:

- Non-specific, non-treponemal markers: antibody to lipoidal antigen (reagin)
- Specific treponemal antibodies.

Treponemal serology is relatively complex with different profiles seen at different stages of infection and depending on whether treatment has been given. Infection with the four major types of pathogenic treponemes cannot be distinguished by serological screening. This is because the major immunodominant epitopes are so similar that the antibodies produced are

detected by any specific antibody assay for syphilis. In general, syphilis assays can be divided into specific and non-specific assays; their use depends on whether the purpose of testing is screening or diagnostic testing.

Specific Assays

Specific assays commonly used for blood screening are *Treponema pallidum* haemagglutination assays (TPHA) and enzyme immunoassays (EIAs). These detect specific treponemal antibodies and thus identify donations from anyone who has ever been infected with syphilis, whether recently or long in the past ,and whether treated or not.

Non-specific Assays

Non-specific assays such as Venereal Diseases Research Laboratory (VDRL) and rapid plasma reagin (RPR) tests identify those individuals who may have been more recently infected. They detect antibodies to cardiolipin or lipoidal antigen (reagin); the plasma levels of these antibodies rise significantly in active infection due to the cellular damage. The use of non-specific assays is of most value in diagnostic testing where it can be used to identify recently infected individuals. When the incidence and prevalence of syphilis in the blood donor population are high and cannot be reduced through donor selection strategies, it may be necessary to consider screening using a non-treponemal assay (e.g. VDRL or RPR) to identify only the highest-risk donors – those with evidence of recent infections. For routine screening, however, this strategy carries a high risk of false negative results as the sensitivity of these assays is lower than specific assays and the test results may not always be positive, even when the infection is recent. To minimize the risk of syphilis infection through the route of transfusion the following recommendations should be adopted:

1. Screening should be performed using a highly sensitive and specific test for treponemal antibodies: either TPHA or enzyme immunoassay.
2. In populations where there is a high incidence of syphilis, screening should be performed using a non-treponemal assay: VDRL or RPR.

2.3 HEPATITIS B VIRUS: HISTORICAL PERSPECTIVE

Hepatitis B virus has been on since 1882. The first recorded cases of "serum hepatitis," or hepatitis B, are thought to be those that followed the administration of smallpox vaccine in Germany in l883. In the early and middle parts of the 20th century, serum hepatitis was repeatedly observed following the use of contaminated needles and syringes. The role of blood as a vehicle for virus transmission was further emphasized in 1943, when Beeson described jaundice that had occurred in seven recipients of blood transfusions. Historically, hepatitis B surface antigen (HBsAg) was formerly called Australia antigen because it was first described in the serum of an Australian aborigine in 1963 by Baruch Blumberg and his colleagues (Blumberg *et al.,* 1967). Okochi and Murakami in 1968 discovered that the Australian antigen was related to type B hepatitis. David Dane found virus-like particles in the serum of patients suffering from type B hepatitis in 1973 (Jonas, 2003). These particles were designated as Dane particle and were shown to be virion of hepatitis B virus (WHO, 2004). Paul Kaplan detected endogenous RNA/DNA-dependent DNA polymerase (reverse transcriptase) within the core of hepatitis B virion. The hepatitis B virus DNA genome was eventually cloned and sequenced by Pierre Tiollais, Patrick Charny, Pablo Valenzuela and William Rutter in 1979. Clinical and epidemiologic studies began to differentiate among various types of acute hepatitis in the decades after World War II (Acheson, 2011).

The ground breaking studies of Krugman and colleagues in 1967 firmly established the existence of at least two types of hepatitis (Krugman *et al.,* 1967), one of which (then called serum hepatitis, and now called hepatitis B) was parenterally transmitted. Links to the virus responsible for this form of hepatitis were derived by serologic studies conducted independently by Prince and colleagues and by Blumberg and colleagues (Blumberg *et al.,* 1965). Blumberg and colleagues, searching for serum protein polymorphisms linked to diseases, identified an antigen (termed Au) in serum from patients with leukemia, leprosy, and hepatitis, though the relationship of this antigen to hepatitis was initially unclear. By systematically studying patients with transfusion-associated hepatitis, Prince and coworkers independently identified an antigen, termed SH, that appeared in the blood of these patients during the incubation period of the disease. And further work established that Au and SH were identical (Prince, 1968a; Prince, 1968b). The antigen represented the hepatitis B

surface antigen (HBsAg) (Prince *et al.*, 1970; Krugman and Giles 1970). These seminal studies made possible the serologic diagnosis of hepatitis B and opened up the field to rigorous epidemiologic and virologic investigation.

2.4 BIOLOGY OF HEPATITIS B VIRUS

The HBV belongs to the family hepadnaviridae and genus orthohepatodnavirus (Brooks, 2004; Acheson, 2011). It is the only HepaDNA virus causing infection in humans (Finlayson *et al.*, 1999). It cannot yet be grown in an artificial medium but can be transmitted to certain primate such as the chimpanzee in which it is able to replicate (Finlayson *et al.*, 1999). It is a resilient virus that can exist on almost any surface for about 1 month (Acheson, 2011). HBV is stable at -20˚C for more than 20 years and stable after repeated freezing and thawing. Infectivity is not destroyed by incubation at 37˚C for 60 minutes and the virus remains viable after desiccation and storage at 25˚C for one week (Bhatia and Ichhpujani, 2008). The Dane particle, but not HBsAg, is inactivated at high temperatures (100 degree Centigrade for one minute) or when incubated for longer periods (60˚C for 10 hours) depending on the amount of the virus present on the sample. Hepatitis B surface antigen (HBsAg) is stable at pH 2.4 for up to 6 hours without loss of viral infectivity. Sodium hypochlorite in concentration of 0.5% (1: 10 house hold bleach), destroys the HBV antigenicity within 3 minutes at low protein concentrations (Brooks, 2004). Hepatitis B surface antigen is not destroyed by UV irradiation of plasma or any other blood product. Hepatitis B virus infected cells produce multiple types of virus related particles (Ganem, 1996). Electron microscopy of partially purified preparations of HBV shows three types of particles (Ganem, 1996). They include:

a. Double shelled particles with diameter of 42-47 nanometer (Known as Dane particles, after their discoverer).

b. Spheres of about 22nm diameter, usually present in a 10,000 to 100,000 fold excess over Dane particles.

c. Relative to complete virus there are smaller quantity of filaments of 20nm diameter and variable length often measuring about 200nm,

All these forms have a common antigen on their surfaces termed hepatic B surface antigen (HBsAg), which is present in large quantity in serum of the infected host (Acheson, 2011). These viral particles in circulation allow for easy antigen detection. The particles containing

HBsAg are antigenically complex. Each contains a group specific antigen, a, in addition to two pairs of naturally exclusive sub determinants, adw, ayw, adr and ayr (Ganem, 1996; Brooks, 2004) These sub types have geographical predilection, For example in the United States of America adw is the predominant subtype, while in Africa it is adr (Brooks, 2004). There is no difference in the pathogenicity between subtypes because cross immunity exists among them due to universal presence of the "a" determinant (Ganem, 1996). Antibody to "a" determinant is used in the diagnostic assay kit for HBsAg detection (Carman *et al.*, 1997). The complete virus or Dane Particles is the infectious viron of HBV (Ganem, 1996); its outer shell is a lipoprotein envelope containing the viral surface glycoprotein (Zuckerman, 1977). The inner core particle (hepatitis B core Antigen) or nucleocapsid has a diameter of 25 - 27nm and its major structural protein is the C protein (Finlayson *et al.*, 1999). Within the core is the viral DNA, a protein kinase and a polymerase known to be centrally involved in genomic replication (Ganem, 1996). The core also contains non-particulate soluble antigen (HBeAg) derived from HBcAg by proteolytic self-cleavage (Zuckerman, 1977; Houseman, 1999).

The viral DNA is a double stranded circular molecule. This molecule has an unusual structure in that its two DNA are not perfectly symmetrical (Ganem, 1996). Replication of HBV occurs predominantly in liver but also occurs in lymphocytes, spleen, kidney and pancreas (Acheson, 2011). Hepatitis B surface antigen is a product of S gene of the HBV genome and the prime constituent hepatitis B particle forms (Brooks, 2004). It is manufactured in the cytoplasm of infected hepatocytes in high quantities, the excess that did not combine with DNA to produce viral particles spill into the serum as 22nm diameter spheres and filaments (Ganem, 1996; Brooks, 2004). Carriage of HBsAg is considered to be chronic when the patient has been HBsAg positive for 6 months or more (Sear, 2000). The spheres and filaments are exclusively of HBsAg and host derived lipids, approximately 30% by weight. These particles lack nucleic acid and hence are noninfectious. Nonetheless, in pure form these particles are highly immunogenic and induce a neutralizing anti-HBs antibody response (Ganem, 1996). Prior to the development of recombinant HBsAg preparations, 20nm spheres as prepared from the serum of HBV carriers, served as HBV vaccine (Brooks, 2004).

2.4.1 Hepatitis B Viral Structure

Hepatitis B virus (HBV) is the prototype member of the Hepadnaviridae (hepatotropic DNA virus) family (Zuckerman, 1996). Hepadnaviruses have a strong preference for infecting liver cells, but small amounts of hepadnaviral DNA can be found in kidney, pancreas, and mononuclear cells. However, infection at these sites is not linked to extrahepatic disease (Acheson, 2011). HBV virions are double-shelled particles, 40 to 42 nm in diameter, with an outer lipoprotein envelope that contains three related envelope glycoproteins (or surface antigens). Within the envelope is the viral nucleocapsid, or core (Locarnini, 2004). The core contains the viral genome, a relaxed-circular, partially duplex DNA of 3.2 kb, and a polymerase that is responsible for the synthesis of viral DNA in infected cells. DNA sequencing of many isolates of HBV has confirmed the existence of multiple viral genotypes, each with a characteristic geographic distribution (Kramvis *et al.*, 2005). In addition to virions, HBV-infected cells produce two distinct subviral lipoprotein particles: 20-nm spheres and filamentous forms of similar diameter. These HBsAg particles contain only envelope glycoproteins and host-derived lipids and typically outnumber virions by 1000:1 to 10,000:1.

2.4.2 Hepatitis B Viral Genes and Proteins

The HBV genome is organized into four long open reading frames (ORFs) in such a manner that the DNA is utilized approximately 1.5 times. These ORFs encode viral polymerase, the X protein, the core and the envelope proteins which form the coat of the infectious virus (Bhatia and Ichhpujani, 2008). The ORF that encodes the envelope proteins is demarcated into three domains, each of which begins with an in-frame initiation codon and encodes three proteins referred to as PreS1+PreS2+S (the large-L-protein), PreS2+S (the middle- M- protein) and S (the major protein, HBsAg). The most abundant protein is the 24-kD S protein (which is known as HBsAg). Initiation at the most upstream start codon generates the M (or preS2) protein, the function of which is unknown. Initiation at the most upstream start codon yields the L (or preS1) protein, which is thought to play key roles in the binding of the virus to host-cell receptors and in the assembly of the virion and its release from the cell (Acheson, 2011). The preC–C (precore–core) region encodes hepatitis

B core antigen (HBcAg) and hepatitis B e antigen (HBeAg). These two proteins are also derived by alternative initiation of translation at two in-frame AUG codons (Acheson, 2011). The internal AUG encodes the 21-kD C protein, the structural polypeptide of the viral capsid, whereas the upstream AUG directs production of the 24-kD preC protein. The preC region encodes a signal sequence, which directs the chain into the secretory pathway. As the chains traverse the Golgi complex, cleavage by cellular proteases generates HBeAg, a 16-kD fragment that is secreted into the blood. Hepatitis B envelop antigen (HBeAg) plays no role in viral assembly, and its function is not clear. It is not required for viral replication; mutants bearing chain-terminating lesions within the preC region replicate well in culture and, in fact, arise frequently during natural infection (Fields *et al*., 1996). The P coding region is specific for the viral polymerase, a multifunctional enzyme involved in DNA synthesis and RNA encapsidation. The X open reading frame encodes the viral X protein (HBx), which modulates host-cell signal transduction and can directly and indirectly affect host and viral gene expression (Beck and Nassal, 2007). The X-protein activity is absolutely required for the in vivo replication and spread of the virus (Bouchard and Schneider, 2004).

2.4.3 Hepatitis B Viral Replication Cycle

The Replication Cycle of HBV shows the main features of the hepadnavirus replication cycle, the cardinal feature of which is the replication of the DNA genome by reverse transcription of an RNA intermediate. Incoming HBV virions are bound by cell-surface receptors, the identity of which remains unknown. After membrane fusion, cores are presented to the cytosol and transported to the nucleus. There, their DNA genomes are converted to a covalently closed circular (ccc) form, which serves as the transcriptional template for host RNA polymerase II. This enzyme generates a series of genomic and subgenomic transcripts (Bruss, 2007). All viral RNA is transported to the cytoplasm, where its translation yields the viral envelope, core, and polymerase proteins, as well as the X and preC polypeptides. Next, nucleocapsids are assembled in the cytosol, and during this process a single molecule of genomic RNA is incorporated into the assembling viral core (Acheson, 2011). Once the viral RNA is encapsidated, reverse transcription begins. The synthesis of the two viral DNA strands is sequential. The first DNA strand is made from the

encapsidated RNA template; during or after the synthesis of this strand, the RNA template is degraded and the synthesis of the second DNA strand proceeds, with the use of the newly made first DNA strand as a template (Bruss, 2007). Some cores bearing the mature genome are transported back to the nucleus, where their newly minted DNA genomes can be converted to cccDNA to maintain a stable intranuclear pool of transcriptional templates (Fields *et al*., 1996). Most cores, however, bud into regions of intracellular membranes bearing the viral envelope proteins. In so doing, they acquire lipoprotein envelopes containing the viral L, M, and S surface antigens and are then exported from the cell.

2.4.4 HBV Genotypes and Its Clinical Significance

Hepatitis B virus has eight genotypes from A-H. The difference in genotypes affects the severity, complication, course of disease and treatment (Olinger *et al*., 2008; Papadakis *et al.*, 2013). HBV genotypes have distinct geographical distributions in which multiethnic population tend to have multiple genotypes (Valsamakis, 2007).Genotype A is distributed globally and is the predominant genotype in North America, North West Europe, India and central Africa (Olinger *et al*., 2008). Genotype B and C are predominant in east and south Asia and Australia (Song *et al*., 2005). Genotype D has been reported globally but mainly found in Middle East, Mediterranean countries and India. Genotype D is the most widespread genotype, while the other HBV genotypes are geographically restricted (Arauz-Ruiz *et al*., 2002; Kidd-Ljungren *et al.*, 2002). Genotype E seems to be predominant in West Africa but genotype F is found in American native, Polynesia, central and South America. Genotype G is found in few patients in France, America, and Germany. Genotype H is found exclusively in central and South America (Mello *et al*., 2007; Mojiri *et al*., 2008). Phylogenetic analysis has shown that B/C recombinants have spread through East Asia and that A/D recombinants exist in Italy and C/D hybrid is present in Tibet (Cui *et al*., 2002). Subgenotypes within many HBV genotypes have been described (Bonino *et al.*, 2010). Infection with multiple genotypes, though infrequent, has been documented (Vivekananda *et al.*, 2004; Rashid and Salih, 2014). An increased prevalence of infection with multiple genotypes has been reported among drug users (Chen *et al.*, 2004).

Hepatitis B Virus genotypes were classified based on the divergence of 8% or entire genome sequence. It is well known that HBV genotypes have distinct geographical distributions.Genotype H was recently identified in Central America (Arauz-Ruiz *et al*., 2002). The prevalent HBV strains in China are genotype B and C (Zhu *et al*., 1999), but the two genotypes distribute unevenly in China. Study by Hou *et al*., (2005) among 1096 Chinese chronic HBV carriers from 9 provinces in Mainland China found four major genotypes A, B, C and D and their prevalence were 1.2%, 41%, 52.5% and 4.3%, respectively. In northern China, genotype C is predominant (85.1%), while in southern China, genotype B is predominant (55.0%). Genotypes A and D are also found in other areas of China. However, the genotypes E-H have not been reported in China. Recently, genotype C/D hybrid was identified in Tibet (Cui *et al*., 2002) and genotype B was found recombinated with preC/C region of genotype C in China (Luo *et al.*, 2004). Accumulated data suggest the importance of genotype, subgroup and recombination that may influence the biological characteristics of virus and clinical outcome of HBV infection.

Several studies reported a correlation of HBV genotypes with HBeAg clearance, liver damage, and the response to IFN treatment. It was reported that HBeAg carrier status tends to be longer and the prevalence of HBeAg appears higher in patients with genotype C than with genotype B (Orito *et al*., 2001; Chu *et al.*, 2002). HBV carriers with genotype B have lower histologic activity scores (Lindh *et al*., 1999), and genotype C is more prevalence in patients with cirrhosis (Ding *et al*., 2001). Furthermore, a retrospective study showed that HBV genotype B is associated with a higher rate of IFN-induced HBeAg clearance compared with genotype C (Kao *et al*., 2000b). However, whether patients with genotype B differ from those with genotype C in development of hepatocellular carcinoma remains controversial. The response of different HBVgenotypes to interferon-alfa treatment is of increasing interest because the benefit of interferon-alfa or its pegylated form in combination with other antiviral agents is being explored in the treatment of chronic hepatitis B. In a homogeneous group of prospectively followed patients from Europe, a recent study demonstrates that genotype A responds better than other HBV genotypes to standard interferon therapy and represents an independent predictor of a therapeutic success, with a greater impact than other pre-treatment characteristics, such as HBV DNA or ALT levels (Hou *et al*., 2005).

2.5 OCCULT HEPATITIS B VIRUS INFECTION (OBI)

2.5.1 Definition of Occult Hepatitis B Virus Infection by Several Scholars

Hepatitis B virus (HBV) remains a major public health problem worldwide (Candotti and Allain, 2009). Among many transmission routes, blood transfusion is the one that should be prevented. Implementation of hepatitis B surface antigen (HBsAg) in routine screening of blood donors in the early 1970s has greatly enhanced transfusion safety. The incidence of transfusion-transmitted hepatitis B has been steadily reduced over the last four decades (Liu *et al.,* 2010). However, it was demonstrated that HBV transmission by blood components negative for HBsAg can still occur (Liu *et al.,* 2006b). HBV transmission remains the most frequent transfusion-transmitted viral infection (Kafi-abad *et al*., 2009); thus, the term occult hepatitis B virus infection (OBI) was introduced.

Occult hepatitis B virus infection (OBI) is simply defined as serologically undetectable hepatitis B surface antigen (HBsAg-ve), despite the presence of circulating HBV DNA (Allain, 2009). Occult Hepatitis B virus Infection was reported for the first time almost 30 years ago in a case report of HBV infection through blood transfusion by an antibody to hepatitis B core antigen (anti-HBc) only positive donor (Allain, 2017). Occult HBV infection was not recognized as a clinical entity until the early 1990s (Sallie *et al.,* 1993). With the extensive use of sensitive molecular techniques for HBV DNA detection there have been an increasing number of studies in the last decade investigating various aspects of occult HBV infection. The residual risk of HBV transfusion transmission is mainly related to blood donations negative for HBsAg that have been collected either during the pre-seroconversion ”window period” (WP). Window period is defined as the time between infection and detection of a viral antigen or antibody marker, or during the late stages of infection (Candotti and Allain, 2009). Additionally, OBI has high significance in management of bone marrow and organ transplantations (Hollinger, 2008; Raimondo *et al.*, 2010). Implementation of HBV DNA screening has the potential to significantly reduce the WP and to reveal OBI or HBV carriage (Allain, 2004a). Allain, (2004b) reported OBI in several clinical contexts including: (1) recovery from past infection indicated by the presence of hepatitis B surface antibody (anti-HBs); (2) chronic hepatitis with surface gene

escape mutants that are not recognized by current assays; (3) chronic carriage without any marker of HBV infection other than HBV DNA (referred to as "seronegative"); and (4) most commonly in endemic areas, chronic carriage stage with HBsAg too low to be detected and recognized by the presence of anti-HBc as the only serological marker (referred to as "anti-HBc alone" or "isolated anti-HBc"). The loss of HBsAg or the presence of detectable anti-HBs indicates resolution from acute HBV infection. Both HBsAg and HBV DNA are detected in the majority of chronically infected patients (Candotti and Allain, 2009). However, a small proportion of individuals have detectable HBV DNA in the serum and/or the liver in the absence of circulating HBsAg (Torbenson and Thomas, 2002; Raimondo *et al*., 2008a). Occult hepatitis B virus infection is defined as the presence of HBV DNA in the liver (with or without detectable HBV DNA in the serum) in HBsAg-negative individuals (Torbenson and Thomas, 2002).

Occult hepatitis B virus infection is classified into two, seropositive and seronegative OBI Seropositive occult hepatitis B virus infection is characterized by the presence of anti-HBc and/or anti-HBs, while neither anti-HBc nor anti-HBs is detected in seronegative occult hepatitis B virus infection (Raimondo *et al.*, 2008a). HBV antibodies, including anti-HBc, anti-HBs, and anti-HBe, are frequently detected in occult HBV infection (Torbenson and Thomas, 2002). In many instances, individuals with occult HBV infection lack hepatitis B virus "e" antigenemia and have virus loads of $<10^3$ copies/ml (Torbenson and Thomas, 2002; Yuan *et al.*, 2010), though exceptions have been reported (Bremer *et al.*, 2009). Hepatitis B virus deoxyribonucleic acid (HBV DNA) levels can vary considerably in occult HBV infection among individuals positive for anti-HBs (Hennig *et al.*, 2002). Apart from above definitions of occult hepatitis B virus infection, other definitions for Occult Hepatitis B virus Infection (OBI) have been proposed by many authors and scholars. Bremer *et al., (*2009) emphasized that the term "occult hepatitis B virus infection" has been introduced to describe a pattern with the presence of replication-competent HBV DNA in the liver but without detectable HBsAg in the serum. This often occurs after progressive disappearance of HBsAg in the years after infection (Raimondo *et al.*, 2007) and persists in low-level carriers (Allain, 2004a). Early phase of HBV infection before appearance of HBsAg is not considered OBI, as the infection becomes eventually non-occult (Kleinman and Busch,

2006). A more specific definition was provided by Allain (2004a), who defined OBI as the presence of HBV DNA without HBsAg, with or without the presence of HBV antibodies outside the acute phase window period. This is in accordance with findings by Gerlich *et al*., (2010) that identified two blood donors whose donations tested HBsAg negative and HBV DNA-negative, but transmitted HBV. Both subsequently developed HBsAg and acute hepatitis. It was confirmed that such cases are transient OBI and should not be considered as true OBI. A true OBI remains HBsAg-negative during the entire course (Gerlich *et al.*, 2010). Nevertheless, a 2008 international workshop on occult hepatitis B virus (HBV) infection (OBI), endorsed by the European Association for the Study of the Liver (EASL) (Raimondo *et al*., 2008b), as well as The Taormina Consensus Conference in 2008, defined "OBI" as the "presence of HBV DNA in the liver of individuals testing HBsAg-negative with currently available assays."

Raimondo *et al*., (2008b) introduced a cutoff value for serum HBV DNA (< 200 IU/mL). Therefore, cases whose serum HBV DNA levels are comparable to those with different serologically evident (overt) HBV infection are generally due to infection with HBV escape mutants and should be labeled as "false" OBI (Raimondo *et al.*, 2008b). As confirmed by Hollinger and Sood (2010), this definition implies that infectious viral clones may be present. However, the detection of HBV DNA does not always correspond to infectivity or to the number of HBV progeny viruses released from hepatocytes. Therefore, the authors suggested a more comprehensive term "occult hepatitis B (OHB)" rather than OBI. Moreover, nosocomial sources should be carefully excluded before speculating that blood donors with OBI were involved in Hepatitis B viral disease transmission (Prati *et al*., 2006).

2.5.2 Occult Hepatitis B Virus Infection in Blood Donors

It is generally admitted that pre-seroconversion window period (WP) infections are most likely to transmit HBV but transmission from occult HBV infection remains a debated subject (Hollinger, 2008). Occult HBV is transmissible through blood transfusion in HBV-naive recipients (Su *et al*., 2011). Post-transfusion hepatitis B virus (HBV) infection still occurs, although its incidence has been found to be substantially reduced since the introduction of screening for HBsAg in blood donors (Liu *et al*., 2010). A similar study was

recently conducted in India and showed that a considerable number of HBV-infected donors remain undetected, if only HBsAg is used for screening (Panigrahi *et al*., 2010). Occult HBV in blood donors has a wide range of potential origin within the natural history of the infection. It may originate from previous infections with development of anti-HBs, but be accompanied by persistent, low-level, viral replication and/or escape mutants undetected by the HBsAg assays or healthy chronic carriage. The latter situation is mostly found with anti-HBc only. Over time, antibody markers may become undetectable leaving HBV DNA as the only marker of the infection (Allain, 2004b). A study conducted by Candotti *et al*., (2008) confirmed that 91% of 77 donor samples of European origin were HBV DNA-positive/HBsAg-negative. Viral load ranged between unquantifiable and 5640 IU/mL (median 25 IU/mL).

Another study conducted in Taiwan showed that in HBV hyperendemic areas, occult hepatitis B transfusion might not lead to HBsAg carriage or post-transfusion hepatitis. The risk of transfusion-transmitted HBV infection was probably lower than that in non-endemic areas because most recipients had already experienced HBV infection (Su *et al*., 2011). Infection of vaccinated individuals favors development of OBI, as was observed in 6 blood donors. HB vaccination may solve the problem of overt HBV infection but may favor OBI (Gerlich *et al.,* 2010). Addition of anti-HBc testing for donor screening, although leading to rejection of a large number of donor units, will definitely eliminate HBV-infected donations and help in reducing HBV transmission with its potential consequences, especially among the immunocompromised population (Panigrahi *et al*., 2010). Blood donors with occult hepatitis B infection have very low HBV replication, and normal liver biochemistry and histology, conferring a favorable prognosis (Yuen *et al*., 2010). Donations carrying anti-HBc only and HBV DNA can be infectious and this is a threat where anti-HBc is not screened. Anti-HBc screening identifies most OBI but not all. HBV NAT needs either extreme sensitivity or to be performed on individual donations to eliminate HBV DNA-containing units (Allain, 2004b). Reduction of HBV residual risk depends upon developing more sensitive HBsAg tests, adopting anti-HBc screening when appropriate, and implementing HBV NAT, either in minipools or more efficiently in individual samples (Candotti and Allain, 2009). Liu *et al*., (2006b) emphasized that anti-HBc screening has the potential to exclude the vast majority of OHBs, leaving only the probably rare cases with

HBV DNA alone undetected. This approach, however, has two main drawbacks: it does not detect the seronegative WP infections; and most importantly, it would not be practical in most parts of the world where the prevalence of anti-HBc is > 10%, as too many otherwise healthy donors will be ineligible (Liu *et al*., 2006b). The transmission risk of OBIs is not well defined, although some cases of OBIs with anti-HBc only which were infectious by transfusion have been described (Satake *et al*., 2007; Gerlich *et al.*, 2007). HBV transmission by blood components from a single anti-HBs-positive OBI donation to two recipients was recognized and it was clearly illustrated that the neutralizing capacity of low-level anti-HBs is limited, reinforcing the validity of considering anti-HBs below 100 IU/L to be poorly protective from infectivity when HBV DNA is present (Levicnik-Stezinar *et al.*, 2008). Authors further emphasized that even in the presence of higher levels of anti-HBs in a severely immunodeficient recipient, HBV DNA-containing blood might be infectious and the clinical expression severe.

However, as emphasized by Candotti *et al*., (2009), iatrogenic sources of infection should be systematically investigated before concluding that HBV-infected blood donors are involved in viral transmission (Liu *et al*., 2006b; Allain, 2006). They further added that adequate donor follow-up and laboratory testing have to be performed, and more importantly, pre- and post-transfusion testing of recipients has to be completed. Definitive evidence of transfusion transmission can be obtained by genomic analysis of the viral strains present in both donor and recipient (Candotti *et al*., 2009). In addition, sequencing, which might be informative, becomes very difficult to perform at levels of viremia below 200 IU/mL (Allain, 2009). Limited but convincing evidence that OBIs can be infectious and can be detected by HBV DNA screening should be carefully considered by the health authorities of countries where neither anti-HBc nor HBV NAT are implemented (Levicnik-Stezinar *et al*., 2008).

2.5.3 Clinical Relevance of Occult HBV Infection

Apart from posing diagnostic challenges, occult HBV infection may often be associated with a variety of clinical conditions. Continuous progress in molecular biology techniques has led to greater recognition and diagnosis of OBI. It has been reported in healthy blood donors, patients with chronic liver disease and patients with hepatocellular carcinoma

(HCC) (Hollinger and Sood, 2010). Also OBI has been reported in viral reactivation following immunosuppression, accidental transmission through transplantation, transfusion or experimental transmission to chimpanzees (Chemin and Trepo, 2005). Therapy should be considered during reactivation and in cirrhotic settings (Ozaslan and Purnak, 2009). As illustrated by Shi and Shi (2009), a dynamic balance between viral replication and host immune response is pivotal to the pathogenesis of liver disease. Most HBV infections are spontaneously resolved in immunocompetent adults, whereas they become chronic in most neonates and infants who are at great risk of developing complications such as cirrhosis, chronic liver disease (CLD) and HCC. Those with chronic HBV infection may present in one of the four phases of infection: immune tolerance, immune clearance (HBeAg-positive chronic hepatitis B), inactive carrier state, and reactivation (HBeAg-negative chronic hepatitis B) (Shi and Shi, 2009).

Occult Hepatitis B virus infection is a complex biological entity with possible relevant clinical implications, mainly related to the intrahepatic persistence of viral covalently closed circular DNA (cccDNA) and to a strong suppression of viral replication and gene expression (Raimondo *et al.,* 2010). Detection of virus-specific nucleic acid does not always translate into infectivity and the occurrence of primer-generated HBV DNA that is of partial genomic length in immunocompetent individuals who have significant levels of anti-HBs may not be biologically relevant (Hollinger and Sood, 2010). Several authors concluded that as a general rule, immune individuals who have recovered from acute hepatitis B have no clinical evidence of liver disease despite the detection of traces of HBV DNA in their blood, PBMC and/or liver decades later (Raimondo *et al.*, 2008a; Hollinger and Sood, 2010). Cross-sectional studies across the spectrum of HBV infection have revealed a marked increase in OBI prevalence towards patients with cirrhosis or HCC (Chemin and Trepo, 2005; Ozaslan and Purnak, 2009). However, data collected in Poland indicated that approximately 50% of OBIs occur in asymptomatic, apparently healthy blood donors carrying anti-HBs (Brojer *et al*., 2006). Levels of DNA and anti-HBs are variable (Levicnik-Stezinar *et al.,* 2008). The role of occult HBV infection in chronic hepatitis C virus (HCV) infection is perhaps the most extensively studied. Cacciola *et al.*, (1999) not only found higher rates of occult HBV infection among patients with HCV-related chronic

liver disease but also found liver cirrhosis more frequently among chronic HCV patients with occult HBV infection (33%) than among monoinfected patients (19%).

In another study, transient lower response rates during interferon (IFN) therapy were seen in anti-HBc-positive chronically HCV-infected patients. Only a fraction of the anti-HBc-positive individuals had demonstrable HBV DNA. Other studies have failed to demonstrate an association between occult HBV infection and lower rates of response to anti-HCV therapy in coinfected patients (Chen *et al.,* 2010). A functional assessment of IFN response was done by Fukuda *et al.,* (1999) by estimation of intrahepatic mRNA expression of the type I IFN receptor gene. Chronic hepatitis C patients with occult HBV infection had lower expression of the IFN receptor gene and a poorer response to IFN than monoinfected patients. It still remains unclear how occult HBV infection affects the treatment of chronic HCV infection (Kao *et al.*, 2002). Occult HBV infection has been associated with liver enzyme flares during chronic HCV infection without changes in HCV RNA levels (Kannangai *et al.*, 2007). Occult HBV infection in chronically HCV-infected patients has been associated with higher histological activity and advanced fibrosis (Mrani *et al.*, 2007), while another study failed to demonstrate this association (Kazemi-Shirazi *et al.*, 2000). Differences in geographical region, infecting HBV and HCV genotypes, and environmental cofactors could partially explain these contrasting findings.

Occult HBV infection is frequently detected in cryptogenic chronic liver diseases, including chronic hepatitis and cirrhosis (Chemin *et al.*, 2001). In individuals with chronic hepatitis, the presence of HBV proteins and HBV DNA has been confirmed by immunostaining and *in situ* hybridization (Chemin *et al.*, 2001). The HBV DNA level in individuals with cryptogenic liver disease is generally less than 10^4 copies/ml (Chemin *et al.*, 2001). While the causal role of occult HBV infection in cryptogenic liver disease and chronic hepatitis is still debated (Chaudhuri *et al.*, 2003), the usefulness of monitoring liver enzymes and HBV DNA levels in the management of occult HBV infection has been demonstrated (Chemin *et al*., 2009). However, specific guidelines for the management of occult HBV-related liver diseases are yet to evolve. Sequence analysis identified mutations in the surface gene, core gene, and polymerase gene of occult HBV genomes from patients with chronic liver diseases. In addition to sequence changes, differences in the ratios of the large and small

surface proteins were also attributed to the loss of HBsAg in serum (Chaudhuri *et al.*, 2003). Another study identified mutations in the X gene which reduced viral replication in occult HBV cases with chronic hepatitis (Saito *et al.*, 1999).

Occult HBV infections have also been reported in patients with nonalcoholic steatohepatitis (NASH) and autoimmune hepatitis (Honarkar *et al.*, 2005). Despite detection of HBV DNA and HBV proteins in patients with chronic liver diseases of unknown etiology, the causal role of occult HBV infection and the underlying pathogenic mechanisms remain elusive. Occult HBV infections are detected in as many as 73% of HCV-related HCC patients (Momosaki *et al.*, 2005) and at a lower frequency (18%) in non-B non-C HCC (NBNC HCC) (Kusakabe *et al.*, 2007). The occult HBV sequences from NBNC HCC lack core promoter mutations that are frequently detected in HBsAg-positive HCC. In addition, diabetes and obesity are frequently detected in the NBNC HCC group compared to the HBsAg-positive HCC group. The authors argue that a higher incidence of nonalcoholic steatohepatitis in the NBNC HCC group weakens the causal association of occult HBV infection in the development of HCC (Kusakabe *et al.*, 2007). The presence of core promoter mutations in occult HBV genomes in HCV-related HCC argues for a contributory role of occult HBV infections in the pathogenesis of HCC (Momosaki *et al.*, 2005).

The detection of transcriptionally active and replication-competent episomal HBV in addition to integrated HBV sequences in occult HBV infection-related HCC further strengthens the causative role of occult HBV infection in HCC (Pollicino *et al.*, 2004). The risk of acquiring post transfusion HBV infection is low with implementation of improved screening procedures. Occult HBV infections and to a lesser extent window-period infections contribute to the risk of transfusion-transmitted HBV infection (Yuen *et al.*, 2010; Zheng *et al*., 2011). Increased rates of occult HBV infection within the high-risk groups do not necessarily imply transmissibility of occult HBV. Nonetheless, it may not be speculative to anticipate transmission of HBV within these groups, considering the risk of multiple exposures in individuals within the high-risk groups. However, large-scale studies confirming transmission rates in high-risk groups from index cases with occult HBV infection are lacking. Intrafamilial horizontal transmission and vertical transmission from individuals with occult HBV infection have been reported (Datta *et al.*, 2006).

2.5.4 Occult Hepatitis B infectivity by Blood Transfusion

It is well known, and confirmed by Candotti and Allain (2009), that the estimated residual risk of HBV transfusion transmission remains significantly higher than the risk of either HIV-1 or HCV. Whether residual risk estimates translate into true rate of infection is largely unknown since estimates are generally based on the simplification that all HBV DNA-containing donations are infectious (Candotti and Allain, 2009). All forms have been shown to be infectious in immunocompromised individuals, such as organ- or bone marrow-transplant recipients. In immunocompetent recipients, there is no evidence that anti-HBs-containing components (even at low titer) are infectious. Anti-HBc only, with HBV DNA, can be associated with infectivity, as can rare cases of HBV DNA without any serological HBV marker (Allain, 2004a). HBV transmission was previously reported from OBI donors who had circulating HBV DNA at a low level (Manzini *et al.*, 2007; Bouike *et al.*, 2011). However, as reported by Candotti and Allain, (2009), in some cases units from WP and OBI donors were not infectious even though viral load ranging between < 20 and> 500 IU/mL (< 100 and > 2500 IU/mL) was transfused (Matsumoto *et al.*, 2001; Satake *et al.*, 2007). These authors emphasized that the lack of a clear relationship between infectivity and viral load in blood components may be related to immune factors affecting the susceptibility to infection in recipients. In addition, HBV infectivity is related to the amount of plasma transfused and the viral load in the product (Candotti and Allain, 2009). Few data regarding the infectivity of blood components or donated organs containing both anti-HBc and anti-HBs are available. Theoretically, if HBV particles are present in the peripheral blood of subjects with high-titer anti-HBs, the anti-HBs may neutralize the infectivity of the viral particles (Liu *et al.*, 2006b). Nevertheless, an OBI carrier with anti-HBs was found to have transmitted HBV to two immunocompetent transfusion recipients (Levicnik-Stezinar *et al.*, 2008 Gerlich *et al.*, 2007). They reported five donors (4 genotype D, one genotype A2) with OBI, also carrying only anti-HBc, transmitting HBV to recipients. Candotti and Allain, (2009) examined the infectivity of HBV-containing blood products according to the immune status of recipients and concluded that:

- WP and anti-HBs-positive and negative OBI units can transmit HBV.

- The confirmed HBV transmission rate of WP-derived donations is higher than by occult carriers (81% *versus* 19%) but may be biased by the large number of Japanese cases identified, with a peculiar set of anti-HBc and DNA screening protocols (Satake *et al.*, 2007).
- Viral transmission can be associated with extremely low levels of HBV DNA in anti-HBc-positive only units (< 20 IU/mL) or blood collected during the very early phase of acute infection (eclipse phase) in which neither HBsAg nor HBV DNA is detectable (Soldan *et al.*, 2002).
- HBV DNA load is similar in infectious and non-infectious anti-HBc-positive donations, suggesting that viral load is not the only factor for infectivity.
- The presence of anti-HBs seems to largely protect from transmission (Gerlich *et al.*, 2007), except in rare cases (Candotti and Allain, 2009).

No transmission of HBV has ever been demonstrated in blood donors who developed anti-HBc and anti-HBs following acute hepatitis B (Hollinger, 2008). Satake *et al.*, (2007) in Japan found that no HBV infections occurred in 22 recipients of HBsAg-negative, HBV DNA-positive blood that contained anti-HBs compared to 10 HBV infections that occurred among 37 recipients (27%) of OHB units that were devoid of anti-HBs (Hollinger and Sood,2010).

2.5.5 Serological Pattern of Occult Hepatitis B Virus Infection

The antibodies produced by the host and proteins released from the virus provide us with valuable information. Within the group of occult hepatitis B infection (OBI) patients, it possible to observe differences based on the results from serological markers (Ocana *et al*., 2011). Occult Hepatitis B virus Infection (OBI) can be classified into 2 groups, seropositive OBI (anti-HBc and/or anti-HBs positive) and seronegative OBI (anti-HBc and anti-HBs negative), on the basis of the HBV antibody profile.

Seropositive subjects

These are OBI subjects with anti-HBc and/or positive anti-HBs in which serum HBsAg is not detected because of the resolution of acute hepatitis B (after a few months of HBsAg carriage) or after years of chronic HBsAg positive infection (Raimondo *et al.*, 2008a). Most

OBIs are seropositive OBIs. Seropositive-OBI develops when serum test results for HBsAg become negative after acute hepatitis or when HBsAg is cleared during the course of chronic hepatitis B. In fact, annual HBsAg seroclearance rates are reported to be 0.50%-2.26% per year in chronic hepatitis B patients, and persistent HBV DNA in the liver was detected in some of these patients (Loriot *et al.*, 1997; Kwak *et al.*, 2011). Studies have shown that thirty-five percent of patients with OBI have positive anti-HBs and forty-two percent of have positive anti-HBc (Torbenson and Thomas, 2002).The HBV DNA detection rate is higher in individuals who are positive anti-HBc but negative for anti-HBs. When patients give a positive result for both antibodies, they have intermediate HBV DNA levels (Urbani *et al.*, 2010). One explanation for this serological pattern is that positive anti-HBc patients with chronic HBV infection clear HBsAg to an undetectable level, with or without anti-HBs: this pattern is associated with older age and anti-HBe (Lok and McMahon, 2007).

Seronegative Individuals

Patients who are not positive for anti-HBc and anti-HBs represent twenty-two percent of OBI patients (Torbenson and Thomas, 2002). They have very low levels of HBV DNA (Hollinger and Sood, 2010). Seronegative-OBI is caused by primary occult of anti-HBs or anti-HBc from the beginning of the infection because of the mutation or due to progressive loss of anti-HBs (Raimondo *et al.*, 2008a; Kim, 2013). This pattern of antibodies may appear from the beginning of the infection when patients have not yet developed positive hepatitis B specific antibody ("primary OBI") or because of clearance of the hepatitis B specific antibodies (Raimondo *et al.*, 2008a). Therefore, this pattern should always be kept in mind, because almost anybody can be a potential carrier of occult B hepatitis. Moreover, there are cases termed as "False" OBI. They are carriers of mutations in HBsAg (in the S gene) that are not recognized by some routine detection assays. In these cases, the DNA result resembles other cases of HBV, because they are usually positive for HBsAg (Raimondo *et al.*, 2008a).

2.6 PATHOGENESIS OF HEPATITIS B VIRUS INFECTION

2.6.1 HBV Transmission

Heepatitis B virus is spread through contact with infected body fluids and the only natural host is human. Blood is the most important vehicle for transmission, but other body fluids have also been implicated, including semen and saliva (Acheson, 2011). Currently, three modes of HBV transmission have been recognized: perinatal, sexual and parenteral/percutaneous transmission. There is no reliable evidence that airborne infections occur and feces are not a source of infection. Hepatitis B virus is not transmitted by contaminated food or water, insects or other vectors (Acheson, 2011).

Perinatal Transmission

Transmission of HBV from carrier mothers to their babies can occur during the perinatal period, and appears to be the most important factor in determining the prevalence of the infection in high endemicity areas, particularly in China and Southeast Asia. Before HBV vaccine was integrated into the routine immunization program, the proportion of babies that become HBV carriers is about 10-30% for mothers who are HBsAg-positive but HBeAg-negative. However, the incidence of perinatal infection is even greater, around 70-90%, when the mother is both HBsAg-positive and HBeAg-positive (Seven *et al.*, 1979; Xu *et al.*, 1985).

There are three possible routes of transmission of HBV from infected mothers to infants: transplacental transmission of HBV in utero; natal transmission during delivery; or postnatal transmission during care or through breast milk. Since transplacental transmission occurs antenatally, hepatitis B vaccine and HBIG cannot block this route. Epidemiological studies on HBV intrauterine infection in China showed that intrauterine infection occurs in 3.7-9.9% pregnancy women with positive HBsAg and in 9.8-17.39% with positive HBsAg/HBeAg (Xu *et al.*, 2002; Wang *et al.*, 2003). And it was suggested that a mother with positive HBeAg (OR = 17. 07) and a history of threatened premature labor (OR = 5.44) are the main risk factors for intrauterine infection. The studies on transplacental transmission of HBV suggested two possible mechanisms (1) hemagenous route: a certain of factors, such as threaten abortion, can make the placental microvascular broken, thus the high-titer HBV maternal blood leak into fetus' circulation (Ohto *et al.*, 1987); (2) cellular

transfer: the placental tissue is infected by high titer of HBV in maternal blood from mother's side to fetus' step by step, and finally, HBV reach fetus' circulation through the villous capillary endothelial cells (Xu *et al.*, 1999; Xu *et al.*, 2002). For neonates and children younger than 1 year who acquire HBV infection perinatally, the risk of the infection becoming chronic is 90% (Hyams, 1995), presumably because neonates have an immature immune system. One of the possible reasons for the high rate of chronicity is that transplacental passage of HBeAg may induce immunological tolerance to HBV in fetus.

Sexual Transmission

Sexual transmission of hepatitis B is a major source of infection in all areas of the world, especially in the low endemic areas, such as North America. Hepatitis B is considered to be a sexually transmitted disease (STD). For a long time, homosexual men have been considered to be at the highest risk of infection due to sexual contact (70% of homosexual men were infected after 5 years of sexual activity) (Alter, 2003). However, heterosexual transmission accounts for an increasing proportion of HBV infections. In heterosexuals, factors associated with increased risk of HBV infection include duration of sexual activity, number of sexual partners, history of sexual transmitted disease, and positive serology for syphilis. Sexual partners of injection drug users, prostitutes, and clients of prostitutes are at particularly high risk for infection (Alter and Mast, 1994).

Parenteral/percutaneous Transmission

The parenteral transmission includes injection drug use, transfusions and dialysis, acupuncture, working in a health-care setting, tattooing and household contact. In the United States and Western Europe, injection drug use remains a very important mode of HBV transmission (Margolis *et al.*, 1991). The risk of acquiring HBV infection increases with duration of injection drug use. Although the risk for transfusion-associate HBV infection has been greatly reduced since the screening of blood for HBV markers and the exclusion of donors who engage in high-risk activities, the transmission is still possible when the blood donors are asymptomatic carrier with HBsAg negative (Luo *et al.*, 1993). Obvious sources of infection include HBV-contaminated blood and blood products, with contaminated surgical instruments and utensils being other possible hazards. Parenteral/percutaneous transmission can occur during surgery, after needle-stick injuries, intravenous drug use, and following procedures such as ear piercing, tattooing,

acupuncture, circumcision and scarification (Alter, 2003). The nosocomial spread of HBV infection in the hospital, particularly in dialysis units, as well as in dental units, has been well described (Margolis *et al.*, 1991), even when infection control practices are followed. As with other modes of transmission, high viral titers have been related to an increased risk of transmission. People at high-risk of infection include those requiring frequent transfusions or hemodialysis, physicians, dentists, nurses and other healthcare workers, laboratory technicians, intravenous drug users, police, firemen, laundry workers and others who are likely to come into contact with potentially infected blood and blood products. The risk of chronicity is low (less than 5%) for transmission through sexual contact, intravenous drug use, acupuncture, and transfusion (Hyams, 1995). Individuals at risk for these transmission modes usually acquire HBV infection during adolescence or adulthood without immune tolerance. Instead, the disease progresses directly to the immune clearance phase and are of short duration, which probably accounts for high spontaneous recovery.

2.6.2 Immune Response to HBV Infection

When an infected blood or body fluid containing Hepatitis B virus comes in contact with a susceptible person the virus moves down to the liver, and replicate thus inhibiting the functions of the vital cells of the liver. At this point, the infected person responds by producing the cytotoxic T-lymphocytes (CTL). The cytolytic T-lymphocytes, in attempt to clear the infection, kill the infected cells. The virus could cause persistent infection, chronic hepatitis, and Hepatocellular Carcinoma (Ganem and Prince, 2004). The strength of the CTL response has been noted to determine symptoms of infection. A vigorous CTL response results in clearance and recovery although with an episode of jaundice. A weak response results in few symptoms and chronic infection and hence higher susceptibility for hepatocellular carcinoma (Baurmert *et al.*, 2007a). Hepatitis B virus specific T- and B- cell responses during acute and chronic infection have been extensively studied (Chisari, 1997). Individuals who clear HBV infection and those who become chronic carriers provide an interesting contrast in the immune responses they make against HBV (Bertoletti and Ferrari, 2003). The antibody response to HBV envelope proteins (HBsAg, PreS1, and PreS2) is thought to play a critical role in viral clearance and in preventing reinfection (Fields *et al.*, 1996; Chisari, 1997). High levels of these anti-envelope antibodies are

present in individuals who have resolved an acute HBV infection but are usually undetectable in the serum of chronic HBV carriers (Baurmert *et al.*, 2007a). In contrast, antibodies to HBV nucleocapsid antigens (HBcAg and HBeAg) and non-structural proteins are readily detected in patients with chronic HBV infection (Chisari, 1997). These findings documenting the selective absence of anti-envelope antibody in carriers have led to the concept of neutralizing antibodies directed to HBV envelope protein play an important role in viral clearance. However, a recent study has shown that anti-envelope antibodies are in fact present in HBV carriers, but they are often missed by conventional techniques because these antibodies are already complexed with viral surface antigens that are present in vast excess in the sera of HBV carriers (Acheson, 2011). This is a potentially important finding and additional quantitative studies should be done to address this issue in greater detail.

Several studies have examined both CD4 and CD8 T- cell responses during acute and chronic HBV infection (Baumert *et al.*, 2007a; Bes *et al.*, 2012). The general consensus is that individuals who resolve the acute hepatitis and eventually clear the infection, the T-cell response to HBV is potent and directed to multiple epitopes (Thimme *et al.*, 2003). However, in individuals who become persistently infected, the T- cell response is relatively weak and oligoclonal (i.e., restricted to a few epitopes). Taken together, these results suggest that both the quality and the magnitude of the T-cell response to HBV are important determinants of viral clearance or persistence (Ganem and Prince, 2004). However, the factors that determine why certain individuals generate an effective response and control the infection whereas others make weaker responses and fail to eliminate the virus are not well understood. Recent studies have documented the presence of CTL escape variants as well as HBV variants that interfere with CTL function (TCR antagonists) in HBV carriers and it is possible that such variants play a role in HBV persistence *in vivo* (Chisari,1997; Baurmert *et al.*, 2007a; Liaw and Chu, 2009;).

2.6.3 Clinical Manifestations of Hepatitis B Virus Infection

The incubation period for acute hepatitis B ranges from 1 to 6 months. Acute HBV infection can be either asymptomatic or symptomatic. Asymptomatic acute HBV infection associated with mild or subclinical disease often goes undiagnosed (Acheson, 2011). Clinically inapparent or asymptomatic acute HBV infections are more common in children

less than 4 years of age than in adults over 30 years of age. Clinically apparent cases have a prodromal phase with nausea, vomiting, malaise, anorexia, fever, and flu-like symptoms (Papadakis *et al.*, 2013).

The prodromal phase may be followed by an icteric phase with jaundice, clay-colored or pale stools, discomfort in the right upper quadrant, and hepatomegaly (Mahoney, 1999). Symptoms in acute HBV infection are clinically indistinguishable from those in other acute viral hepatitis infections. Several HBV markers, including HBV DNA, HBsAg, hepatitis B virus e antigen (HBeAg), and anti-hepatitis B virus core IgM (anti-HBc IgM), are detectable in the serum during acute HBV infection (Said, 2011; Samal *et al.*, 2012). Biochemical abnormalities such as increased transaminase levels and increased bilirubin levels may also be detected (WHO, 2009a). Seroconversion to antibody to HBsAg (anti-HBs) indicates resolution from acute HBV infection. Fulminant hepatitis B virus infection is associated with high mortality. Mutations in the precore region of the HBV genome have been associated with small outbreaks of fulminant hepatitis B (Liang *et al.*, 1991). Extrahepatic manifestations occur in up to 20% of HBV-infected individuals and may involve the gastrointestinal, renal, and nervous systems (Cacoub and Terrier, 2009). The mechanisms leading to extrahepatic manifestations of hepatitis B virus infection are poorly understood. Circulating immune complexes containing HBV have been demonstrated in patients with extrahepatic manifestations of chronic HBV infection. Circulating immune complexes and complement-mediated injury are implicated in extrahepatic manifestations of HBV infection, including arthralgia, serum sickness-like syndrome, polyarteritis nodosa, and glomerulonephritis (Cacoub and Terrier, 2009).

Chronic HBV infection is defined as the persistence of HBsAg for 6 months or longer. Chronic HBV infections are more common following anicteric disease (Said, 2011). The risk of developing chronic HBV infection decreases with age at infection; about 80 to 90% of neonates, 30% of children less than 6 years of age, and fewer than 5% of adults infected progress to chronicity (WHO, 2009a). In addition to age at infection, host genetic factors such as polymorphisms in the interleukin-18 gene, tumor necrosis factor, alpha promoter polymorphisms and human leukocyte antigen-DP gene variants have been linked to persistence of HBV (Cheong *et al*., 2010; Guo *et al*., 2011). It is well accepted that $CD8^+$ T

cells play a crucial role in the clearance of acute HBV infection (Thimme *et al.*, 2003). Chronic HBV infection may be classified into 3 phases, consisting of the immune tolerant phase, the immune active phase, and the inactive carrier phase (McMahon, 2009). The immune tolerant phase is associated with the presence of HBsAg, HBeAg, and high levels of HBV DNA in the serum. Alanine transaminase (ALT) levels are normal, and no major pathological changes are observed in liver biopsy specimens. The immune tolerant phase may last for several years and is characterized by mild disease. The progression of chronic liver disease is very slow in the immune tolerant phase despite high HBV DNA levels (Hui *et al*., 2007); this phase is also referred to as the "highly replicative phase."

The immune active phase is associated with elevated ALT levels, detectable HBV DNA, and inflammation of the liver with or without fibrosis. The "tolerance" to HBV is lost, and damage to the hepatocytes is mediated by the host immune responses to HBV; for this reason, the immune active phase is also referred to as the "immune clearance" phase. The immune active phase is seen in patients with or without HBeAg. The HBV DNA levels are generally higher in HBeAg-positive patients than in HBeAg-negative patients in the immune active phase. Patients with an HBeAg-negative status in the immune active phase are also classified as having "HBeAg-negative chronic hepatitis B." The loss of HBeAg may be associated with mutations in the precore region or the core promoter regions (Lindh *et al.*, 1999). About 70% of chronic HBV patients seroconvert from HBeAg to antibody to HBeAg (anti-HBe) within 10 years of diagnosis. However, reversion to HBeAg-positive status occurs in a small proportion of HBeAg-negative individuals. The inactive carrier phase is characterized by an HBeAg negative- and anti-HBe positive status with low HBV DNA levels (typically <2,000 IU/ml) and minimal or no fibrosis (McMahon *et al.*, 2001). The rates of spontaneous seroclearance of HBsAg among inactive carriers range from 0.5% per year to as high as 40% in 25 years of follow-up (MaMahon *et al.*, 2001). Symptoms in chronic HBV infection may range from mild nonspecific symptoms such as fatigue and right upper quadrant discomfort in patients with minimal liver damage to ascites, peripheral edema, and encephalopathy in patients with advanced liver disease (Papadakis *et al.*, 2013). Progression to cirrhosis is associated with multiple episodes of severe acute exacerbations, hepatic decompensation, and reversion to HBeAg-positive status from an anti-HBe positive status (Liang *et al.*, 1991). The 5-year survival rate in chronic HBV patients with cirrhosis

is less than 60%. Hepatocellular carcinoma (HCC) is a major global problem. In areas with a high prevalence of HBV infection such as Southeast Asia, higher rates of HCC are documented (Okuda, 2000). The risk of developing HCC may be increased up to 100-fold in patients with chronic HBV infection (Beasley, 1988). Hepatocellular carcinoma is a leading cause of cancer-related deaths. The morbidity and mortality associated with HBV-related HCC necessitate improved surveillance measures for early diagnosis and newer therapeutic options. Direct and indirect mechanisms of hepatocarcinogenesis have been identified in HBV-related HCC. The inflammation associated with chronic active hepatitis is a major contributor in hepatocarcinogenesis (Beasley *et al.*, 1981).

Integration of HBV DNA fragments into host chromosomal DNA is detected in the majority of HBV-related HCCs (Murakami *et al.*, 2005). The precise time of HBV DNA integration remains unclear, though it may occur as early as the acute phase of HBV infection (Murakami *et al.*, 2004). Integration of subgenomic fragments of HBV DNA into host chromosomal DNA was initially thought to be a random process (Matsubara and Tokino, 1990). Subsequent studies have identified recurrent HBV DNA integration sites on human chromosomes, including the human telomerase reverse transcriptase (RT) gene. The other HBV DNA integration sites identified include cancer-related genes, tumor suppressor genes, and genes involved in key signaling pathways (Murakami *et al*., 2005). Integration of HBV DNA could potentially modulate expression profiles of genes involved in carcinogenesis, including tumor suppressor genes and oncogenes. Furthermore, HBV DNA integration could be associated with chromosomal aberrations such as translocations, inversions, and deletions leading to chromosomal instability (Matsubara and Tokino, 1990). Interestingly, the expression of HBx protein (a protein that is not an integral part of HBV) is preferentially retained in HBV-related HCC tissue compared to the expression of HBsAg and hepatitis B virus core antigen (HBcAg). The role of HBx protein in HBV-related HCC has been extensively studied. It is generally accepted that HBV is not directly cytopathic and that liver injury is immune mediated. However, direct cytopathic effects of HBV have been demonstrated in individuals with an impaired immune system. Increased production of the large surface protein is associated with severe hepatic injury, triggering a cascade of events, including dysregulation of cellular genes and secondary genetic events leading to hepatocarcinogenesis in transgenic mice (Dunsford *et al.*, 1990). Defective HBV particles

are associated with singly spliced HBV RNA that encodes the hepatitis B virus splice-generated protein (HBSP), a novel protein associated with a frameshift during splicing in HBV. An increased relative abundance of defective HBV particles is associated with inflammation and fibrosis of the liver. Hepatitis B virus splice-generated protein modulates apoptosis and secretion of inflammatory cytokines, suggesting a potential role for the protein in hepatocarcinogenesis.

2.6.4 Reactivation of Hepatitis B Virus Infection

Reactivation of hepatitis B refers to the abrupt increase in hepatitis B virus (HBV) replication in a patient with inactive or resolved hepatitis B (Hoofnagle, 2009). Reactivation can occur spontaneously, but more typically is triggered by immunosuppressive therapy of cancer, autoimmune disease, or organ transplantation. Reactivation can be transient and clinically silent, but often causes a flare of disease that can be severe resulting in acute hepatic failure. Hepatitis B virus DNA persists in the body after infection and in some people the disease recurs (Vierling, 2007). Resolution of acute HBV infection is marked by the appearance of anti-HBe in the serum followed by the appearance of anti-HBs. A pool of HBV cccDNA persists in hepatocytes after resolution of infection (Reaiche *et al.*, 2010). Although rare, reactivation is seen most often following alcohol or drug use, (Villa *et al.*, 2011) or in people with impaired immunity (Katz *et al.*, 2008). HBV goes through cycles of replication and non-replication. Approximately 50% of overt carriers experience acute reactivation (Hoofnagle, 2009). Males with baseline ALT of 200 U/L are three times more likely to develop a reactivation than people with lower levels. Reactivation of HBV is characterized by a sudden rise in HBV DNA and serum transaminase levels in patients with past exposure to HBV infection (Hoofnagle, 2009). Reactivation of HBV is frequently associated with immunosuppressive therapy (Berger *et al.*, 2005), cancer chemotherapy (Hui *et al.*, 2006), sudden withdrawal of antiviral therapy (Dore *et al.*, 2010), and progressive immunodeficiency in Human Immunodeficiency Virus (HIV) infection (Bloquel *et al.*, 2010). Although reactivation can occur spontaneously, people who undergo chemotherapy have a higher risk (Mastroianni *et al.*, 2011). Immunosuppressive drugs favor increased HBV replication while inhibiting cytotoxic T cell function in the liver (Bonacini, 2009). The risk of reactivation varies depending on the serological profile; those

with detectable HBsAg in their blood are at the greatest risk, but those with only antibodies to the core antigen are also at risk. The presence of antibodies to the surface antigen, which are considered to be a marker of immunity, does not preclude reactivation ((Mastroianni *et al*., 2011). Treatment with prophylactic antiviral drugs can prevent the serious morbidity associated with HBV disease reactivation ((Mastroianni *et al*., 2011). The rate of HBV reactivation varies greatly across different clinical groups (Berger *et al.*, 2005; Manzano-Alonso *et al.*, 2011). Reactivation of HBV occurs in individuals who are HBsAg positive and also in HBsAg-negative individuals with occult HBV infection (Hoofnagle, 2009).

Among HBsAg-positive patients on chemotherapy for hematological malignancies, the prophylactic use of lamivudine in preventing reactivation of HBV infection is well recognized (Rossi *et al.*, 2001). Reactivation of occult HBV infection leading to clinical hepatitis may occur in HBsAg-negative patients undergoing chemotherapy (Hui *et al.*, 2006); however, the need for early identification of occult HBV infection in this group of patients is not widely appreciated. The role for anti-HBV prophylaxis in occult HBV patients undergoing chemotherapy needs further investigation. Emergence of drug resistance following prophylactic antiviral treatment among occult HBV-infected chemotherapy recipients is another potential problem. HBV DNA contains a glucocorticoid response element and patients receiving corticosteroids had a demonstrable increase in HBV DNA levels (Lai *et al.*, 1989). The use of glucocorticoids in chemotherapeutic regimens is linked to increased risk of HBV reactivation (Cheng *et al.*, 2003). Reactivation of HBV in HIV-infected patients with serological markers of past HBV infection is well documented (Gupta and Singh, 2010). Occult HBV infection is reported in 0.6% of HIV-infected patients with isolated anti-HBc reactivity (Neau *et al.*, 2005). Reactivation of both overt HBV infection (Dore *et al.*, 2010) and occult HBV infection (Bloquel *et al.*, 2010) has been reported in HIV-infected patients, especially after cessation or interruption of antiretroviral therapy. Recurrent monitoring of HBV DNA levels in HIV-infected patients with markers of past HBV infection may assist in early diagnosis and better management of HBV reactivation in this group of patients.

2.7 MOLECULAR AND IMMUNOLOGICAL MECHANISMS LEADING TO OCCULT HEPATITIS B VIRUS INFECTION

Occult HBV infection is characterized by the presence of HBV DNA in the absence of detectable HBsAg (Said, 2011). Occult HBV infection is a complex clinical entity documented worldwide (Samal *et al*., 2012). Significant advances in understanding the pathogenesis of occult HBV infection have been reported in the last decade. Several possible mechanisms have been hypothesized for the pathogenesis of OBI and the condition is probably multifactorial. Both host and viral factors are important in suppressing viral replication and keeping the infection under control (Hollinger, 2008; Hollinger and Sood 2010). The following are some of the molecular and immunological mechanisms leading to occult HBV infection as postulated by renowned scholars (Said, 2011; Samal *et al.*, 2012);

- Mutation and Deletions in the HBV Genome
- Coinfection with other Microbes
- Host Immune responses and occult HBV infection
- Host Genome integration
- Immune complexes in occult hepatitis B virus infection.
- Epigenetic changes
- Apolipoprotein B mRNA editing enzyme catalytic polypeptide and occult HBV infection

2.7.1 Mutation and Deletions in the Viral Genome

Sequence variation in HBV genomes which have been linked to occult HBV infection include;

- Mutations in the "a" determinant of HBsAg
- Treatment-associated mutations
- Splicing, and
- Mutations in the pre-S region

A mutation in the "a" determinant of the surface antigen was one of the earliest recognized mechanisms leading to occult HBV infection. Mutations in HBsAg lead to conformational changes rendering the protein undetectable by some of the commercially available HBsAg

assays (Raimondo *et al.*, 2008a). Recently, the term "false" occult hepatitis B virus infection has been used to describe occult hepatitis B virus infections in individuals with HBV DNA levels comparable to those in individuals with overt HBV infection. "False" occult hepatitis B virus infections are usually associated with surface gene mutants that are not detectable by some commercial HBsAg assays (Lledo *et al.*, 2011). Individuals with isolated anti-HBc-positive status with virus loads of greater than 10^4 copies/ml frequently harbor HBsAg mutants (Launay *et al.*, 2011). The "a" determinant of HBsAg is a 2-loop structure that includes amino acids (aa) 124 to 147 (Carman *et al.*, 1995). It is rich in cysteine residues which are involved in disulfide bond formation and maintain the conformation of this region. Carman *et al.*, (1990) first reported the sG145R mutation in the "a" determinant of HBsAg in a child who became infected with HBV despite active and passive immunoprophylaxis. This sG145R mutant has lower binding affinity to monoclonal antibody against HBsAg. Subsequently, several other mutations within the "a" determinant and mutations in the surface gene outside the "a" determinant (Carman *et al.*, 1995) were reported to have reduced binding affinity to monoclonal anti-HBs. The inability of some but not all commercial assays to detect HBsAg from samples associated with mutations in the "a" determinant is well documented (Laulu and Roberts, 2006). The emergence of "a" determinant mutants is a serious health concern not only because they are not detectable by some commercial HBsAg assays but also because they can infect both unvaccinated and vaccinated individuals.

Treatment-associated mutations are another factor that can cause sequence variation in HBV genome. Double mutations in the HBV polymerase associated with the emergence of a mutation in YMDD motif during lamivudine treatment result in amino acid changes in both the HBV polymerase and the surface gene (Q563S in the polymerase and sS207R in the surface gene) (Wakil *et al.*, 2002). In addition, other lamivudine-induced mutations that result in synonymous changes in the polymerase gene open reading frame (ORF) but nonsynonymous changes in the surface gene ORF were reported by Wakil *et al.*, (2002). They also reported (i) another novel mutation (V539I) in the "C" domain of the HBV polymerase that was associated with a premature stop codon in the surface gene and (ii) the emergence of a substitution within the "a" determinant of HBsAg (sS143L) in lamivudine-treated patients. Lamivudine-associated polymerase gene mutations M204I and

L180M/M204I, corresponding to sI195M and sW196S in HBsAg, have been shown to be associated with reduced binding to anti-HBs antibodies, suggesting that these mutants may escape detection in some of the commercially available assays for HBsAg (Torresi *et al.*, 2002a). These mutants may potentially escape neutralization by vaccine-induced anti-HBs. Mutations that confer resistance to lamivudine also reduce the affinity of the HBV polymerase to natural deoxynucleoside triphosphate (dNTP) substrates, resulting in reduced replication competence (Gaillard *et al.*, 2002). Therefore, lamivudine-selected mutants have reduced replication fitness compared to wild-type HBV.

Mutations in the pre-S region, especially deletions, have also been associated with a lack of detectable HBsAg in the serum. Deletions in the pre-S region are associated with reduced expression of HBV surface proteins and also help in viral persistence by eliminating HLA-restricted B-cell and T-cell epitopes. Pre-S1/pre-S2 mutations are frequently detected in occult HBV infection (Chaudhuri *et al.*, 2004; Vivekanandan *et al.*, 2008). Mutations in the pre-S2/S promoters were detected in patients with occult HBV-related chronic liver disease; serum HBsAg was not detectable in these patients (Chaudhuri *et al.*, 2004). In another study, a 183-bp deletion (nt 3019 to 3201) in the pre-S1 region was detected in occult HBV patients. The deletion covered the CCAAT element that is required for transcription factor binding. Other point mutations in the pre-S genes were also detected. The association of mutations and deletions in the pre-S gene with a lack of secreted HBsAg and low levels of HBeAg and HBV DNA was demonstrated using functional analysis by transfection into hepatocyte cell lines (Fang *et al*., 2009). Escape mutation is another mechanism which also leads to decreased reactivity in HBsAg detection assays (El Chaar *et al*., 2010). This is confirmed by Gerlich *et al*., (2010). Van Hemert *et al*., (2008) proposed an evolutionary scenario for occult HBV infection. They identified a novel RNA splicing event (deleting nucleotides 2986-202) that abolishes surface protein gene expression without affecting polymerase, core or X-protein related functions. This 2986-202 splicing generates intracellular virus particles devoid of surface protein, which subsequently accumulate mutations due to relaxation of coding constraints. Such viruses are deficient in autonomous propagation and cannot leave the host cell until it is lysed (van Hemert *et al*., 2008). Additional mechanisms not related to the host response were also extensively studied by many authors, where it was shown that the low level of viral replication was a result of the

presence of defective interfering particles or of mutations in transcription control regions or the polymerase domain leading to decrease in HBV DNA replication and HBsAg expression (Hollinger, 2008; Fang *et al*., 2009; Hollinger and Sood 2010).

2.7.2 Coinfection with other Microbes

Coinfection with microorganisms such as Hepatitis C virus, HIV and *Schistosoma masoni* have been reported as an underlying mechanism for occult hepatitis B virus infection (Samal *et al.*, 2012).

Coinfection with HCV

Coinfection with hepatitis delta virus or hepatitis C virus (HCV) which results in down regulation of HBV replication and a reduction in HBsAg synthesis has been reported (Hollinger and Sood 2010). Sagnelli *et al.*, (2000) showed an inhibitory effect of HCV on HBV replication. This inhibitory activity of HCV on HBV replication has also been reported by other investigators in a follow-up study of 6 years duration, where it was shown that the rate of HBsAg clearance is 2.5 times higher in HBsAg/anti-HCV-positive cases than in those with HBV infection alone. It was suggested that HCV is the most important hepatotropic virus that enhances HBsAg clearance in chronic hepatitis B (Sheen *et al.*, 1994). The underlining molecular mechanism responsible for this suppressive effect has been extensively studied both *in vitro* (Bellecave *et al.*, 2009) and *in vivo* studies (Guido *et al.*, 1999). Indirect mechanisms mediated by innate and/or adaptive host immune responses have also been postulated as being involved (Chu and Lee, 2008). Recent study on the prevalence of occult HBV among children and adolescents with hematological diseases with or without HCV in an area of high endemicity of HCV infection has shown that HCV RNA was a significant predictor for OBI, with an increased frequency of HBV DNA in those who were HBsAg-negative and HCV RNA positive compared with patients negative for HCV RNA (Said *et al*., 2009).

Coinfection with HIV

Human immunodeficiency virus (HIV) and HBV share modes of transmission. Occult HBV infection in HIV-infected individuals is well recognized. The reported prevalence of occult HBV infection among HIV-positive patients varies based on the methods used for the

detection of HBV DNA, the endemicity of HBV in the geographical region studied, and the history of antiretroviral treatment. In a longitudinal follow-up study, HBV DNA was detected at least once in almost 90% of a cohort of Swiss HIV-positive patients, compared to 25% detection rates for HBsAg (Hofer *et al.*, 1998). This finding suggests that (i) occult HBV infections are common among HIV-positive individuals and (ii) HBV DNA is intermittently detected in HIV-positive individuals, necessitating multiple sampling for HBV DNA in this group of patients. Another study, by Gupta and Singh (2010), found occult HBV infection in treatment-naïve HIV-infected patients; interestingly, about a fifth of the patients with occult HBV infection had detectable anti-HBs. However, the specific mechanisms leading to occult HBV infection in HIV-infected individuals still remain unknown.

Coinfection with *Schistosoma mansoni*

Schistosoma mansoni is a parasite that affects over 200 million people worldwide, particularly in Asia and Africa (Ross *et al.*, 2002). Coinfection with HBV and *Schistosoma* occurs frequently in areas where both agents are endemic (Berhe *et al.*, 2007). When transgenic mice supporting HBV replication were infected with *Schistosoma mansoni*, a Th1-type response was followed by both Th1 and Th2 responses. HBV replication levels remained suppressed during both the Th1 response and the subsequent Th1 and Th2 responses (McClary *et al.*, 2000). In gamma interferon (IFN-γ) knockout mice coinfected with *Schistosoma mansoni*, the suppression of HBV replication was minimal, suggesting that IFN-γ is the major antiviral cytokine in *Schistosoma mansoni* infection. HBV replication remained inhibited when both Th1 and Th2 responses were detected, suggesting that Th2 cytokines do not interfere in the antiviral activity of IFN-γ (McClary *et al.*, 2000).

2.7.3 Host Immune Responses and Occult HBV Infection

The majority of OBI cases is secondary to overt HBV infection and represents a residual low viremia level suppressed by strong immune response together with histological derangements occurring during acute or chronic HBV infection (Ozaslan and Purnak 2009). It was previously suggested that long-term maintenance of an active anti-viral T cell

response several years after clinical recovery from acute hepatitis B could be important, not only for protection against reinfection, but also for keeping the persisting virus under tight control where detection of minute amounts of virus in some recovered subjects was confirmed (Penna *et al*., 1996). Also, in a study to characterize the features of the HBV-specific T-cell response in patients with OBI, 2 different profiles were defined. Anti-HBc-positive patients showed a T-cell response typical of protective memory, suggesting that this condition represents a resolved infection with immune-mediated virus control. In contrast, HBV-specific T cells in anti-HBc-negative patients did not readily expand, suggesting the possibility of a low-dose infection insufficient to allow maturation of protective memory (Zerbinet *et al*., 2008). Humoral and cellular immune pressure on the HBV envelope proteins are major mechanisms generating OBI. Amino acid substitutions are significantly concentrated in the immunologically active parts of the Pre-S/S proteins affecting both cellular CD8 T-cell epitopes and B-cell neutralizing major hydrophilic region epitopes (Candotti *et al*., 2008).

2.7.4 Immune Complexes in Occult Hepatitis B Virus Infection

Masking of HbsAg by HbsAg-anti-HBs immune complexes is another postulated mechanism for the development of OBI (Hu KQ, 2002; Zhang *et al.*, 2007). Entrapment of HBsAg in immune complexes with anti-HBs can impair HBsAg detection by conventional serological assays. In general, the appearance of detectable anti-HBs correlates with a decrease or complete disappearance of HBsAg-containing immune complexes (Pernice *et al.*, 1979). Interestingly, immune complexes containing HBsAg have been detected in HBsAg-negative occult HBV infection among patients with HCC (Brown *et al.*, 1984). HBV DNA was detected in about 40% of HBsAg-negative but anti-HBc total positive blood donors, with HBV-containing immune complexes present in a majority of the HBV DNA-positive donors (Yotsuyanagi *et al.*, 2001).

Sequencing of HBV DNA from these donors demonstrated the lack of nucleic acid changes that alter major epitopes of HBsAg, confirming the role of circulating immune complexes in occult HBV infections. Michalak *et al.*, (1994) demonstrated that HBV DNA from convalescent-phase sera cosedimented with HBsAg in a sucrose gradient column representing naked core particles or intact virions in circulating immune complexes. These

data suggest that hepatitis B virus can persist despite recovery from acute infection. Circulating HBsAg-containing immune complexes have been demonstrated in patients with acute HBV infection, in asymptomatic HBsAg carriers, and also in chronic HBV infection (Anh-Tuan and Novak, 1980). HBsAg-containing immune complexes have also been found in the presence of detectable anti-HBs (Sansonmo *et al.*, 1986). Madalinski *et al*., (1999) found anti-pre-S1 antibodies to be frequently involved in the formation of HBV-containing immune complexes in patients with chronic hepatitis B virus infection. Levya *et al.*, (1988) correlated the presence of HBsAg-containing immune complexes and the course of hepatitis. Interestingly, they found that HBsAg-containing immune complexes are consistently present in patients with chronic hepatitis B virus infection compared to those with acute resolving hepatitis. Other studies have shown the opposite finding. Differences in the methods used could in part account for these contrasting observations. Increases in levels of immune complexes containing woodchuck hepatitis virus surface antigen (WHsAg) and anti-WHs correlated with the peaks of serum viremia and antigenemia, indicating the presence of surface antigen-containing complexes in WHV similar to those in HBV (Glebe *et al.*, 2009). It is clear that circulating HBsAg-containing immune complexes can be associated with occult HBV infection; however, the magnitude of the problem in the settings of acute resolving hepatitis and chronic hepatitis remains poorly understood.

2.7.5 Host Genome Integration

Additional mechanisms for OBI have been thoroughly investigated; emphasizing that integration of viral sequence may alter HBsAg expression and decrease HBV replication (Chemin and Trepo 2005). Meanwhile, reduced HBV viremia may result from extra-hepatic HBV replication such as that takes place in peripheral blood mononuclear cells (PBMCs). Patients with long-standing abnormal results of liver function tests with unknown etiology may have HCV RNA or HBV DNA in their PBMCs in the absence of anti-HCV antibodies, HBV markers, serum HBV DNA and serum HCV RNA (Zaghloul and El-Sherbiny 2010). Integration of HBV DNA sequences into the host genome is frequently detected in patients with chronic HBV infection and is found to precede HCC (Urashima *et al.*, 1997). Disruption and rearrangement of genes during integration into chromosomal DNA can result in (i) loss of HBsAg in the serum, (ii) reduction in virion production, and (iii) loss of

detectable HBV DNA in serum. Therefore, integration of HBV DNA represents a key mechanism underlying occult HBV infection, especially following several years of chronic HBV infection. Integrated HBV DNA rather than episomal HBV is frequently detected in HBV-related HCC (Raimondo *et al.*, 2008b). Integrated HBV DNA is often defective (Brechot *et al.*, 2010), and integration of certain HBV genes is observed more frequently than that of others. While high rates of HBV DNA integration have been reported from HBsAg-positive HCC (Kawai *et al.*, 2001), HBV DNA integration in HBsAg-negative HCC is also widely reported (Tamori *et al.*, 2003; Momosaki *et al.*, 2005), especially among anti-HCV-positive patients. Specific integration patterns disrupting the expression of HBsAg have not been reported in HBV-HCV dual infection. The inhibition of HBV replication and HBV proteins has been discussed above.The HBV core gene may often be lost during integration of HBV DNA, resulting in reduction or loss of the HBV core protein. The loss of the HBV core protein is associated with suboptimal virus assembly and the accumulation of unencapsidated HBV DNA within the hepatocyte. This may explain why patients with HCC related to HBV lack detectable HBV DNA in blood though HBV DNA may be readily detected in the liver (Raimondo *et al.*, 2008a). Overexpression of large HBs protein prevents secretion of all forms of surface protein and leads to intrahepatic accumulation of surface proteins as granules (Chisari *et al.*, 1986). The large HBs protein constitutes a small proportion of total surface proteins. Huang and Yen, (1993) studied the role of disruptions and rearrangements in HBV DNA that occur during integration in regulating HBsAg expression. Replacement of a region downstream of the S gene ORF containing enhancer I and II with the pre-S1 promoter resulted in decreased S gene transcripts without affecting the levels of pre-S transcripts. Changes in the ratios of S gene transcripts to pre-S1 transcripts blocked the secretion of S protein. Similarly, other rearrangements resulting in the loss of HBV enhancers lead to intracellular retention of surface proteins that remain undetectable in serum.

2.7.6 Epigenetic Changes

Methylation

In the human genome, regions rich in CpG dinucleotides are referred to as CpG islands. Methylation of cytosines in CpG dinucleotides within CpG islands in gene promoters leads

to gene silencing (Portela and Esteller, 2010). Methylation is a key mechanism for regulation of transcriptional activity. Methylation of HBV DNA represents a novel epigenetic mechanism that impairs HBV proteins, HBV replication, and HBV virion production, leading to occult HBV infection. Nearly 2 decades ago it was demonstrated that HBV DNA integrated into the host genome is methylated. Methylation of HBV DNA encoding the HBV core protein leads to loss of HBV core protein in PLC/PRF/5, a human hepatoma cell line with integrated HBV DNA sequences (Miller and Robinson, 1983). It was generally accepted that only integrated HBV DNA sequences are methylated. However, the observation of chromatin-like minichromosomes during replication prompted the search for CpG islands in episomal DNA, and three were recently identified (Vivekanandan *et al.*, 2008a). Interestingly, key regulatory elements of the HBV genome were located within or adjacent to the 3 CpG islands (Vivekanandan *et al.*, 2008a). Episomal HBV DNA from human liver tissue and from cell culture can be methylated. Of note, methylation of CpG island 2 in the HBV genome is frequently detected in occult HBV infection (Vivekanandan *et al.*, 2008a). Hepatitis B virus cccDNA is frequently methylated in human liver tissues (Vivekanandan *et al.*, 2009). Transfection of *in vitro*-methylated HBV DNA constructs into hepatocyte cell lines was associated with a >90% decrease in secreted HBsAg. In addition, HBeAg and HBcAg expression was markedly reduced by methylation of HBV, clearly demonstrating the role of CpG islands in regulating HBV gene expression (Vivekanandan *et al.*, 2009).

Hepatitis B virus replication in cell culture induced the expression of DNA methyltransferases (DNMTs), enzymes vital for DNA methylation. The HBV-induced DNMTs could methylate HBV DNA, resulting in the inhibition of HBV transcription and HBV replication (Vivekanandan *et al.*, 2010). Hypermethylated HBV DNA sequences are frequently detected in HCC patients with occult HBV infection (Kaur *et al.*, 2010). Methylation of cccDNA is associated with low serum HBV DNA levels and decreased virion production in patients with liver cirrhosis (Kim *et al.*, 2011). The association between HBeAg and high virus loads is well known (Chu *et al.*, 2003). It was recently demonstrated that a higher ratio of methylated cccDNA to total cccDNA is detected in HBeAg-negative individuals than that in HBeAg-positive individuals. In addition, methylation of cccDNA correlated with reduced HBV replication (Guo *et al.*, 2009). Recent

studies have demonstrated a pivotal role for HBV methylation in occult HBV infection. However, additional studies will throw more light on the role of this recently identified epigenetic mechanism in occult HBV infection.

Acetylation

The role of acetylation of histones bound to HBV DNA in regulating HBV replication and transcription has been convincingly demonstrated in several studies. In the mid-1990s, Newbold *et al.*, (1995) discovered that the hepatitis B viral nucleoprotein complex is arranged in the form of a minichromosome composed of nucleosomes, suggestive of transcriptional regulation of HBV DNA. Hyperacetylation of cccDNA-bound histones is associated with increased HBV replication in cell culture. In the presence of histone deacetylase inhibitors (valproic acid or trichostatin A), high HBV transcript levels and increased HBV replication are correlated with an increase in acetylated histones bound to cccDNA. Furthermore, acetylation of H3 and H4 bound to cccDNA in liver tissues from patients with chronic hepatitis B virus infection correlated with serum HBV DNA levels. Hypoacetylation of histones bound to cccDNA seen in liver tissue from patients with low virus loads is linked to recruitment of histone deacetylase (Pollicino *et al.*, 2006). Subsequently, the recruitment of HBx protein to the cccDNA minichromosome along with histones has been demonstrated (Belloni *et al.*, 2009).

Interestingly, an HBx mutant with a single nucleotide substitution is associated with rapid hypoacetylation of histones bound to cccDNA, impairing recruitment of p300, a transcriptional coactivator. The HBx mutant was associated with reduced HBV pregenomic RNA and reduced HBV replication, suggesting a key role for the HBx protein in regulating HBV replication. Recently, remodelling of the HBV minichromosome by phosphorylation and methylation of histones has been shown to regulate HBV replication (Gong *et al.*, 2011). However, this recently reported mechanism and its potential role in occult HBV infection have not yet been investigated by use of molecular epidemiological studies.

2.7.7. Apolipoprotein B mRNA Editing Enzyme Catalytic Polypeptide and Occult HBV Infection

The physiological function of apolipoprotein B mRNA-editing enzyme catalytic polypeptides (APOBECs) is cytidine deamination (Teng *et al.*, 1993). The role of

APOBECs in inhibiting and editing HIV replication has been well studied (Holmes *et al.*, 2007; Holmes *et al.*, 2007). Subsequently, the ability of APOBEC3G to inhibit HBV replication was demonstrated. The expression of APOBEC3G in cells replicating HBV resulted in a 50-fold reduction in HBV DNA levels (Turelli *et al.*, 2004). Both deamination-dependent and deamination-independent mechanisms of inhibition of HBV replication have been reported for APOBECs.

Deamination-Dependent Inhibition of HBV Replication

Initially it was believed that only the cytidines in the minus strand of the HBV genome are edited, which is reflected as G-to-A mutations in the plus strand of HBV (Noguchi *et al.*, 2005). However, Suspene *et al.*, (2005) demonstrated that the plus strand of HBV is also deaminated by APOBEC enzymes. The overexpression of APOBEC3G is associated with hyperedited HBV genomes and a reduction in the replicative intermediates of HBV. Regions of the HBV genome encoding the surface proteins, polymerase, and the HBx protein are hyperedited by APOBECs (Noguchi *et al.*, 2005). Normal human livers express low levels of APOBEC proteins (Bonvin *et al.*, 2006). Several APOBEC3 genes are upregulated in HBV-related cirrhotic livers compared to cirrhotic tissue from alcoholic liver disease (Vartanian *et al.*, 2010).

It has been demonstrated that up to 35% of the HBV genomes in the liver may be edited by APOBECs (Vartanian *et al.*, 2010). Of note, common mutations leading to the loss of HBeAg (G1896A) and lack of detectable HBsAg (sG145R) result from G-to-A mutations; these mutations have also been linked to APOBEC editing of HBV (Vartanian *et al.*, 2010). APOBEC hyperedited sequences have been reported from both occult and nonoccult cases of chronic HBV infection (Vivekanandan *et al.*, 2008b). It remains clear that the majority of HBV genomes in occult HBV infection are not hyperedited (Vivekanandan *et al.*, 2008b). However, minimal editing by APOBEC proteins, for example, the sG145R mutation that results due to a G-to-A mutation, is sufficient to cause occult HBV infection. Therefore, APOBEC-mediated occult HBV infection can occur in the absence of extensive HBV DNA editing. A growing body of literature in the last few years has demonstrated the role of APOBEC proteins in noncytolytic clearance of HBV infection. An increase in serum ALT levels is linked to an increase in hyperediting of the HBV genome, loss of HBeAg, and a

reduction in HBV DNA levels, suggesting that hyperediting and the associated reduction in virus loads represent a strong host immune response (Noguchi *et al.*, 2009).

Deamination-Independent Inhibition of HBV Replication

Although deamination-inactive APOBEC3G lacked the ability to hyperedit the HBV genome, it still retained the ability to inhibit HBV replication (Noguchi *et al.*, 2007), suggesting the presence of deaminase-independent mechanisms inhibiting HBV replication. Rosler *et al.*, (2005) reported increased nuclease susceptibility of HBV core-protein-associated pregenomic RNA. In another study, Nguyen *et al.*, (2007) demonstrated the inhibition of HBV reverse transcriptase (RT) activity by APOBEC3G. They also showed that APOBEC3G inhibited early stages of HBV DNA synthesis by targeting HBV DNA-RNA hybrids and single-stranded HBV DNA. APOBEC3G copellets with HBV capsids, and encapsidation of APOBEC3G by replication-competent HBV nucleocapsids renders it resistant to proteinase K digestion. Further, it has been suggested that multiple copies of APOBEC3G may be packaged into HBV nucleocapsids by its interaction with HBV RT and the HBV RNA packaging signal (ε) (Nguyen *et al.*, 2007).

APOBEC proteins bind to the HBV core protein and hinder HBV capsid formation (Baumert *et al.*, 2007b). Apart from APOBEC3G, other APOBEC proteins, including APOBEC3F and APOBEC3B, can inhibit HBV replication (Bonvin *et al.*, 2006). In addition to inhibiting HBV replication, APOBEC3B can inhibit secretion of both HBsAg and HBeAg. The mechanisms underlying APOBEC-mediated inhibition of HBV proteins remain unclear, but interference with protein synthesis or processing has been suggested as a potential mechanism (Bonvin *et al.*, 2006; Zhang *et al.*, 2008). While the inhibitory mechanisms of APOBEC proteins that could produce occult infection are being increasingly recognized *in vitro*, large-scale studies comparing patients with and without occult infection are required to confirm and better understand the role of these proteins *in vivo* (Samal *et al.*, 2012).

2.8 EPIDEMIOLOGY AND RISK FACTORS OF HEPATITIS B VIRUS INFECTION

2.8.1 General Epidemiology

The prevalence of chronic HBV infection varies greatly in different part of the world. The prevalence of chronic HBV infection worldwide could be categorized as high (>8%), intermediate (2- 7%) and low (<2%) endemicity (Hou *et al.*, 2005). The age at the time of infection is associated with the endemicity of HBV infection.

High Endemicity

The prevalence of HBV infection varies markedly throughout regions of the world (Margolis *et al.*, 1991). Hepatitis B is highly endemic in developing regions with large population such as South East Asia, China, sub-Saharan Africa and the Amazon Basin, where at least 8% of the population are HBV chronic carrier. In these areas, 70–95% of the population shows past or present serological evidence of HBV infection. Most infections occur during infancy or childhood. Since most infections in children are asymptomatic, there is little evidence of acute disease related to HBV, but the rates of chronic liver disease and liver cancer in adults are high (Alter, 2003). Sub-Saharan Africa, Asia, the Pacific, the Amazon and southern part of eastern and central Europe are areas of high endemicity with the prevalence rate of above 7% (WHO, 2004).

Intermediate Endemicity

Hepatitis B is moderately endemic in part of Eastern and Southern Europe, the Middle East, Japan, and part of South America. Between 10–60% of the population have evidence of infection, and 2-7% is chronic carriers. Acute disease related to HBV is common in these areas because many infections occur in adolescents and adults. However, the high rates of chronic infection are maintained mostly by infections occurring in infants and children (Toukan, 1990). In these areas, mixed patterns of transmission exist, including infant, early childhood and adult transmission.

Low Endemicity

The endemicity of HBV is low in most developed areas, such as North America, Northern and Western Europe and Australia. In these regions, HBV infects 5–7% of the population, and only 0.5–2% of the population is chronic carriers (Alter, 2003). In these areas, most HBV infections occur in adolescents and young adults in relatively well-defined high-risk

groups. These groups include injection drug user, homosexual males, and health care workers, patients who require regular blood transfusion or hemodialysis. Chronic infection varies from less than 1 % in USA and Western Europe to 5% in the Indian subcontinent and Middle East (WHO, 2004). Chronic infection with HBV occurs in 90% of infants infected at birth, 30% of children infected at 1-5yrs and 6% of persons infected above 5years (CDC, 2003). Thus there is an inverse relationship between chronic infection and age due to maturation of the immune system.

In Nigeria, many studies have been done on prevalence of HBV infection. In Awka, Ezegbudo *et al*., (2004) found that the prevalence of HBsAg among pregnant women decreases with increasing social status. Mustapha and Jibrin (2004) and Siresena *et al.*, (2002) in Gombe and Jos respectively found that having multiple sex partners increased the carriage of HBsAg. Ola *et al*., (1994) in Ibadan found that 57.1 % of patients with primary liver cell carcinoma were positive for HBsAg. Also in Ibadan, Olubyide *et al.*, (1997) found that a high (39 %) prevalence of HBsAg infection was associated with Surgeons and Dentists, with a high potential of transmissibility. They speculated that it was due to lack of vaccination and infrequent application of universal precaution. In Benin, Obiaya *et al.*, (1982) in their study noted that blood transfusion was hazardous in view of the high prevalence of HBsAg infection in donor blood.

Multimer *et al*., (1994) found that blood transfusion clearly increased the risk of HBV infection as shown by significantly higher markers of HBV infection (HBsAg and anti HBc) in subjects who were transfused. Abiodun *et al.*, (1986) in Benin observed that HBV infection increased with increasing units of blood transfused. Agumadu and Abiodun (2002) studying 213 children with sickle cell anaemia, showed that markers of HBV infection (HBsAg and anti HBc) increased with age. Amazigo and Chime (1990) in Eastern Nigeria found that HBsAg carriage and exposure rate to HBV were significantly higher in rural than in urban populations. This was attributed to overcrowding and clustering. They also demonstrated that by 40 years of age 87% of indigenous population of Eastern Nigeria has at least one HBV marker in their serum. Most of studies done in Nigeria on HBsAg did not find significant differences between male and female subjects (Kaine and Okafor, 1983; Abiodun and Omoike 1990). The increasing surface antigenaemia with age has been demonstrated by several workers in Nigeria (Agumadu and Abiodun 2002; Chukwuka *et*

al., 2003). Amazigo and Chime (1990) found a significantly higher HBsAg prevalence among prisoners in Eastern Nigeria, which was attributed to overcrowding and clustering. Recent studies on HBsAg prevalence in Jos (Ukaeje *et al.*, 2005) and Gombe (Mustapha and Jibrin 2004) among patients with human immune deficiency syndrome (HIV) showed a prevalence of 25.9% and 26.5% respectively. These high values could be because HIV and HBV share similar modes of transmission and risk factors.

2.8.2 Prevalence of Occult Hepatitis B Virus Infection

Occult hepatitis B is defined by the presence of HBV DNA in serum or liver in the absence of HBsAg (Samal *et al.*, 2012). Although occult HBV infection has been identified in patients with chronic liver disease two decades ago, its precise prevalence remains to be defined. Occult HBV infection has been found in patients with HCC, past HBV infection, or chronic hepatitis C, and individuals without HBV serological markers. The frequency of the diagnosis depends on the relative sensitivity of HBV DNA assays and the prevalence of HBV infection in the population.

Collectively, around 30% to 35% of HBsAg-negative subjects with chronic hepatitis with or without HCC have positive serum HBV DNA (range from 5% to 55%). The prevalence of HBV DNA is higher in anti-HBc-positive, but anti-HBs-negative patients, ranging from 7% to 60% in populations highly exposed to HBV (Brechot *et al.*, 2001). HBV DNA is much less frequently identified in HBsAg-negative patients with acute and particularly fulminant hepatitis at around 10% and 7% in serum and liver samples (Brechot *et al.*, 2001). Viral DNA persistence is not, however, restricted to patients with liver disease and may be observed in subjects with normal liver parameters, including blood and/or organ donors. Overall, occult HBV infection is seen in 7%-13% of anti-HBc-positive and/or anti-HBs-positive subjects, and in 0% to 17% of blood donors. The clinical significance of occult HBV infection remains unclear. Occult HBV infection represents a potential transmission source of HBV via blood transfusion or organ transplantation. In addition, occult HBV infection has been associated with cryptogenic chronic hepatitis and hepatocellular carcinoma. Furthermore, some studies suggested that occult hepatitis B might affect responsiveness of chronic hepatitis C to interferon therapy and disease progress. The prevalence of occult HBV is unclear and depends in part on the sensitivity of

the HBsAg and DNA assays used as well as the prevalence of HBV infection in the study population (Schmeltzer and Sherman, 2010).

Occult Hepatitis B virus infection varies significantly between different geographical regions (Hollinger, 2008). Studies have shown that the prevalence of occult HBV infection is closely related to the endemicity of HBV infection (Brechot *et al*., 2001; Zervou *et al.*, 2001 Hollinger, 2008). Patients from countries highly endemic for HBV are more likely to develop occult HBV infections (Chemin and Trepo, 2005). As in highly endemic countries, the majority of infections are contracted perinatally or in early childhood; a higher proportion of the infected adults have late chronic HBV with undetectable HBsAg. This may account for the higher rate of OHB in anti-HBc-positive populations in these areas (Liu *et al.*, 2006a). Prevalence may also vary depending on the nature of biological material tested, with a higher proportion for liver compared to serum specimens (Chemin and Trepo, 2005). Occult HBV infection has been reported in 0.1%-2.4% of HBsAg-negative, anti-HBc-positive (± anti-HBs) blood donors in Western countries such as the United States, where only 5% of the population has prior exposure to HBV, and in up to 6% of a similar cohort of donors who reside in endemic areas where 70%-90% of the population has been exposed to HBV (Hollinger, 2008; Hollinger and Sood, 2010). When anti-HBc only data is evaluated, the rates range from 0% to 15%, median of 1.1% (Hollinger, 2008).

2.9 LABORATORY METHODS FOR DIAGNOSIS OF OCCULT HEPATITIS B VIRUS INFECTION

Evaluation of different diagnostic techniques is important in detection and management of occult HBV infection. Most OBIs are asymptomatic and would only be detected by systematic screening of large populations (Allain, 2009). No published guidelines are provided up till now, categorizing those who should be screened for OBI. However, such investigations should be considered in the following situations:

- HCV-infected patients with flares in viral replication and liver damage (Kannangai *et al.*, 2007).
- Infected patients becoming immune deficient mainly by receiving immunosuppressive regimens for various clinical conditions (Allain, 2009).

- Screening of blood donations for immunocompromised recipients (Said *et al*., 2009).
- Subjects with unexplained liver diseases.

Candotti and Allain (2009) further clarified that OBIs are mainly found in older donors, nearly 100% carry anti-HBc, and approximately 50% also carry anti-HBs, suggesting that OBIs occur largely in individuals having recovered from the infection but unable to develop a totally effective immune control (Candotti *et al*., 2008).

2.9.1 Liver Biopsy

Detection of HBV DNA in liver biopsy is the best way for diagnosis of OBI. However, liver biopsy tissue is not always available as it requires invasive procedure, and standardized and valid assays for detection of HBV DNA in liver tissue are not FDA approved (Hollinger and Sood, 2010). A recent Italian study investigated the prevalence of occult HBV in the general population by examining 98 liver specimens from liver disease-free individuals who were HBsAg-negative, and detected HBV DNA in sixteen of them (16.3%); 10/16 (62.5%) were anti-HBc positive (Raimondo *et al*., 2008a).

2.9.2 HBsAg Testing

Chronic HBV infection is generally ruled out in the absence of detectable HBsAg in the serum. Differences among commercial assays in their ability to detect HBsAg associated with mutations in the "a" determinant are well recognized. Assays using polyclonal tracer antibodies for the detection of HBsAg vary in their ability to detect mutant HBsAg. However, assays using a polyclonal tracer antibody perform better than assays using a monoclonal tracer for the detection of mutant HBsAg (Coleman, 2006). Furthermore, HBsAg assays differ in their lower limits of detection. Highly sensitive commercial assays for HBsAg that consistently detect frequently encountered "a" determinant mutants (Coleman *et al.*, 1999; Coleman, 2006) should be preferentially used for HBsAg testing. The main target for antibodies used in diagnostic tests is the major hydrophilic loop (MHL, amino acids 100-160) that contains the "a" determinant (amino acids 124-147) and is coded by the envelope (S) gene. The existence of mutations in this region could cause diagnostic

failure (Katsoulidou *et al.*, 2009). Current HBsAg screening assays are enzyme immunoassays (EIAs), including enzyme-linked immunosorbent assays (ELISAs), and chemiluminescence immunoassays (CLIAs) (Candotti and Allain, 2009). These different assays have sensitivity ranging between < 0.1 and 0.62 ng of HBsAg per mL (1 ng/mL corresponds to approximately 2 IU/mL) (Biswas *et al.*, 2003; Scheiblauer *et al.*, 2006). Performance of commercial assays would be improved by the incorporation of OBI mutants in reagent development (El chaar *et al.*, 2010). The course of HBV markers during the early phase of true OBI is not well known. In spite of transient strong HBV replication, much less HBsAg in the serum than the normal courses is shown (Bremer *et al.*, 2009). This has been previously confirmed in a Japanese study by Yoshikawa *et al*., (2007), where 17 million donations were tested for occult infection and 328 HBV DNA-positive donations were found. From 26 of these donors, sequential samples were examined for the dynamics of viral markers in acute HBV infection. Six of the 26 donors were infected with mutant viruses, and 3 of these 6 donors did not develop detectable HBsAg during the entire observation period, despite a moderately high viral load of 10^4 to 10^5 HBV DNA copies per mL. The authors concluded that HBV nucleic acid amplification test (NAT), even in minipool (MP) configuration, is more effective than HBsAg testing. This is also capable of excluding infected donors in the pre- and post-HBsAg window periods (Yoshikawa *et al.*, 2007).

A novel immunoassay that detects simultaneously HBV PreS1 and/or core-related antigens was developed and evaluated for its potential value for detecting HBsAg variants. The detection limits of the assay were 10 (2.9 ± 0.5) copies/mL (mean ± SD) for HBsAg-positive sera with different genotypes, and 10 (3.5 ± 1.2) copies/mL for HBsAg variants containing sera. The specificity of the assay was 99.9% (95% CI: 99.7-99.9, 4551 healthy individuals). The sensitivities were 93.9% (95% CI: 92.8-94.9), 59.3% (95% CI: 38.7-77.6) and 80% (95% CI: 44.4-97.5) in three independent groups which included: 2065 hepatitis patients, 27 patients with OBI and 10 HBsAg variants, respectively. In addition, a novel premature stop code mutation at position 112 of HBsAg was observed in two patients with chronic hepatitis B with different genotypes (Yuan *et al*., 2010).

2.9.3 Anti-HBc Testing

Different screening methods are used across the world for screening blood and blood products (Kuhns and Busch, 2006). A good proportion of blood donors with occult hepatitis B virus infection have anti-HBc as the only serological marker of HBV infection (Katsoulidou *et al.*, 2009). Blood donors with isolated anti-HBc status are more infectious than those with low titers of anti-HBs (Allain and Cox, 2011). However, high rejection rates in areas of high HBV endemicity preclude anti-HBc screening of blood donors (Chaudhuri *et al.*, 2003; Chaudhuri *et al.*, 2004). Serological profiling of HBV infection showed that OBI may be antibody (anti-HBc alone or together with anti-HBs) positive (seropositive OBI) or antibody negative (seronegative OBI) (Raimondo *et al.*, 2010).

The HBV DNA detection rate is highest in subjects who are anti-HBc-positive but anti-HBs-negative, and these individuals are more likely to be infectious (Hollinger and Sood, 2010). Recently, Urbani *et al.*, (2010) illustrated that the serological assay for the long-lasting antibody response to the highly immunogenic HBV core antigen (anti-HBc) represents a qualified candidate as a surrogate for DNA amplification, or for increasing overall sensitivity when assessing the risk of occult hepatitis in peripheral blood. The risk of occult hepatitis associated with anti-HBc seropositivity has been demonstrated extensively, and the presence of antibody response to HBc can be considered a sentinel marker of occult HBV infection (Urbani *et al.*, 2010). In a study conducted by Candotti and Allain, (2009), it was emphasized that approximately 90% of blood donors carrying anti-HBc also carry anti-HBs, indicating recovered HBV infection (Allain *et al.*, 2003). The remaining 10% are either false-positive anti-HBc due to poor assay specificity and the lack of confirmatory assays, or true anti-HBc (anti-core antigen alone) (Roth and Seifried, 2002; Kleiman *et al.*, 2003). Anti-HBc only samples may originate either from recovered infections having lost detectable anti-HBs or from late stage chronic infections having lost detectable HBsAg (Candotti and Allain, 2009). Recent studies have confirmed the existence of occult HBV infection in samples with anti-HBc alone (Huang *et al.*, 2010; Panigrahi *et al.*, 2010). Nevertheless, low levels of HBV DNA were reported not only in anti-HBc alone positive blood donations but also in some blood units carrying low-level anti-HBs (Candotti and Allain, 2009). A serologic testing algorithm with anti-HBc followed by anti-HBs (anti-

HBs ≥ 100 IU/L probably non-infectious) or implementation of highly sensitive HBV DNA screening are adopted in different countries; however, this is still an area of debate by many authors. Recent study has shown that OBI was detected in blood units from healthy volunteer blood donors showing adequate level of anti-HBs (Hou *et al.*, 2005). OBI is observed in anti-HBc-positive patients with chronic HBV infection following the decline of HBsAg to an undetectable level that is sometimes associated with the appearance of anti-HBs. This serological pattern occurs at a rate of 0.7%-1.3% per year and is associated with older age and hepatitis B e antibody (anti-HBe) reactivity (Gigi *et al.*, 2007; Hollinger and Sood, 2010). In an experimental study to determine the relationship between anticore detection and the molecular status of virus replication in a primary woodchuck hepatitis virus (WHV) surface antigen (WHsAg)-negative infection or long after resolution of WHV hepatitis, it was shown that the long-term presence of anticore antibodies alone is a consequence of sustained restimulation of the immune system by virus nucleocapsid produced during low-level hepadnaviral assembly (Coffin *et al.*, 2004). On the other hand, it was shown that about 20% of OHB sera are negative for all serological markers of HBV infection except HBV DNA (Hollinger and Sood, 2010).

2.9.4 HBV Nucleic Acid (DNA) Testing by PCR

Detection of HBV DNA from serum or liver samples is considered the gold standard for the diagnosis of occult HBV infection (Urbani *et al.*, 2010). At present, the optimal standard for diagnosis is the analysis of HBV DNA extracts from plasma performed by real-time or nested polymerase chain reaction (PCR) techniques (Hollinger and Sood, 2010). Experts have recently recommended the use of highly sensitive nested PCR or real-time PCR assays that can detect fewer than 10 copies of HBV DNA for the diagnosis of occult HBV infection. In addition, testing for multiple targets on the HBV genome increases HBV DNA detection rates (Hassan *et al.*, 2011) in patients with occult HBV infection. False results of these assays could be avoided by choosing PCR primers that span at least three genomic regions of the HBV genome such as the S, X and core genes, and validation should require detection from at least two regions of the genome (Raimondo *et al.*, 2008b). Unfortunately, this suggestion is not usually fulfilled, and only one segment of a region is amplified. The preferred lower limit of detection (LLOD) for HBV DNA is 5 IU/mL (Hollinger and Sood,

2010). Some investigators prefer to repeat extraction and testing under the assumption that according to Poisson distribution, repeated testing increases the chances of detecting a low number of template sequences (Allain, 2009). Intermittent viremia can occur in occult HBV infection, and periodic testing of HBV DNA will improve detection of occult HBV infection (Kannangai *et al.*, 2007). Periodic sampling for HBV DNA testing may be particularly appropriate for clinical groups at risk for occult HBV infection. When available, testing of a liver biopsy specimen for HBV DNA will further augment the diagnosis of occult HBV infections, as HBV DNA is frequently detected in the liver in the absence of HBV DNA in the serum (Hassan *et al.*, 2011). Individuals with occult hepatitis B virus infection can potentially transmit the infection through blood transfusions (Levicnik-Steznar *et al.*, 2008).

Occult HBV infection is a major cause of post transfusion hepatitis B (Lledo *et al.*, 2011). The prevalence of chronic HBV infection and availability of resources are major determinants of the screening methods used for HBV. For example, HBsAg with anti-HBc (total) and/or NAT are used for screening in resource-rich areas with a low prevalence of chronic HBV infection (Ocana *et al.*, 2011). Although NAT testing for HBV DNA will reduce the risk of HBV transmission, its cost-effectiveness is still being questioned (Jackson *et al.*, 2003). The suitability of anti-HBc testing and NAT for screening blood and blood products is determined by (i) endemicity of HBV infection, (ii)) rates of anti-HBc detection in the population, and (iii) availability of resources. Nucleic acid testing (NAT) for HBV DNA detection that combines simultaneous detection of human immunodeficiency virus (HIV) RNA, HCV RNA, and HBV DNA (‘‘multiplex” NAT assays) and use of an automated testing platforms have made HBV NAT blood screening feasible (Candotti and Allain, 2009). In order to standardize these newly developed assays, the World Health Organization International Standard for hepatitis B virus DNA (NAT)-based assays was created (code 97/750) with a potency of 10^6 IU/mL (500 000 IU/vial) (Baylis *et al.*, 2008). Biswas *et al.*, (2003) showed that pooled-sample NAT would reduce the WP by 9 to 11 days; and single-sample NAT would reduce the WP by 25 to 36 d, compared to currently licensed HBsAg tests (Biswas *et al.*, 2003). This leaves WPs of 40-50 d and 15-34 d with minipool (MP) and individual donor (ID) HBV NAT, respectively (Candotti and Allain 2009). As emphasized by Candotti and Allain (2009) the ability of

NAT to reduce the WP depends not only on the sensitivity of both the molecular and serological tests, but also on the sample volume (200 or 500 μL) as well as the dilution factor introduced by pooling samples, the prevalent HBV genotype at the location and the level of HBV endemicity (Biswas *et al.*, 2003; Allain, 2009). Beyond shortening the WP, NAT screening, particularly in individual units, has uncovered a relatively large number of HBsAg-negative ‘‘occult” HBV infection or carriage (Allain, 2004b; Hollinger, 2008). Occult Hepatitis B virus Infection (OBI) is usually characterized by very low HBV DNA load in plasma < 200 IU/mL (Candotti and Allain, 2009). Detection of OBI requires assays of the highest sensitivity and specificity with a lower limit of HBV DNA detection of less than 10 IU/mL and < 0.1 ng/mL for hepatitis B surface antigen (HBsAg) (Hollinger and Sood, 2010). Regarding estimation of HBV residual transfusion transmission risk, Candotti and Allain (2009) in their recent review clarified that HBV DNA yield appears directly related not only to the analytical sensitivity and serum pool size used for the HBV NAT assay, but also to the analytical sensitivity of the HBsAg test used for screening and to the general HBV prevalence in the donor population. They further added that HBV NAT yields reported from countries with low, moderate, and high HBsAg prevalence range between 1:4 000 and 1:730 000 (Kleiman *et al.*, 2005; Stramer, 2007), 1:4000 and 1:20 300 (Brojer *et al.*, 2006; Manzini *et al.*, 2007), and 1:192 and 1:5200 (Li *et al*., 2008; Vermeulen *et al.*, 2009), respectively.

2.9.5 Role of Anti HBs Testing

Occult HBV carriers without detectable antibodies to the surface antigen could be infectious (Katsoulidou *et al.*, 2009). Indeed, Candotti and Allain (2009) emphasized that the presence of anti-HBs following natural infection, vaccination, or passive immunoprophylaxis prevents *de novo* HBV infection in transplanted patients receiving anti-HBc positive livers (Roche *et al*., 2003; Barcena *et al.*, 2006). Experiments in chimpanzees showed no HBV infection in animals transfused with blood from three anti-HBs positive human plasma samples, despite exposure to an HBV DNA dose known to be infectious in the absence of anti-HBs (Prince *et al.*, 2001). However, it has been reported by many authors that among individuals positive for anti-HBs, 0.5%-15% still tested positive for serum HBV DNA, though at a very low titer (Matsumoto *et al.*, 2001; Liu *et al*., 2006b).

Countries such as Germany, Austria and Japan allow transfusion of units with anti-HBs titers higher than 100 IU/L (Minuk *et al*., 2005).

2.9.6 Non Specific Tests- Liver Function Tests

These tests are useful for assessing liver damage/ function but do not distinguish between various forms of hepatitis. In acute phase or in exacerbation of chronic aggressive hepatitis, the level of alanine aminotransferase (ALT) and other liver enzymes are raised and prothrombin is depressed. Serum bilirubin is also increased (Collier and Oxford, 2000). Studies have shown that the liver enzymes are not elevated in occult hepatitis B virus infection (WHO, 2009a).

2.10 PREVENTION AND CONTROL OF HEPATITIS B VIRUS INFECTION

Prevention of HBV infection can be simply divided into primary, secondary and tertiary prevention. Primary prevention includes those activities that are intended to stop the onset of the disease (Gupta and Ghai, 2007). The classic example of primary prevention is immunization with vaccines. Vaccination against hepatitis B Virus (HBV) in West African nation of Nigeria is lower than many Sub-Saharan African countries (Musa *et al.*, 2015). In addition to HBV vaccines for immunization, health education and health promotion are important tools to mitigate HBV transmission in the community. Health education aims at informing the population on various modes of HBV transmission and risk factors so that people will take adequate preventive measures to avoid HBV infection.

Secondary prevention is simply arresting the progression of established disease. It refers to techniques that find health problems early in their course so that action can be taken to minimize the risk of progression of the disease in individuals or the risk of communicable illnesses will be transmitted to others (Gupta and Ghai, 2007). Tertiary Prevention is focused on rehabilitation in an effort to prevent the worsening of an individual's health in the face of a chronic disease like HBV disease. Basically there are five main strategies available for the prevention and control of HBV infection. They include;

- Behavior modification to prevent disease transmission
- Interrupting transmission
- Passive Immunoprophylaxis

- Active Immunization, and
- Treatment of infected persons with antiviral drugs.

2.10.1 Behavior Modification

Behavioral HBV transmission prevention strategies are those strategies designed to induce and promote behavioral changes within individuals and communities through a variety of educational and motivational approaches. Behavioral interventions are based on the observation that behavior change can be encouraged by raising greater awareness of risks and benefits associated with certain behaviors. This is done in conjunction with increasing access to the necessary equipment or materials e.g. Condoms to support the desired behavior change. This education is provided in various formats including mass media dissemination, entertainment (e.g. the theater), school-based sex education and peer education. Changes in sexual practice and improved screening measures of blood products have reduced the risk of transfusion-associated hepatitis. Behavior modification is thought to be more beneficial in developed countries than in developing countries, where neonates and children in early childhood are at the greatest risk of acquiring infection. In this group, immunoprophylaxis, both passive and active, will be more effective (Hou *et al.*, 2005).

HBV infection in Nigeria can be prevented or drastically reduced through health education of the general population on the various mode of transmission of HBV and preventive measures (Sirisena *et al.*, 2002). Such measures include careful handling of blood and body fluids by health care workers since they are potentially infectious. Also discouraging communal sharing of blade/sharp instruments used for shaving, barbing, manicure and body piercing/cutting and high level sexual networking (Househam, 1999). Prechewing of solid for children by an adult, especially those at risk for HBV infection should be discouraged because saliva is known to transmit HBV (Immunization Action Coalition, 2005). World Healh Organization recommends universal screening of blood and plasma for HBsAg by sensitive method before transfusion to recipients. Even when all blood donations are screened for HBsAg, donations from volunteered non- remunerated donors have been proved to be safest (WHO, 2009b). About 2 out of 1000 units screened plasma donations, negative for HBsAg using a very sensitive test are still infectious because the sensitivity of the third generation test is not 100%. Addition of a low dose hepatitis B immunoglobulin to

potentially infectious plasma appears to be reliable measure to eliminate the hepatitis B transmission. This is preferred to other methods for labile plasma derivatives. It has been advocated that only donations from immunized donors with a detectable amount of anti-HBs should be collected either for transfusion or for preparation of plasma derivative. Pasteurization of plasma derivatives like albumin, factors iii and viii at 60°C for at least 10 hours is essential for the elimination of HBV (Brummechuis *et al.*, 1983; Alter, 2003). In Nigeria most of these control measures, are poorly observed (Olubuyide *et al.*, 1997; Sirisena *et al.,* 2002), safe blood for transfusion are not easily accessible (Multimer *et al.*, 1994). Socio-economic and living condition of most Nigerians encourage transmission of HBV (Amazigo and Chime, 1990). Because of risks of blood transfusions, it should be given only when it is absolutely necessary as it was said that most blood transfusions were not necessary (Obiaya and Ebohom, 1982).

2.10.2 Interrupting Transmission

Studies have shown that interrupting transmission is a powerful mode of prevention and control of HBV infection. This can be achieved by preventing entry of infectious materials into the body of susceptible person from a hepatitis B patient or HBV carrier (Gupta and Ghai, 2007). This can be accomplished by simple precautions while dealing with people, who are known or presumed to be infectious. Within the Health care setting, transmission can occur by;

- Accidental inoculation of infectious material, usually blood or plasma via contaminated needles.
- Visible or invisible skin lesions exposed to infectious material directly by splashing or indirectly via hand or inanimate object, and
- Contact of infectious material with mucous membrane by splashing or indirectly via the hand or inanimate object.

As a result of these, all blood and blood contaminated materials should therefore be handled carefully adopting WHO universal precautions. Instruments including dialysis machine should be cleaned and disinfected or sterilized before re-use. Health care workers should adopt hand washing procedures as stipulated by World Health Organization (WHO, 2009c).

Outside of Health care setting, precautions such as nose or ear piercing or scarification may transmit HBV, if proper precautions are not taken. Simple heating of the instruments over flame would eliminate the risk of HBV infection (Acheson, 2011).

2.10.3. Passive Immunoprophylaxis

Conventional immune globulin is not recommended for passive immunization against HBV infection. Hepatitis B immune globulin (HBIG) has been shown to be effective in pre and post-exposure prophylaxis (Gupta and Ghai, 2007). Hepatitis B Immune Globulin (HBIG) is a sterile solution of ready-made antibodies against hepatitis B. HBIG is prepared from human blood from selected donors who already have a high level of antibodies to hepatitis B and used in passive immunoprophylaxis. The main indication for the use of HBIG is for post-exposure prophylaxis in these four situations;

- Newborns of mothers infected with hepatitis B
- After needle stick exposure
- After sexual exposure
- After liver transplantation.

Hepatitis B Immune Globulin (HBIG) must be given as soon as possible after an accidental inoculation (ideally within 6 hours and not later than 48 hours) since the efficacy of HBIG in preventing the disease and the development of carrier state rapidly decreases with time (Gupta and Ghai, 2007). Administration of hepatitis B immune globulin at birth and repeated during the first year of life prevents the development of the persistent carrier state in infants born to HBV carrier mothers. Neonates born to virus carrier mothers should be given anti-hepatitis immunoglobulin (HBIG) (0.5ml IM, to be given within 24 hours of birth) and three doses of the vaccine at 0, 1-2 and 6 months of age. This is followed by post vaccine testing for HBsAg and anti-HBs at 9-15 months of age. Administer a second complete series of hepatitis B vaccine, if the child is negative for both HBsAg and anti-HBs (Gupta and Ghai, 2007). The combination of immunoprophylaxis and vaccine treatment results in a higher-than-90% level of protection against perinatal acquisition of HBV (Sevens *et al.*, 1979). Studies have shown that 3.7% to 9.9% of infants still acquire HBV infection perinatally from HBV infection mothers, despite immunoprophylaxis (Xu *et al.*, 1999; Xu *et al.*, 2001; Xu *et al.*, 2002). Failure of passive and active immunoprophyxis in

this setting may be the result of in utero transmission of HBV infection, perinatal transmission related to a high inoculum, and/or the presence of surface gene escape mutants. Hepatitis B immune globulin remains a central component of prophylaxis in HBV-infected patients undergoing liver transplantation.

Hepatitis B Immune Globulin monotherapy given at a high dosage can prevent recurrence in 65% to 80% of patients. Because the cost of long-term prophylaxis with high-dose HBIG is extremely high and combination therapy using HBIG with a nucleoside analog is more uniformly effective, the current protocol is combination HBIG with a nucleoside analog after liver transplantation. These combination protocols have reduced the rate of virologic breakthrough to 10% or less (Terranet and Vyas, 2003).

2.10.4 Active Immunization

Immunization is the most effective means of controlling HBV infection world-wide (Kire, 1993). The vaccine has an outstanding record of safety and efficacy, and it is 95% effective in preventing development of the chronic carrier state (WHO, 1998). In Africa, vertical transmission accounts for 1-5% of cases, (WHO,1998) while most children are infected with HBV between ages of 2-1l years through horizontal transmission, hence universal immunization at birth has been adopted. As cost effective measure it has been incorporated into WHO expanded programme on immunization (EPI) on global basis according to Yaounde declaration at the International conference on the control of HBV held in 1991. Babies born to HBsAg positive mother should be given hepatitis B immunoglobulin at birth and active immunization should commence immediately. Post exposure prophylaxis with hepatitis B immunoglobulin should be given promptly in all cases of suspected blood or body fluid inoculation as this could reduce HBV infection (Immunization Action Coalition, 2005).Prevention of primary infection by vaccination is an important strategy to decrease the risk of chronic HBV infection and its subsequent complications. The first-generation hepatitis B vaccine, an inactive plasma-derived vaccine, became available in 1982. Consequently, the second generation of HB vaccine, a DNA recombinant HB vaccine was also available for general use in 1986. Both of the vaccines are highly immunogenic and were proven to be safe and efficacious in preventing HBV infection with seroconversion rates of 96% reported after 3 doses (Gupta and Ghai, 2007). In 1991, the World Health

Organization (WHO) recommended that hepatitis B vaccination should be included in national immunization system in all countries with a hepatitis B carrier prevalence (HBsAg) of 8% or greater by 1995 and in all countries by 1997. By May 2002, 154 countries had routine infant immunization with hepatitis B vaccine (Lavanchy, 2004).

The world's first universal vaccination program for HBV infection was launched in 1984 in Taiwan (Ni *et al.*, 2001). During the first 2 years of the program, coverage was provided mainly for infants whose mothers were carriers of HBsAg. Vaccination was subsequently extended, first to all newborns and then to unvaccinated preschool-age and elementary school-age children. Since 1991, catch-up vaccinations have been given to children in the first grade. This program reduced the overall HBsAg prevalence rate from 9.8% in 1984 to 1.3% in 1994 among children <15 years of age. The HBV carrier population was further reduced through improved maternal screening (Chen *et al.*, 1996; Ni *et al.*, 2001).

Universal HB vaccination was proven to be effective in the prevention of HCC in several large cohort studies in Southeast Asia. Chang *et al.*, (1997) reported that the average annual incidence of HCC in children 6 to 14 years of age declined from 0.70 per 100,000 children between 1981 and 1986 to 0.57 between 1986 and 1990, and to 0.36 between 1990 and 1994 ($P<0.01$) in the first vaccinated cohort in Taiwan. The corresponding rates of mortality from Hepatocellular Carcinoma (HCC) also decreased. After universal vaccination against Hepatitis B in 1987 in Long'an, Guang Xi, a highly endemic area in Southern China, a birth cohort study was used to evaluate the efficacy of hepatitis B vaccination. The incidence of HCC dropped from 3.27/10,000 to 0.17/10,000, a 94.8% decrease, in the group of 0-19 year-olds. The average incidence of HCC in general population for the period from 1996 to 2002 dropped to 27.86/100, 000 from 48.18 for the period from 1969 to 1988 (Li *et al.*, 2004). The protective effect of HBV vaccination against liver cancer in adults was investigated in a cohort study in Korea. This study suggested that the immunization with Hepatitis B vaccine, even in adulthood, could reduce the risk of liver cancer (Lee *et al.*, 1998). The decrease in the rate of HCC after universal vaccination against hepatitis B provides further evidence that HBV is a cause of HCC. The effectiveness of routine infant hepatitis B immunization in significantly reducing or eliminating the prevalence of chronic HBV infection has been demonstrated in a variety of countries and settings. However, there are still many challenges to achieve the goal of

universal childhood immunization against hepatitis B, such as poor immunization delivery infrastructure, low coverage and lack of financial sustainability. Therefore, to continue to promote access to hepatitis B vaccines worldwide, great efforts are needed to support countries to ensure sustained funding for immunization programs.

2.10.5 Treatment of Infected Persons with Antiviral Drugs

Treatment with Antiviral drugs (lamivudine, adeforvir and dipivoxil) and immunostimulatory therapy with alpha-interferon for those that are affected is another way of prevention and control of HBV infection and disease (Levinson, 2014). The acute infection does not normally demand treatment, but the threat of an HBeAg positive carrier state demands immediate action (Bhatia and Ichhpujani, 2008). Some, at least of those who become carrier in adult life are naturally deficient in interferon, and some cures have been obtained with large doses of *IFN-α*. Treatment should be prolonged for at least six months. Unfortunately, it is of no use in those who became infected in infancy (Collier and Oxford, 2000). Studies have shown that various DNA polymerase inhibitors such as lamivudine and famciclovir may be useful in those receiving liver transplant, but reduces viraemia temporarily. Currently, six antiviral drugs are effectively used to treat chronic carriers of human hepatitis B virus. One is pegylated interferon, an immune modulator that works by stimulating cytokine synthesis and enhancing host antiviral responses. Lamivudine (3TC), a nucleoside analogue that was originally developed for HIV was also shown to be active against hepatitis B virus. It interferes with DNA polymerase activity of the P protein of HBV. Other effective nucleoside analogues that have been approved include entecavir, telbivudine, tenefovir and adefovir. Development of viral resistance against 3TC was initially a problem but a combination of drug approach alleviated this problem substantially. Antiviral treatments can reduce viral load to base levels in 48 weeks and are generally administered indefinitely to prevent reemergence of the virus (Acheson, 2011). Interferon can have some secondary effects such as nausea and fever, which makes this treatment uncomfortable. There is no still cure for individuals infected with hepatitis B virus, and the infection remains a major world-wide health problem, particularly because it can be a persistent, chronic infection that can eventually lead to a serious liver disease and hepatocellular carcinoma (WHO, 2009a). Chronic hepatitis can be controlled by nucleoside

inhibitors to a large extent, but treatments are expensive and only available in developed nations (Acheson, 2011). Availability of vaccines worldwide especially in poorer countries is still a major problem. More effective vaccines and antiviral agents are currently being developed by a number of laboratories and pharmaceutical companies.

2.11 GAPS OBSERVED IN THE LITERATURE

Many studies have been done on Hepatitis B virus infection all over the world including Nigeria. However, there is paucity of data on occult hepatitis B virus infection among blood donors at University of Abuja Teaching Hospital and Nnamdi Azikiwe University Teaching Hospital, Nnewi as these Teaching Hospitals rely on screening for only HBsAg marker and bleed blood donors based on its negativity. It is against this background, we sought to investigate the prevalence of occult HBV infection in these two teaching Hospitals, to enable us make evidence-based recommendations for effective HBV screening of blood donors to prevent HBV transmission from donors with OBI to recipients during blood transfusion.

The present study is therefore intended to characterize occult hepatitis B virus infection based on prevalence, viral markers, viral load and genotypes. Previous studies on occult HBV infection among blood donors in Nigeria were able to identify the prevalence and significance of anti- HBc marker in occult HBV infection. No study has been done in Nigeria to indicate whether the identified HBcAb is of IgG or IgM origin. This study is designed to identify the IgM anti-HBc and its association with occult HBV infection. Other gaps this study has identified and intend to fill that will make useful contribution to advancement of knowledge include;

- Determination of Alpha Feto Protein (AFP) and Alanine aminotransferase (ALT) levels among blood donors with occult and overt HBV infection.
- Determination of vaccination status of blood donors in the studied population.
- Association and impacts of some risk factors (previous blood transfusion, surgeries, domestic/occupational accident, multiple sex partners etc) on HBV transmission and infection.

- Identification of viral markers pattern that could be used as surrogate marker for occult HBV infection among blood donors.
- Determination of prevalence of occult HBV infection among HBV seronegative blood donors.
- Studies on blood values of PCV, HGB, WBC, RBC, Platelets and CD4 of blood donors with occult HBV infection compared with seronegative blood donors.
- Detection of HBV Genotypes among blood donors at UATH, Abuja and NAUTH, Nnewi and its clinical and epidemiological significance.
- Detection of coinfection of transfusion transmissible viral infectious agents such as HBV, HIV and HCV among blood donors' participants.
- The study also intends to perform PCR on some samples negative for HCV and HIV by rapid test/ELISA to determine the safety of blood units for transfusion at these two teaching Hospitals.

At the end of this study, knowledge will be added that will help to mitigate HBV and other viral agents that could be transmitted through Blood transfusion as well as minimize the prevalence of transfusion transmissible viral infections in Nigeria. Finally this study will generate ideas for further studies on Hepatitis B Virus infection, particularly molecular mechanisms leading to occult HBV infection.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 DESCRIPTION OF STUDY SITES

The study sites comprise of University of Abuja Teaching Hospital Blood bank and Nnamdi Azikiwe University Teaching Hospital, Nnewi Blood bank. University of Abuja Teaching Hospital (UATH) is situated in Gwagwalada in the Federal Capital Territory (FCT). It is about 50 kilometers from the city Centre. The Hospital is a tertiary health institution and referral Centre for the whole of the FCT and neighboring states like Kogi, Nasarawa, Niger and Kaduna States. University of Abuja Teaching Hospital, Gwagwalada is located in the North Central Geo-political zone of Nigeria. The Hospital was established in 1987 as a Specialist Hospital and subsequently upgraded to a Teaching Hospital in 2006. It has a bed capacity of over 350 with 14 clinical departments including Medicine, Surgery, Pediatrics, Obstetrics and Gynecology with full support from well-equipped laboratories. The laboratory has four major Departments, Microbiology & Immunology, Chemical Pathology, Haematology & Blood Bank and Histopathology & Cytology. The Blood Bank is a Unit of Haematology Department that has the responsibility of screening blood donors before donations for transfusion services. The Blood bank on the average screens and bleeds over 500 blood donors monthly.

Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi is located in the South East Geo-political zone of Nigeria. It is a tertiary health institution established in 1991. Nnamdi Azikiwe University Teaching Hospital is located at Nnewi and shares boundary with Nnobi, Ozubulu, Ukpor Orafite and Awka Etiti, all in Anambra State of Nigeria. It has several outstation clinics/health centers and serves as a major referral center for all these clinics and private hospitals in the State. Like any other Teaching Hospital, Nnamdi Azikiwe University Teaching Hospital is primarily established for specialized clinical service, teaching and research purposes.

3.2 STUDY DESIGN

The research is a descriptive, cross sectional, laboratory based study done at Nnamdi Azikiwe University Teaching Hospital, Nnewi and University of Abuja Teaching Hospital

Blood banks. Participants included healthy blood donors who have been previously screened and tested negative to HBsAg marker and other transfusion transmissible infections (HIV, HCV and Syphilis) using rapid test devices and Enzyme Linked Immuno-Sorbent Assay (ELISA) technique.

3.3 ETHICAL CONSIDERATIONS

Informed consent (Appendix 1) of each participant was obtained prior to sample collection and testing. Ethical approval was also obtained from Research Ethics Institutional Review Board of these two Teaching Hospitals (University of Abuja Teaching Hospital, Abuja and Nnamdi Azikiwe University Teaching Hospital, Nnewi) where the study was conducted. The approval letters from Health Research Ethics Committees with reference numbers for UATH Abuja- FCT/UATH/HREC/PR/514 and NAUTH Nnewi - NAUTH/CS/66/VOL.9/40 are attached (Appendix 2 & 3) respectively.

3.4 SAMPLE SIZE DETERMINATION

The minimum sample size for this study was 100 healthy blood donors who have been tested negative for HBsAg, HIV, HCV and Syphilis antibodies by rapid test device/ELISA technique as it is done in these Hospitals. This was derived from the formula below. It is estimated that this sample size will enable us to achieve a 5% precision at 95% confidence interval with a mean prevalence rate of HBsAg among prospective blood donors at 6% across the two study sites. The sample size was calculated using Fisher's formula as described by Araoye, (2004).

$(n) = z^2p\ (1\text{-}p)/d^2$

Where

n = sample size (87 stepped up to 100)

z score = 1.96 at 95% Confidence Interval

p = 6% (the arithmetic mean of the prevalence of HBsAg among blood donors at both sites (7%, at Nnamdi Azikiwe University Teaching Hospital Nnewi and 5% at University of Abuja Teaching Hospital, Abuja)

d (Precision of the study) = 5%

Thus n= $(1.96)^2$ X 0.06 X (1- 0.06)/ $(0.05)^2$ = 86.6 approx. 87.

To adjust for expected losses that may occur due to withdrawal of some blood donors from the study or blood donors failed to qualify because of inclusion criteria, the sample size was stepped up to 100.

3.5 STUDY POPULATION, SAMPLING TECHNIQUE AND DURATION OF SAMPLING

A total of 212 subjects (healthy blood donors) were enrolled into the study. One hundred and eight (108) from University of Abuja Teaching Hospital and one hundred and four (104) from Nnamdi Azikiwe Teaching Hospital, Nnewi participated. The selection of the subjects was done with the support of the staff of the hospitals' blood banks. Blood donors who have been certified fit for donations were selected after they have consented by signing the consent form. These subjects were screened and tested negative for HIV antibodies, HBsAg, Syphilis antibodies and anti-HCV by rapid test device/ELISA technique as done in these Teaching Hospitals. Sampling was done randomly until the expected number was sampled. Healthy blood donors who had been screened and found eligible by the respective blood banks for donation were recruited over a period of five months, from 1st June, 2016 to 30th October, 2016. In addition, known positive samples for HBV, HIV and HCV among blood donors were collected during this period. These positive samples and those detected in the course of testing using ELISA technique were used as controls to compare with those identified as occult hepatitis B virus (OBI) and blood donors seronegative for these viral agents.

3.6 INCLUSION AND EXCLUSION CRITERIA

All subjects who gave informed consent by signing the consent form were included in the study. Subjects who declined to offer consent were excluded from the study.

3.6.1: Inclusion Criteria

The inclusion criteria for the subjects were as follows:

- ✓ Age range of 18 to 60 years

- ✓ Not on medication for Hepatitis B virus infection
- ✓ Healthy Blood donors
- ✓ Blood donors who have been tested negative for HIV antibodies, HBsAg, anti-HCV and Syphilis antibodies by rapid test device/ELISA technique.

3.6.2: Exclusion Criteria

The exclusion criteria for all subjects included;

- Pregnant Women
- Children < 18 years; adult >60 years old
- On HBV Medication
- People that declined informed consent
- Subjects who have tested positive to any of the following; HIV, HCV, HBsAg and Syphilis antibodies.

3.7 SOCIODEMOGRAPHIC INFORMATION AND HBV RISK FACTORS ASSESSMENT

With the aid of a structured questionnaire (Appendix 4), relevant sociodemographic information and hepatitis B virus associated risk factors were obtained from blood donors that participated in the study. These included age, sex, marital status, academic attainment/social status, number of sexual partners, history of Sexually Transmitted Diseases (STDs), presence of tatoos/scarification marks, history of alcohol/drug abuse, sharing of sharps/occupational or domestic accident with sharp and hepatitis B vaccination status. Other information obtained from questionnaire includes history of blood transfusion and surgery as well as previous infection with viral hepatitis and knowledge of HBV infection. The questionnaires were completed by participants and any subject who needed help in filling the questionnaires were assisted by trained research assistants who were medical laboratory scientists and laboratory technicians that work in these blood banks.

3.8 SPECIMEN COLLECTION, PROCESSING AND PRESERVATION

Blood specimens were collected by venipuncture from all individuals enrolled in the study for the following assays;

1. Hepatitis B surface antigen 4th. generation ELISA

2. HIV 1 & 2 (Antigen +Antibody) 4th. generation ELISA
3. Anti-HCV (4th. Generation) ELISA
4. Hepatitis B Virus serological markers- 5 Panel Assay
5. Anti-HBc Total ELISA
6. IgM Anti- HBc ELISA
7. Anti-HBs ELISA
8. Liver Enzyme (ALT)
9. Alpha Feto-Protein (AFP) assay by ELISA
10. Complete Blood Count (HGB, HCT, RBC, WBC and Platelets)
11. CD4 Cell Count
12. Conventional PCR to detect HBV DNA
13. HBV DNA (Viral Load) Quantification using Real Time PCR
14. DNA Sequencing to determine HBV Génotypes
15. RT-PCR for detection of HIV RNA and HCV RNA

Blood Specimens (10ml) were collected from participants and appropriately separated as whole blood for Complete Blood Count (CBC) and CD4 cell count; serum for serological assays such as liver enzyme (ALT) and Alpha feto protein; plasma for molecular assays such HBV DNA, HIV RNA, HCV RNA and gene sequencing. The specimens for serological assays were put in plain tubes while samples for CBC, CD4 counts and molecular assays were collected into Ethylene Diamino Tetramino- Acid (EDTA) tubes. Plasma samples and sera were obtained by centrifugation and separated into cryovials. Complete Blood count and CD4 cell count were performed same day as prescribed by Standard Operating procedures (Dacie and Lewis, 1999; WHO, 2007b). Plasma samples for molecular assays and sera for serological/biochemical (Liver enzyme and Alpha –Feto Protein) tests were frozen at -70◦C before tests were performed.

3.9 LABORATORY PROCEDURES

3.9.1 Preliminary Testing

ABO Blood group/ type of all blood donors recruited were determined using cell and serum grouping methods as described by Contreras *et al.*, (2005). ABO grouping consists of cell grouping in which red cells are tested for antigen A and B using anti-A and anti-B sera and

serum grouping (reverse grouping) in which the serum is tested for anti-A and anti-B antibodies using known A and B cells. Anti A and anti B reagents from Spectrum Biotech, USA were used for cell grouping while serum grouping was performed using pooled washed known group A cell and B cell. The principle of ABO blood group testing is based on antigen and antibody reaction resulting to agglutination. The procedure for cell grouping is as follow:

A drop of Anti A, Anti B and Anti D sera were placed on a clean white tile. Thereafter, a drop of donor's red blood cells were added on each of the anti-sera and mixed with a clean plastic stick. The tile containing the specimen and antisera was shook using a rotary shaker for 2 minutes and results read. The test method for ABO grouping, tube method is as follow; five small test tubes were labeled as numbers 1 to 5. Each of these tube were added the following: Tube 1: One volume anti-A serum and one volume of 5% donor's red blood cells. Tube 2: One volume anti-B serum and one volume of 5% donor's red blood cells. Tube 3: One volume of donor's serum and one volume of 5% A cells. Tube 4: One volume of donor's serum and one volume of 5% B cells. Tube 5 (Auto-control): One volume of donor's serum and one volume of donor's 5% red cells. The contents of the tubes were mixed gently by tapping the base of the tubes with the finger. The tubes were incubated at room temperature for 5 minutes after which they were centrifuged at 150g for 1 minute. The results were read by tapping gently the base of each tube for agglutination or hemolysis. Results were recorded accordingly. The procedure for preparation of pooled A cell and B cell including interpretation of the result is contained in Appendix 5.

3.10 DETECTION OF HBV SEROLOGICAL MARKERS USING HEPATITIS B VIRUS 5 PANEL ASSAY

The serological markers for HBV infection were determined on all 212 blood donor samples using the method of Levinson, (2014). The CTK Biotech HBV 5 panel test kit was used for this assay. It is one step cassette style HBV rapid test, which is based on the principle of immunoassay combined with conjugated colloid gold technology and lateral flow. This kit is devised to detect five serological markers (HBsAg, HBeAg, HBsAb, HBcAb & HBeAb) associated with hepatitis B infection. The HBsAg strip and HBeAg strip are antibody based sandwich immunoassay. HBsAb strip is an antigen sandwich

immunoassay. HBeAb and HBcAb strips are competitive immunoassay. The procedure of testing is as follow:

The test kit was removed from the pouch and labeled with the specimen identifier. Two to three drops of serum were placed in each of the sample well using the disposable pipette accompanying the test kit. The reading of result was taken after 15 minutes according to manufacturer's instruction. In house positive and negative controls were performed before testing the blood donors' samples to validate the reagent kit.

3.11 DETECTION OF HEPATITIS B SURFACE ANTIGEN (HBsAg) BY 4th GENERATION ELISA

Surface antigen for hepatitis B was tested by ELISA technique on all specimens that were negative for HBsAg with rapid test device using the method of Burtis *et al.*, (2006). Fortress Diagnostics 4th generation ELISA kit UK was used. It is a solid-phase simultaneous sandwich immunoassay, which employs monoclonal antibodies and polyclonal antibodies specific for HBsAg. Microtiter wells are coated with monoclonal antibodies specific for HBsAg. Test procedures are as follow:

A 50ul volume of positive control, negative control and serum specimens were added in respective wells after which 50ul of Horseradish perioxidase (HRP) Conjugate was added to each well and mixed by tapping the plate gently. The plate was then covered with selotape provided and incubated at 37ºC for 1 hour. The microplate was removed from incubator and washed with buffer for five times. The reminant of the buffer was removed using toilet tissue. 50ul of chromogen A and 50ul of chromogen B were dispensed in all wells and mixed by tapping. The microplate was incubated for 15 minutes in the dark and thereafter 50ul of stop solution was added into each well and mixed gently. The absorbance was measured within 5 minutes after stopping the reaction at 450nm and 630nm as differential filter using Stat Fax 2100 Plate Reader. The cut off was calculated according to the kit manufacturer instructions and results evaluated. The presence or absence of HBsAg was determined by using the recorded absorbance and comparison with the calculated cut-off values. The calculation of cut-off values for controls samples were done according to kit manufacturer instructions Samples with an optical density (OD) less than the cut-off values

were considered as negative. Samples with OD higher than, or equal to, the cut-off value were also considered as positive. If the sample contains no HBsAg, the labelled antibody cannot be bound specifically and only a background color develops. The assay procedure is contained in Appendix 6.

3.12 DETECTION OF HIV 1 & 2 (Ag/Ab) BY 4th GENERATION ELISA

HIV 1 & 2 (Ag/Ab) was tested by ELISA technique on all specimens that were negative for HIV antibodies with rapid test device using the method of Constantine *et al*., (2005). Fortress Diagnostics 4th generation ELISA kit UK was used. The HIV (1+2) Ag/Ab ELISA kit used is a two-step incubation sandwich enzyme immunoassay which uses polystyrene microwell strips precoated with recombinant HIV antigens (recombinant HIV-1 gp41, gp120, and recombinant HIV-2 gp36 and anti-HIV p24 antibodies. As a first step, biotinylated anti-HIV (p24) antibodies together with patient's serum sample are added into the wells. During incubation, the specific HIV 1/2 antibodies was captured inside the wells. Simultaneously, if HIV p24 antigen is present in the sample, it would also be captured as a double antibody sandwich complex comprising of the coated antibodies p24-biotinylated antibodies. The microwells were then washed to remove unbound serum proteins. The detection of the captured HIV p24 antigen-biotinylated antibodies or HIV 1/2 antibodies was achieved during the second incubation step by adding of the Horseradish Peroxidase (HRP) which has been conjugated to second HIV 1+2 recombinant antigens and to avidin. The microwells were washed manually to remove unbound materials. Finally, a solution of substrate (Chromogen) was added to the wells and incubated for 15 minutes at room temperature. A blue color developed in proportion to the amount of HIV Ag/Ab present in the specimen. The enzyme-substrate reaction turns to yellow when the reaction is stopped with sulphuric acid. Assay procedures are as follow:

A 20ul volume of biotinylated anti-HIV p24 antibodies was added into each well except the blank. A 100ul of positive control, negative control and specimens were added in respective wells. This was covered with selotape provided and incubated at 37ºC for 60 minutes after which it was manually washed 5 times with buffer. A 100ul HRP conjugate was added to each well except blank and mixed by tapping the plate gently. The plate was covered and incubated at 37ºC for 30 minutes after which it was washed 5 times with buffer. A 50ul of

chromogen A and 50ul of chromogen B were dispensed in all well and mixed by tapping. The plate was then incubated for 15 minutes in the dark. A 50ul of stop solution was added into each well and mixed gently. The absorbance was measured within 5 minutes after stopping the reaction at 450nm and 630nm as differential filter using Stat Fax 2100 Plate Reader. The cut-off value was calculated and results evaluated. The presence or absence of HIV antigen/antibodies was determined by using the recorded absorbance and comparison with the calculated cut-off values. Samples with an optical density (OD) less than the cut-off values were considered as negative. Samples with OD higher than, or equal to, the cut-off value were also considered as positive. Wells containing samples negative for anti-HIV 1/2 or p24 remain colorless. The step by step procedure as contained in manufacturer guideline is presented in Appendix 7.

3.13 DETECTION OF ANTI-HCV (4th GENERATION) BY ELISA

Anti-HCV was detected from blood donor samples using the method of Burtis *et al.*, (2006). The 4th generation Fortress Diagnostics ELISA kit UK was used. It is a solid-phase simultaneous sandwich immunoassay, which employs monoclonal antibodies and polyclonal antibodies specific for hepatitis C virus antibodies. Microtiter wells are coated with monoclonal antibody specific for HCV. A serum specimen was added to the antibody coated microtiter wells together with enzyme conjugated polyclonal antibodies. Hepatitis C virus, if present, will form an antibody-HCV-antibody-enzyme complex. The plate was then washed manually to remove unbound material. Finally, a solution of substrate was added to the wells and incubated for 15 minutes at room temperature. A blue color developed in proportion to the amount of HCV antibodies present in the specimen. The enzyme-substrate reaction turns to yellow when the reaction was stopped with sulphuric acid. Test result was read using Stat Fax 2100 ELISA Plate reader at 450nm and 630nm as differential wavelength. Test procedures are as follow:

A 100ul volume of specimen diluent was added to all wells after which 10ul of positive, negative and specimen were added in respective wells. The plate was covered and incubated at 37^{o}C for 30 minutes. The plate was washed 5 times with buffer and 100ul of HRP conjugate was added to each well except the blank and mixed by tapping gently. The

plate was covered with selotape provided and incubated at 37ºC for 30 minutes after which it was manually washed 5 times with buffer. A 50ul volume of chromogen A and 50ul of chromogen B were dispensed into all wells and mixed by tapping.The plate was incubated at 37ºC for 15 minutes in the dark after which 50ul of stop solution was added into each well and mixed gently. Intense yellow color develops in positive control and HCV positive sample wells. The absorbance was measured within 5 minutes after stopping the reaction at 450nm and 630nm as differential filter using Stat Fax 2100 Plate Reader. The cut-off was calculated as described by kit manufacturer intructions and results evaluated. The detailed procedure of the kit manufacturer was followed and is contained in Appendix 8.

3.14 DETECTION OF ANTI-HBc TOTAL (4th GENERATION) BY ELISA

All specimens that were positive for HBV DNA and some seronegative samples were tested for Anti-HBc as described by Winn *et al*., (2006) using 4th generation Fortress Diagnostics ELISA kit UK. It is a solid-phase simultaneous sandwich immunoassay, which employs monoclonal antibodies and polyclonal antibodies specific for Hepatitis B Virus HBc antibodies. Microtiter wells are coated with monoclonal antigen specific for HBV anti-HBc marker. A serum specimen was added to the antibody coated microtiter wells together with enzyme conjugated polyclonal antibodies. Hepatitis B virus anti-HBc, if present, will form an antibody-HBV-antibody-enzyme complex. The plate was then washed manually to remove unbound material. Finally, a solution of substrate was added to the wells and incubated for 15 minutes at room temperature. A blue color developed in proportion to the amount of HBcAb present in the specimen. The enzyme-substrate reaction turned to yellow when the reaction is stopped with sulphuric acid. Test result was read using STAT Fax 2100 ELISA Plate reader at 450nm and 630nm as differential wavelength. Test procedures are as follow:

A 100ul volume of specimen diluent was added to all wells after which 10ul of positive, negative and specimen were added in respective wells. The plate was covered with selotape provided and incubated at 37ºC for 30 minutes. The plate was manually washed 5 times with buffer and 100ul of HRP conjugate was added to each well except the blank and mixed by tapping gently. The plate was covered and incubated at 37ºC for 30 minutes after which

it was washed 5 times with buffer. A 50ul volume of chromogen A and 50ul of chromogen B were dispensed into all wells and mixed by tapping.The plate was incubated at 37ºC for 15 minutes in the dark after which 50ul of stop solution was added into each well and mixed gently. Intense yellow color develops in positive control and anti-HBc positive sample wells. The absorbance was measured within 5 minutes after stopping the reaction at 450nm and 630nm as differential filter using Stat Fax 2100 Plate Reader. The cut-off was calculated as described by kit manufacturer intructions and results evaluated. The presence or absence of anti- HBc antibodies was determined by using the recorded absorbance and comparison with the calculated cut-off values. Samples with an optical density (OD) less than the cut-off values were considered as negative. Samples with OD higher than, or equal to, the cut-off value were also considered as positive. Wells containing samples negative for anti-HBc Total remained colorless. Appendix 9 contains the assay procedure.

3.15 DETECTION OF ANTI-HBc IgM (4th GENERATION) BY ELISA

All specimens that were positive for Anti-HBc total and some seronegative samples were tested for IgM Anti-HBc using 4th generation Fortress Diagnostics ELISA kit UK as described by Burtis *et al.*, (2006). It is a solid-phase simultaneous sandwich immunoassay, which employs monoclonal antibodies and polyclonal antibodies specific for Hepatitis B Virus HBc IgM antibodies.

A 100ul volume of specimen diluent was added to all wells after which 10ul of positive, negative and specimen were added in respective wells. The plate was covered with selotape provided and incubated at 37ºC for 30 minutes. The plate was manually washed 5 times with buffer and 100ul of HRP conjugate was added to each well except the blank and mixed by tapping gently. The plate was covered and incubated at 37ºC for 30 minutes after which it was washed 5 times with buffer. A 50ul volume of chromogen A and 50ul of chromogenB were dispensed into all wells and mixed by tapping.The plate was incubated at 37ºC for 15 minutes in the dark after which 50ul of stop solution was added into each well and mixed gently. Intense yellow color develops in positive control and anti-HBc IgM positive sample wells. The absorbance was measured within 5 minutes after stopping the reaction at 450nm and 630nm as differential filter using Stat Fax 2100 Plate Reader. The cut-off was calculated as described by kit manufacturer intructions and results evaluated.

The presence or absence of HBc IgM antibodies was determined by using the recorded absorbance and comparison with the calculated cut-off values. Samples with an optical density (OD) less than the cut-off values were considered as negative. Samples with OD higher than, or equal to, the cut-off value were also considered as positive. Wells containing samples negative for anti-HBc IgM remained colorless. The assay procedure as outlined by manufacturer was strictly followed and is contained in Appendix 10.

3.16 DETECTION OF ANTI-HBs (4th GENERATION) BY ELISA

All specimens that were positive for HBV DNA and some seronegative samples were tested for Anti-HBs using 4th generation Fortress Diagnostics ELISA kit UK as described by Burtis *et al.*, (2006). It is a solid-phase simultaneous sandwich immunoassay, which employs monoclonal antibodies and polyclonal antibodies specific for Hepatitis B Virus HBs antibodies. Microtiter wells are coated with monoclonal antigen specific for HBV Anti-HBs.

A 100ul volume of specimen diluent was added to all wells after which 10ul of positive, negative and specimen were added in respective wells. The plate was covered with selotape provided and incubated at 37ºC for 30 minutes. The plate was washed 5 times with buffer and 100ul of HRP conjugate was added to each well except the blank and mixed by tapping gently. The plate was covered and incubated at 37ºC for 30 minutes after which it was washed 5 times with buffer. A 50ul volume of chromogen A and 50ul of chromogen B were dispensed into all wells and mixed by tapping.The plate was incubated at 37ºC for 15 minutes in the dark after which 50ul of stop solution was added into each well and mixed gently. Intense yellow color develops in positive control and anti-HBs positive sample wells. The absorbance was measured within 5 minutes after stopping the reaction at 450nm and 630nm as differential filter using Stat Fax 2100 Plate Reader. The cut-off was calculated as described by kit manufacturer intructions and results evaluated. The presence or absence of anti- HBs antibodies was determined by using the recorded absorbance and comparison with the calculated cut-off values. Samples with an optical density (OD) less than the cut-off values were considered as negative. Samples with OD higher than, or equal to, the cut-off value were also considered as positive. Wells containing samples negative for anti-HBs remained colorless. The assay procedure is contained in Appendix 11.

3.17 DETECTION OF HEPATITIS B VIRUS DNA BY CONVENTIONAL POLYMERASE CHAIN REACTION (PCR)

The presence of HBV-DNA was examined in one hundred (100) samples negative for HBsAg, HIV, HCV and syphilis using a routine Conventional PCR to detect occult HBV infection. HBsAg positive samples were also assayed as control. HBV DNA was quantified using Roche COBAS Ampliprep/Taq man 48 analyzer; a Real-Time PCR on some samples positive by conventional PCR. .

3.17.1 DNA Extraction from Plasma Samples

DNA extraction was performed according to method of Kwok and Higushi (1989) using Zymo extraction kit manufactured by Zymo Research Corporation, USA and marketed by Ingaba Biotech Ltd Pretoria, South Africa. The extraction was done according to the guidelines of the manufacturer. DNA was extracted from HBsAg negative/positive samples and then PCR was used for detection of HBV-DNA. The protocol of DNA extraction is as follows;

A 400ul volume of genomic lysis buffer was added to 100ul of plasma. This was completely mixed by vortexing for 4-6 seconds and then left to stand for 5-10 minutes at room temperature. The mixture was transferred to a zymo-spin column in a collection tube. This was centrifuged at 10,000g for 1 minute using microcentrifuge and the collection tube discarded with the flow through. The content was transferred into a new collection tube. A 200ul volume of DNA pre-washed was added and centrifuged at 10000g for 1 minute. Thereafter, a 500ul DNA wash was added and centrifuged at 10000 for 1 minute. The content was transferred to a clean microcentrifuge tube and 50ul DNA elution buffer added. This was allowed to stand for 2-5 minutes at room temperature and then centrifuged at top speed for 30 seconds to elute the DNA. The eluted DNA was stored at $\leq 20^{\circ}C$ for future use and for molecular based applications.

3.17.2 Assessing the Quality and Quantity of DNA Extract

The quality of DNA extract was assessed using Thermo Scientific NanoDrop 1000 Spectrophotomer. It combines the principle of light and wave as it uses ultra violet light. The amount of light absorbed by sample is proportional to the concentration of DNA in the sample. The process is to assess the sample integrity, purity of extract as well as estimate or quantify the amount of DNA in the plasma samples. This optimizes the system making it possible to implement quality control checks throughout the procedure or process.

3.17.3 PCR Amplification using Thermal Cycler for Detection of HBV DNA

PCR amplifications were carried out in 20µl reaction volumes with the following PCR components: Master Mix (1X) 10ul, forward primer 0.4ul, Reverse primer 0.4ul, DNA Template 2ul and 7.2 ul of water making it up to 20ul on an Applied Biosystems GeneAmp PCR System 9700 Thermal cycler. Primer pairs were designed from the highly conserved overlapping regions of the *S* and *P* regions of the HBV genome. A conventional PCR was performed: Forward primer sequence: TCA CCA TAT TCT TGG GAA CAA GA and Reverse Primer sequence: CGA ACC ACT GAA CAA ATG GC. PCR conditions include the following: Thermal cycling parameters were: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 30 seconds at 95°C denaturation, 30 seconds at 53°C annealing temperature, 30 seconds at 72°C extension, followed by a final extension of 2 minutes at 72°C. Thermal cycling parameters remained the same as in the all PCR round done. A positive control (HBV plasmid DNA) and a negative control of the master mix were integrated to each run to validate the PCR products that produce a 340bp fragment. The detection limit of the HBV DNA by conventional PCR is approximately 2.5 copies per reaction (between 30-40 copies/mL)

3.17.4 Detection of PCR Amplified Product by Agarose Gel Electrophoresis

For analyses of the PCR amplification, 10ul of the amplified samples was electrophoresed on a 2% agarose gel made in Tris acetated EDTA buffer (pH=8.0-8.5) and visualized by UV illumination after ethidium bromide (10 g/ml) staining. Positive and negative controls

were also treated as samples. The detailed procedure of preparation of 2% Agarose gel used for electrophoresis of amplified product is contained in Appendix 12.

3.18 QUANTIFICATION OF HBV DNA BY REAL TIME PCR TECHNIQUE

Fourteen (14) samples suspected of OBI cases with known HBsAg positive samples were subjected to PCR using Roche CAP-CTM technologies. CAP-CTM is an automated real-time PCR based on a dual-labeled hybridization probe targeting the precore and core regions associated with an HBV DNA automated extraction based on the affinity of DNA for silica gel-covered magnetic beads. The procedure processes 1100 ul of plasma and consists of subsequent steps of lysis with chaotropic agents and proteinase K, DNA capture by use of glass particles, and purification. After DNA elution at high temperature (80°C), a robotic arm loads nucleic acids in microvials containing the PCR master mix prepared for each sample by the robotic arm. An internal quantitation standard (QS) was added to each sample during the processing step. After HBV DNA extraction with the COBAS AmpliPrep instrument, a real time PCR test was performed by the COBAS TaqMan 48 analyzer with a multiplex TaqMan assay. Two targets are amplified: HBV DNA and the internal QS. The QS is a noninfectious construct containing fragments of HBV sequences with primer binding regions identical to those of the HBV target sequence but with detection probe different from that for HBV. The results were expressed as international unit per milliliter with 5.82 copies per IU conversion factor. Prevention of carryover contamination and sample integrity was provided by the use of the Amperase system based on uracil-N-glycosylase and dUTP incorporation. The sensitivity of CAP-CTM is 12 IU/ml with a dynamic range from about 54 to 1.1 x 10^2 IU/ml and is designed for extraction of 24 plasma specimens in about 2 hours (Gurdiollo *et al.*, 2005; Hochberger *et al.*, 2006).

3.19 DEOXYRIBONUCLEIC ACID SEQUENCING AND PHYLOGENETIC ANALYSIS

3.19.1 Deoxyribonucleic Acid (DNA) Sequencing

PCR-positive samples (HBV DNA positive) representing five OBI samples and five PCR-positive samples from HBsAg positive carriers totalling ten samples were successfully sequenced. Also four samples positive for HIV were sequenced. The Pre s gene of hepatitis

B virus and V3 gene portion of HIV were successfully sequenced. The DNA sequencing was done using Sanger sequencing method as described by Sanger *et al.*, (1977). The PCR purified products were directly used for sequencing. The BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit was used on ABI 3510 Genetic analyzer (Applied Biosystems, Foster City, CA). The sequencing was done at Inqaba Biotechnological and Molecular Laboratories, Pretoria South Africa. Gene Sequencing was performed according to manufacturer's instructions.

3.19.2 Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Centre for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969). Sequences were deposited in GenBank, accession numbers are MG562502- MG562503.

3.20 NUCLEIC ACID TESTING FOR HIV and HCV

Positive samples and negatives for HIV and HCV by ELISA technique were tested to detect viral RNA. The HIV and HCV are RNA virus. First and foremost the RNA were extracted from the plasma samples and then converted to cDNA by RT-PCR before amplification was performed by conventional PCR.

3.20.1 RNA Extraction from Plasma Specimens

RNA extraction was performed using Zymo extraction kit manufactured by Zymo Research Inc. USA and marketed by Ingaba Biotech Ltd Pretoria, South Africa. The extraction was done according to the guidelines of the manufacturer. RNA was extracted from HIV and HCV negative/positive samples and then PCR was used for detection of HCV RNA and HIV RNA. The protocol for RNA extraction from plasma samples is as follow:

A 300ul volume of viral RNA buffer was added to 100ul of plasma. This was completely mixed by vortexing briefly. The content was transferred to Zymo-spin IIC colunm in a collection tube and centrifuged at 10000g for 1-2 minutes using microcentrifuge. The flow through was discarded. A 500ul viral wash buffer was added to the column and centrifuged for 2 minutes at 10000g. The column was carefully transferred into DNase/RNase- free tube. Then 15ul of DNase/RNase free water was directly added to the column matrix and centrifuged for 30 seconds to elute the RNA. The eluted RNA was stored at -70◦C for HIV RNA and HCV RNA detection.

3.20.2 Detection of HCV RNA by Conventional RT-PCR

Three different regions of HCV RNA genome namely 5′ UTR, core and NS5B were PCR amplified with genome specific primers by conventional RT-PCR method. The primer sequences for Forward primer: ACTGTCTTCACGCAGAAAGCGTCTAGCCAT and Reverse Primer: CGAGACCTCCCGG GGCACTCGCAAGCACCC. Conventional RT-PCR for the detection of HCV RNA was performed with normal ABI *Taq* polymerase. For rapid amplification of HCV RNA, RT-PCR was carried out in a total volume of 20ul with the following PCR components: Master mix (1X) 10ul, Forward Primer (0.2uM) of 0.16ul volume, Reverse Primer (0.2uM) of 0.16ul, Template 1ul and water 8.68ul making the final volume to 20ul. The RT-PCR conditions were initial denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds. Annealing was done at 50°C for 30 seconds, extension at 72°C for 30 seconds and the final extension was carried out at 72°C for 2 minutes in an ABI 9700 thermal cycler. The PCR products were electrophoresed in 2% agarose gel stained with ethidium bromide and observed under gel documentation system (Bio-Rad, USA) for HCV bands.

3.20.3 Amplification of HIV V3 Region (Nested PCR)

Conventional nested RT-PCR for the detection of HIV RNA was performed with normal ABI *Taq* polymerase. The primers for the primary or 1st round or nest are: Forward primer: GGCATCAAACAGCTCCAGGCAAG and Reverse Primer: AGCAAAGCCCTTTCTAAGCCCTGTCT. For rapid amplification of HIV RNA, 1st round

one-step RT-PCR was carried out in a total volume of 20 µl containing PCR components: Master Mix (1X) of 10ul volume, Forward Primer (0.2uM) of 0.16ul, Reverse Primer (02.uM) of 0.16ul, Template 1ul and H2O 8.68ul Final volume to 20ul. The PCR Conditions: Initial denaturation 95°C for 5 minutes, Denaturation 95°C for 30 seconds, Annealing 55°C for 30 seconds, Extension 72°C for 30 seconds, Final Extension 72°C 2minutes. The total number of cycles is 25 cycles. This was followed by the 2 nd round nested PCR with this primers sequence: Forward primer: TCCTGGCTGTGGAAAGATACCTA and Reverse Primer: GTCCCCTCGGGGCTGGGAGG in a 20 µl total reaction volume containing the following PCR components: Master Mix (1X) of 10ul, Forward Primer (0.2uM) of 0.16ul volume, reverse Primer (0.2uM) of 0.16ul, Template 0.5ul volume, H2O 9.18ul making the final volume to 20ul. The PCR conditions are as follow: initial denaturation 95°C for 5 minutes, Denaturation 95°C for 30 seconds, Annealing 58°C for 30 seconds, Extension 72°C for 30 seconds, Final Extension 72°C at 2minutes. The total number of cycles was 35 cycles. The PCR products were electrophoresed in 2% agarose gel stained with ethidium bromide and observed under gel documentation system (Bio-Rad, USA) for HIV V3 bands.

3.21 DETERMINATION OF ALANINE AMINOTRANSFERASE (ALT) BY SPECTROPHOTOMETRIC METHOD

Alanine Aminotransferase (ALT), a liver enzyme was determined by a standard method as described by Reitman and Frankel, (1957) using Randox reagents and Stax Fax 1904 Spectrophotometer. Alanine Aminotransferase (ALT) determination was measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine. The kit manufacturer instructions and procedures were strictly followed. Test procedure is as follow:

A 50ul volume of serum samples were added to their respective test tubes and 50ul of distilled water in the blank tube. A 250ul of reagent 1 (Buffer) was added into all the tubes, mixed and incubated at 37ºC for 30 minutes. Thereafter, 250ul volume of reagent 2 (2,4-dinitrophenylhydrazine) was added into all the tubes. The tubes were mixed by tapping gently and incubated in the dark for exactly 20 minutes at 20-25ºC. A 2.5ml volume of 0.4N Sodium Hydroxide solution was added into all tube, mixed and absorbance of samples

read against reagent blank after 5 minutes using stat fax Sphectrophotometer 1904 at 545-600nm wavelength. The activity of ALT in the specimen was obtained from the chart provided by the kit manufacturer. ALT assay procedure and the chart for determination of ALT results of blood donors as prescribed by the manufacturer are contained in Appendix 13.

3.22 DETERMINATION OF SOME HAEMATOLOGICAL PARAMETERS USING SYSMEX AUTO-HAEMATOLOGY ANALYZER

Full blood count of all blood donors enrolled in this study was done using Sysmex 3 part differential hematology analyzer as described by Dacie and Lewis, (1999). The Sysmex KN 21 auto-hematology analyzer used was able to determine the haematological indices of the blood donors like HGB, RBC, WBC total and differential, HCT and Platelets.

The Sysmex KN 21X Auto-hematology analyzer is a quantitative, automated hematology analyzer and leucocyte differential counter for in-vitro diagnostic use in clinical laboratories. Blood sample collected in EDTA anticoagulant (50µl) was diluted with Cellpack in the WBC counting chamber. A fixed volume of Stromatolyser- WH solution (1 volume of Stromatolyser-WH to 2 volumes of Cellpack) was added automatically to obtain a final dilution of 1:500. The addition of Stromatolyser-WH lyses the RBC and so the remaining cell stroma was at a level undetectable by the instrument. At the same time, the WBC membrane was preserved and WBC was stabilized at a level detectable by the instrument. The cells were then counted by Direct Current method. Hemoglobin was released during RBC lyses, and was converted to the red methemoglobin and read photo metrically at 555nm. A portion of this diluted sample was transferred automatically to the hemoglobin detector where the absorbance of the red pigment was measured to give blood hemoglobin level.

Direct Current Detection Method: Blood sample was aspirated, measured to a predetermined volume, diluted at the specified ratio, and then fed into each transducer. The transducer chamber has a minute hole called the aperture. On both side of the aperture, there are the electrodes between which flows direct current. Blood cells suspended in the diluted sample passed through the aperture, causing direct current resistance to change between the electrodes. As direct current resistance changes, the blood cell size was

detected as electric pulses. Blood cell count was calculated by counting the pulses, and histogram of blood cell sizes was plotted by determining the pulse sizes. Also, analyzing a histogram makes it possible to obtain various analysis data. The manufacturer's instructions and Standard Operating Procedures including the start up and shut down procedures were adhered strictly. The assay procedure is contained in Appendix 14.

3.23 DETERMINATION OF CD4 CELL COUNT USING PARTEC CYFLOW COUNTER

The CD4 cell count was performed on all blood donor participants following the method as described by (WHO, 2007a). This was done using Cyflow counter machine by Partec Germany. The principle is based on flow cytometry. Flow cytometry is a method by which cell or micro particles in suspension is differentiated and counted according to the cell size, fluorescence emission and internal structure. In the Cyflow counter, the florescence monoclonal antibody (CD4 mAb PE) binds to the CD4 antigen on the mononuclear cell (T lymphocytes and monocytes) and in a buffer suspension the complex is passed through the flow cuvette in a single stream of flow. The complex is excited by the solid state laser light (green laser) at a wave length of 532nm causing the complex to emit light which is captured by a photomultiplier tube and transmitted into digital read out as count. The test methodology is as follow:

A 20ul of CD4 Antibody was added into respective labeled Rohren tubes after which 20ul of well mixed EDTA whole blood of blood donors were added into their respective tubes. The contents of the tubes were mixed gently by tapping and incubated in the dark for 15 minutes at room temperature. A 800ul of CD4 non-lyse buffer was added, mixed and read on the Cylow counter machine. The step by step procedure including the start up and shut down operational procedures as contained in Appendix 15 were strictly followed.

3.24 DETERMINATION OF ALPHA FETO PROTEIN (AFP) BY ELISA

Alpha feto protein level of blood donor participants was determined using Perfemed Diagnostics ELISA Microwells Kit on all specimens that are positive for HBV DNA and negative samples using the method of Abelev, (1974). The principle of AFP quantitative test kit is based on a solid phase enzyme linked immunosorbent assay. The assay system

utilizes one anti-AFP antibody for solid phase (microtiter wells) immobilization and another mouse monoclonal anti-AFP antibody in the antibody-enzyme (horseradish peroxidase) conjugate solution. Test procedure is as follow:

A 20ul volume of the standard, specimens and control were dispensed into appropriate well. A 100ul of zero buffer was added into each well, thoroughly mixed and incubated at room temperature for 30 minutes. The incubation mixture was discarded into a waste container and plate washed 5 times with washing buffer. A 150ul Enzyme conjugate was dispensed in all well and mixed by tapping gently. The plate was then incubated at room temperature for 30 minutes. Thereafter, the microtititer wells were washed for 5 times with washing buffer. A 100ul TMB substrate was added into each well, mixed gently by tapping and incubated at room temperature for 20 minutes. The reaction was stopped by adding 100ul of stop solution to each well. Optical Density (OD) of the reaction mixture was read at 450nm with differential filter of 630nm using Stat Fax 2100 microplate reader. The concentration of AFP is directly proportional to the color intensity of the test sample. The calculations and interpretations of AFP results were done according to kit manufacturer's instructions. The detail of the procedures is contained in Appendix 16.

3.25 STATISTICAL ANALYSIS

Data obtained from this study were entered on Microsoft excel and analyzed on SPSS version 20 for windows. Also Graph pad prism software version 7.0 was used for some data analysis. Descriptive statistics (frequency, means, and standard deviation) was used to estimate participants' socio-demographic characteristics. The student t-Test and Analysis of variance (ANOVA) were used to determine relationship between the means of various variables while Chi-Square was used to establish association within the variables. Research hypotheses/assumptions were tested using Chi-square. Value was considered statistical significant at $p< 0.05$ at 95% confidence interval.

33 CHAPTER 4

4.0 RESULTS

Findings from this study revealed that out of the 212 blood donors recruited from two Teaching Hospitals for this study, 14 were invalidated because 8 samples tested positive to Hepatitis B Virus (HBsAg) and 6 samples were positive for Hepatitis C virus (anti-HCV) after preliminary screening and were excluded from the study. This is presented in Table 4.1. However, these samples were used as positive controls, in comparing occult and overt HBV infections. Table 4.2 presents frequency of occurrence of HBV serologic markers' pattern among blood donors' participants and its relationship with HBV DNA value. Out of 204 participants tested, 173 representing 84.8% were seronegative for HBV serologic markers while 31 representing 15.2% were seropositive for HBV markers. From the table, four serologic patterns were observed. They included; the seronegative, those positive for only anti-HBs, Anti-HBc, and those that were positive for both anti-HBc and anti-HBs. It was observed that none of the blood donor participants was positive for HBeAg and Anti-HBe markers. None was also positive for more than two HBV serologic markers.

Pattern of HBV markers among blood donors that tested negative to HBsAg at the study population is presented in Table 4.3. The data showed that 22 out of 204 blood donors representing 10.8% were positive for HBsAb marker while 12 blood donor participants representing 5.9% were positive for HBcAb marker. The blood donors with HBV serological markers were more at UATH Abuja than donors at NAUTH, Nnewi ($p<0.05$).

Figure 4.1 presented the prevalence of Occult HBV infection among blood donors in the two Teaching Hospitals studied. The overall prevalence of OBI among blood donors at two Teaching Hospitals under study was 14%. The prevalence rate is more at UATH Abuja than at NAUTH, Nnewi. The findings showed that out of the 50 samples tested for OBI at each study, 11 (22%) and 3 (6%) were positive indicating a prevalence occult HBV infection at UATH Abuja and NAUTH Nnewi stood at 22% and 6% respectively. This is statistically significant ($p=0.02$).

Table 4.1: Frequency of Blood Donors Based on HBsAg, HCV and HIV Assay Positivity by Rapid Test Device at UATH, Abuja and NAUTH, Nnewi

Study Sites	Total No. of Blood Donors Tested	No. (%) of HBsAg Negative Donors	No. (%) of HBsAg Positive Donors	No. (%) of HCV Positive Donors	No. (%) of HIV Positive Donors	Chi-square (p-value)
UATH Abuja	108	101 (93.5%)	7 (6.5%)	5 (4.6%)	0 (%)	*10.15 (0.1185)*
NAUTH Nnewi	104	103 (99%)	1(1%)	1 (1%)	0 (%)	
Total	**212**	**204 (96.2%)**	**08 (3.7%)**	**06 (2.8%)**	**0 (%)**	

Statistically Significant (p< 0.05)

Key:

UATH: University of Abuja Teaching Hospita, Abuja, **NAUTH:** Nnamdi Azikiwe Teaching Hospital, Nnewi, **HBsAg:** Surface antigen of Hepatitis B Virus, **HCV:** Hepatitis C Virus, **HIV:** Human Immunodeficiency Virus, <: Less than, %: Percentage

Table 4.2: Frequency of HBV Serologic Markers Pattern among Blood Donor Participants in the Study Population and its Relationship with HBV DNA Assay

HBV Serological Pattern	Frequency (%) of Occurrence	No. of Samples Tested for HBV DNA	No. (%) Positive for HBV DNA	Chi-square (p-value)
HBsAg- HBsAb- HBeAg- HBcAb- HBeAb- (Seronegative)	173 (84.8%)	70	2 (2.9%)	*44.3 (<0.0001)**
HBsAg-HBsAb+ HBeAg- HBcAb HBeAb-	19 (9.3%)	19	7 (36.8%)	
HBsAg- HBsAb HBeAg-HBcAb+ HBeAb-	09 (4.4%)	08	3 (37.5%)	
HBsAg-HBsAb+ HBeAg-HBcAb+ HBeAb-	03 (1.5%)	03	2 (66.7%)	
Total	**204 (100%)**	**100**	**14 (14%)**	

Statistically significant (p < 0.05)

Key:

+: Positive, **-:** Negative, **%:** Percentage, **<:** Less than, **HBV DNA:** Hepatitis B Virus Deoxyibonucleic Acid

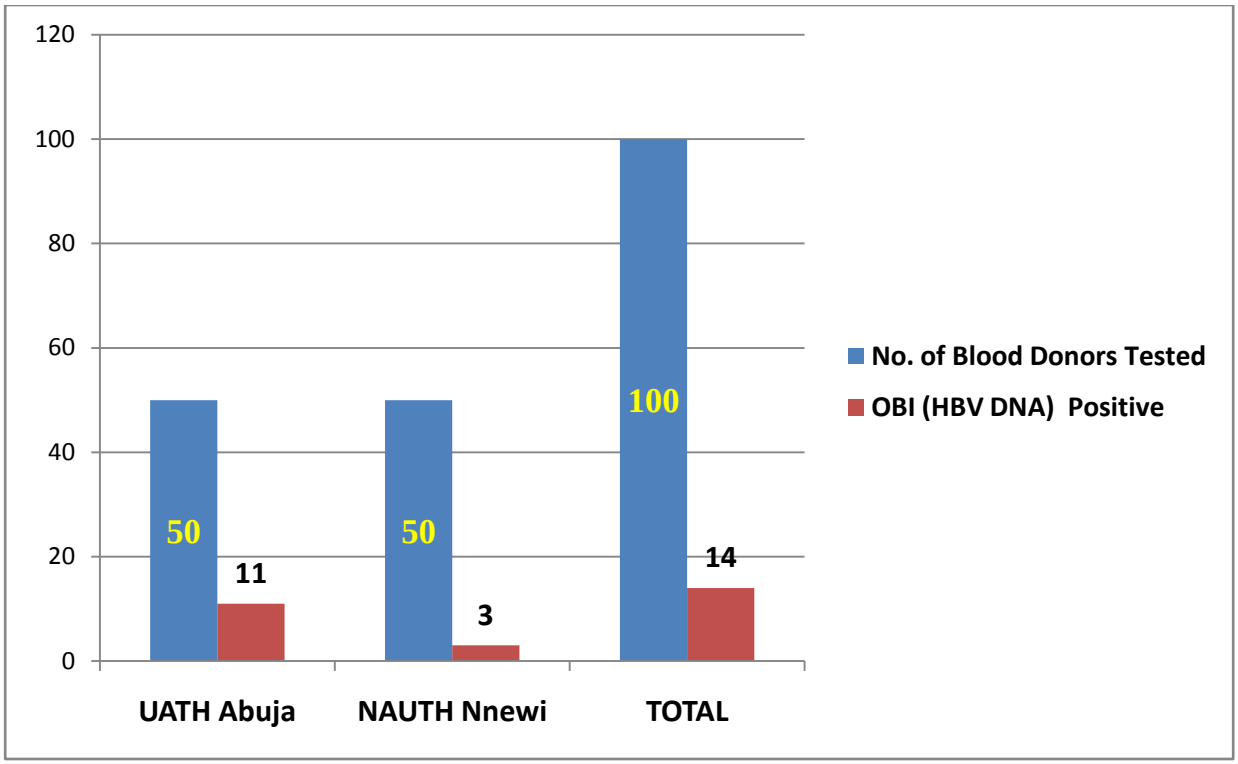
Table 4.3: Prevalence of HBV Serologic Markers among Blood Donors that Tested Negative to HBsAg at UATH and NAUTH using HBV 5 Panel Assay

HBV Serologic Markers	UATH n=101 No. (%) of Positive Samples	NAUTH n=103 No. (%) of Positive Samples	Total n=204 No. (%) of Positive Samples	Chi-Square (p-value)
HBsAb	18 (17.8%)	4 (3.9%)	22 (10.8%)	*7.6 (0.006)**
HBeAg	0 (0%)	0 (0%)	0 (0%)	*NA*
HBeAb	0 (0%)	0 (0%)	0 (0%)	*NA*
HBcAb	9 (8.9%)	3 (2.9%)	12 (5.9%)	*2.16 (0.141)*
Total	27 (26.7%)	7 (6.8%)	34 (16.7%)	*10.45 (0.001)**

**** Statistically Significant (p< 0.05)***

Key:

n: Number of samples, **NA:** Not Applicable



Statistically Significant (p< 0.05) Chi-Square = 5.3; p-value= 0.02

Figure 4.1: Prevalence of Occult HBV Infection among Blood Donors at UATH, Abuja and NAUTH, Nnewi

Table 4.4 presented the prevalence of HBV serologic markers among blood donors with occult HBV infection. The result showed that out of 14 blood donors with OBI, 5 (35.7%), 3 (21.4%) and 9 (64.3%) were positive for Anti-HBc total, Anti-HBc IgM and Anti-HBs respectively. This is statistically significant. We recorded 0% prevalence for HBeAg and anti-HBe serologic markers among blood donors with OBI. Figure 4.2 shows the classification of OBI into serological groups. Out of 14 OBI blood donors isolated in this study, 12 were seropositive for HBV markers representing 85.7% while the remaining 2 blood donors were seronegative for HBV serologic markers representing 14.3% prevalence.

The pattern of HBV serologic markers among blood donors with OBI is presented in Figure 4.3. Out of 12 seropositive OBI blood donors identified, 7 blood donors have Anti- HBs marker representing 58.3%, 3 blood donors haveAnti-HBc marker representing 25% and 2 blood donors with OBI have a combination of Anti-HBc and Anti-HBs markers representing 16.7%. None of OBI blood donor was positive for more than two serologic markers.

Table 4.5 shows the HBsAg positivity by ELISA among blood donors that tested negative by rapid test device. Out of 202 samples tested for HBsAg by ELISA Technique, 28 (13.9%) samples were positive. The rate of positivity among blood donors at NAUTH and UATH is statistically significant p=0.0003. In the table is also shown HBV DNA positivity among blood donor participants that tested positive by ELISA. Out of 28 samples positive for HBsAg by ELISA, 20 samples, representing 71.4% were positive for HBV DNA by conventional PCR. There is no statistical significant difference between blood donors at UATH Abuja and NAUTH Nnewi.

Table 4.6 presents HIV positivity by ELISA technique among blood donors that tested negative by rapid test devices at UATH and NAUTH. Out of 197 samples tested for HIV (Ag + Ab) by ELISA Technique, 27 (13.7%) were positive. No statistical significant difference was observed in the degree of positivity between the two study sites. The Table 4.6 also shows HIV RNA positivity rate among blood donors that tested positive by ELISA technique but negative by rapid test method. Out of 22 samples positive by ELISA, 8 (36.4%) was positive for HIV RNA by PCR. No statistical significant difference was observed between the two study sites.

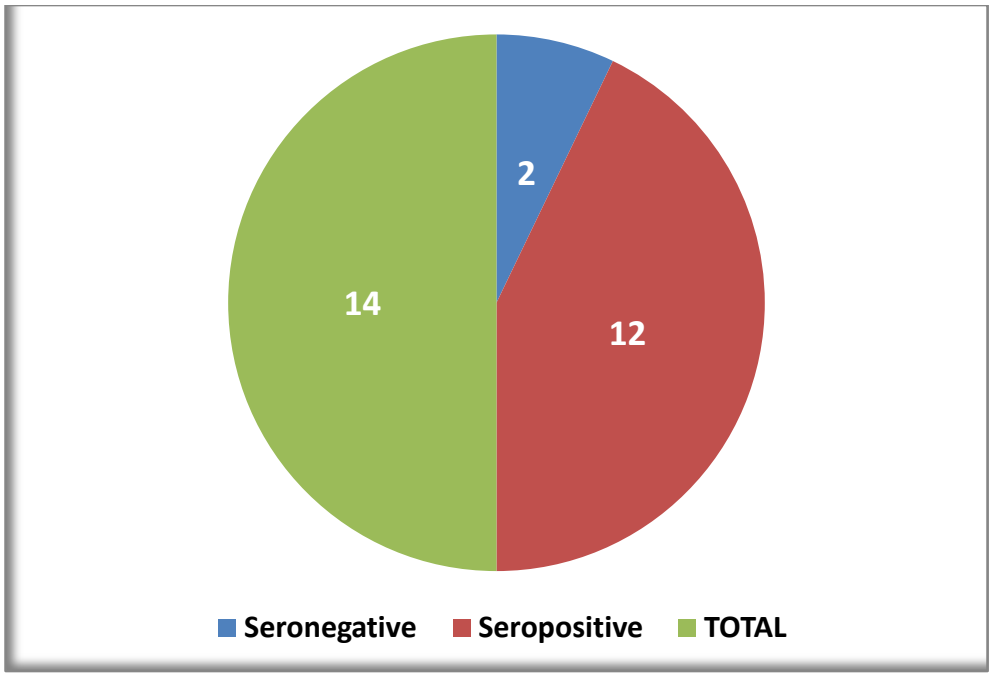
Table 4.4: Prevalence of HBV Serologic Markers among Blood Donors with Occult HBV Infection (n= 14) in the Study Population

HBV Serologic Markers	**No. Positive**	**% Positive**	**Chi-Square (p-value)**
Anti-HBc Total	5	35.7	*8.4 (0.003)**
Anti-HBc IgM	3	21.4	*6.2 (0.04)**
Anti-HBs	9	64.3	*18.2 (0.015)**
Anti-HBe	0	0	*2.517 (0.4721)*
HBeAg	0	0	*2.517 (0.4721)*

* ***Statistically significant (p<0.05)***

Key

HBV: Hepatitis B Virus, **OBI:** Occult hepatitis B virus infection, **IgM:** Immunoglobulin M



Statistically significant (p<0.05) ***Chi-square: 0.1977 p-value: 0.9965***

Figure 4.2: Serological Classification of OBI among Blood Donor Participants in the Study Population

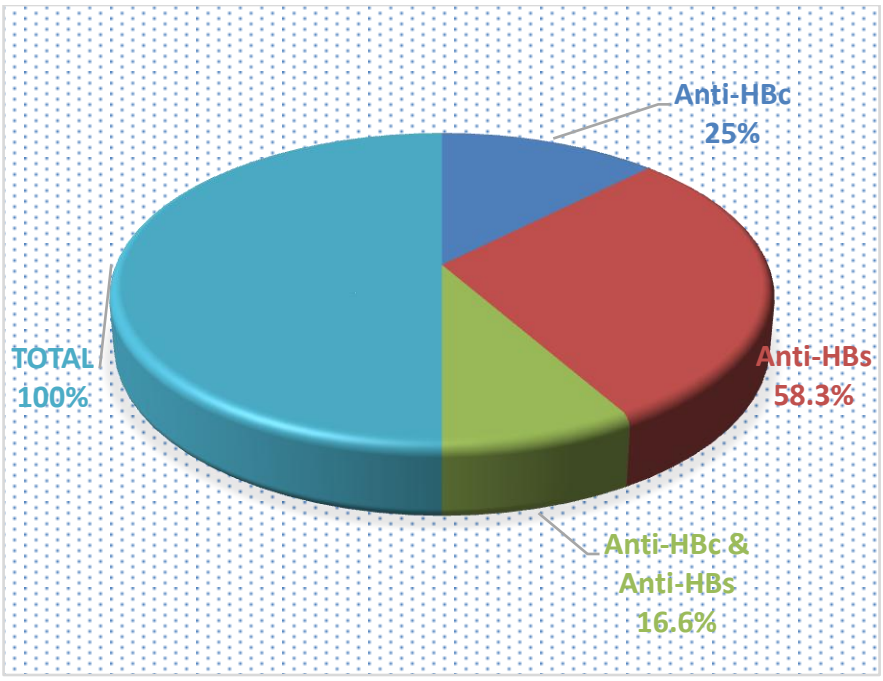


Figure 4.3: Pattern of HBV Serological Markers among Blood Donors with Occult HBV Infection in the Study Population

Table 4.5: HBsAg Positivity by ELISA Technique among Blood Donors at UATH, Abuja and NAUTH, Nnewi that Tested Negative by Rapid Test Device and its Relationship with HBV DNA Assay

Study Sites	ELISA No. of Samples Tested	No. (%) Positive	Chi-Square (p-value)	PCR No. of Samples Tested	No. (%) Positive	Chi-Square (p-value)
UATH Abuja	100	5 (5%)	*12.95 (0.0003)* *	5	0 (0%)	*0.70 (0.4)*
NAUTH Nnewi	102	23 (22.5%)		23	20 (87%)	
Total	**202**	**28 (13.9%)**		**28**	**20 (71.4%)**	

* ***Statistically Significant (p< 0.05)***

Key:

ELISA: Enzyme Linked Immunosorbent Assay, **PCR:** Polymerase Chain Reaction

HBsAg: Surface antigen of Hepatitis B Virus, **DNA:** Deoxyribonucleic Acid

Table 4.6: HIV Positivity by ELISA Technique among Blood Donors at UATH, Abuja and NAUTH, Nnewi that Tested Negative by Rapid Test Device and its Relationship with HIV RNA Assay

Study Sites	ELISA No of Samples Tested	No. (%) Positive	Chi-Square (p-value)	PCR No. of Samples Tested	No. (%) Positive	Chi-Square (p-value)
UATH Abuja	99	10 (10.1%)	*2.1 (0.14)*	10	4 (40%)	*0.0487 (0.9759)*
NAUTH Nnewi	98	17 (17.3%)		12	4 (33.3%)	
Total	**197**	**27 (13.7%)**		**22**	**8 (36.4%)**	

Statistically Significant (p< 0.05)

Key:

HIV: Human Immunodeficiency Virus, **ELISA:** Enzyme Linked Immunosorbent Assay

PCR: Polymerase Chain Reaction, **RNA:** Ribonucleic Acid

Table 4.7 revealed that out of 185 samples tested for anti-HCV by ELISA, 15 samples representing 8.1% were positive. There is statistical significant difference in the degree of positivity between the two Teaching Hospitals studied. Also the rate of HCV RNA positivity among blood donor participants at UATH and NAUTH is presented in Table 4.7. Out of 14 samples positive by ELISA, 4 (28.6%) samples were positive for HCV RNA by conventional PCR.

Coinfection of transfusion transmissible viral infections among blood donors' participants is presented in Figure 4.4. Out of 198 blood donor samples that tested negative for HBV, HCV and HIV by rapid test kit, it was found that 12 samples representing 6.1% had coinfection of TTIs when retested with ELISA 4th generation kit. One (1) sample out of the 12 samples was co-infected with these three viral (HBV, HCV and HIV) agents representing 8.3% population of coinfection. HCV/HIV co-infection was the most prevalent with 5 out of 12 samples co-infected with these viral agents representing 41.7% prevalence.

Table 4.8 presents HBV risk factors among blood donors with overt and occult HBV infections. HBV risk factors assessed included: knowledge of HBV/HBV infection, HBV vaccination status, occupation/domestic accidents, previous blood transfusion, multiple sexual partner, history of STDs to mention but a few. We observed no statistical significant difference between blood donors with occult and overt HBV infection among the risk factors assessed. The principal obsevations from Table 4.8 indicated that most of the blood donor participants with occult and overt HBV infection lacked knowledge of HBV infection and have not received HBV vaccination.

Table 4.7: HCV Positivity by ELISA Technique among Blood Donors at UATH, Abuja and NAUTH, Nnewi that Tested Negative by Rapid Test Device and its Relationship with HCV RNA Assay

Study Sites	ELISA No. of Samples Tested	No. (%) Positive	Chi-Square (p-value)	PCR No. of Samples Tested	No. (%) Positive	Chi-Square (p-value)
UATH Abuja	93	2 (2.2%)	*8.8 (0.003)**	2	1 (50%)	*0.2571 (0.8794)*
NAUTH Nnewi	92	13 (14.1%)		12	3 (25%)	
Total	**185**	**15 (8.1%)**		**14**	**4 (28.6%)**	

* ***Statistically Significant (p< 0.05)***

Key:

HCV: Hepatitis C Virus, **ELISA:** Enzyme Linked Immunosorbent Assay, **PCR:** Polymerase Chain Reaction, **RNA:** Ribonucleic Acid

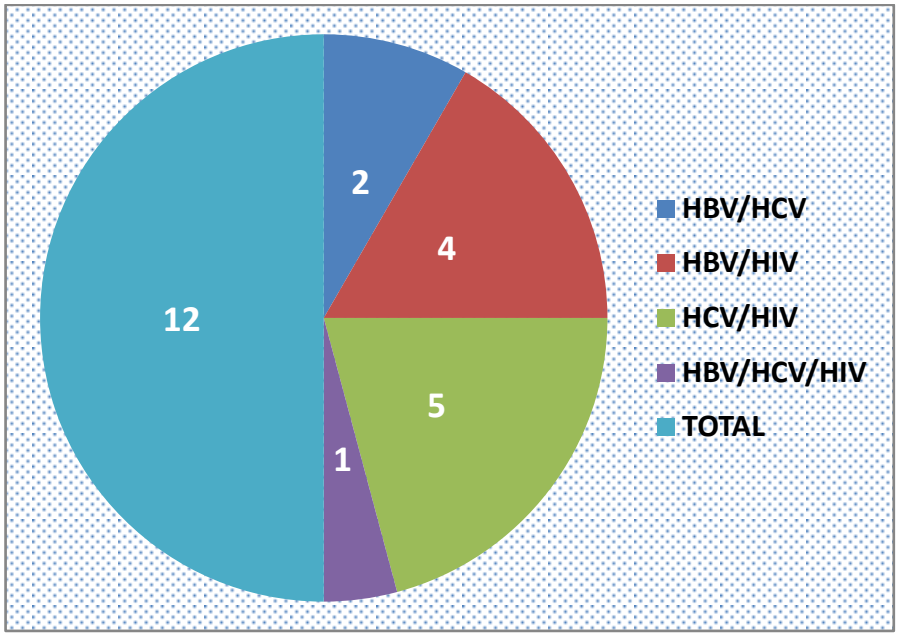


Figure 4.4: Coinfection of Transfusion Transmissible Viral Infections among Blood Donors in the Study Population

Table 4.8: Risk Factors of Blood Donors with Occult and Overt HBV Infection in the Study Population

HBV Risk Factors	No. (%) of OBI Donors affected n=14	No. (%) of Donors with Overt HBV Infection n= 20	Chi-Square (p-value)
Lack of knowledge of HBV	10 (71.4%)	10 (50%)	*1.5 (0.21)*
No HBV Vaccination	12 (85.7%)	20 (100%)	*2.9 (0.08)*
Occupational/Domestic Accident (Needle prick/injuries)	8 (57.1%)	17 (85%)	*3.1 (0.07)*
Previous Blood Transfusion	2 (14.3%)	0 (0%)	*2.9 (0.08)*
Multiple Sexual Partner	1 (7.1%)	0 (0%)	*1.4 (0.23)*
History of Sexually Transmitted Diseases	1 (7.1%)	0 (0%)	*1.4 (0.23)*
Alcohol/Drug Abuse	2 (14.2%)	0 (0%)	*2.9 (0.08)*
Previous Surgeries/Dialysis	1 (7.1%)	1 (5%)	*0.06 (0.79)*
Tribal Marks/Tatoo	1 (7.1%)	2 (10%)	*0.08 (0.77)*
Visiting Commercial Barber/Manicurer/Pedicurer	11 (78.6%)	13 (65%)	*0.7 (0.39)*
Organ Transplant	0 (0%)	0 (0%)	*1.75 (0.18)*

p< 0.05 (statistically significant)

Key:

n: Number of samples, **OBI:** Ocult Hepatitis B Virus Infection

Table 4.9 showed the demographic characteristics of blood donors with occult HBV infection. The demographics studied include gender, age, marital status, academic status and occupation/social status. From the Table, it is shown that (13) out of 14 OBI blood donors were males representing 92.9% while only one OBI case was a female representing 7.1% of the population. There is no statistical significant difference observed. Eight (8) out of 14 blood donors with OBI were married representing 57.7% while 6 were single representing 42.9% of the population. No widower/single parent was associated with occult HBV infection. The academic status of blood donors with occult HBV infection showed that 2 (14.2%) of 14 blood donors with OBI have attained primary school level of education while 6 each have attained secondary and tertiary level representing 42.9% prevalence.

Age distribution of blood donors with occult HBV infection showed that 12 (85.7%) out of 14 blood donors with OBI are within age 18-40 years. Age group 18-25 years had 2 blood donors with OBI; 26 – 40 years had 9 blood donors each with OBI representing 64.3% prevalence. Age group 41-60 years had 2 blood donors with OBI. No statistical significant difference was observed among the various age groups (p= 0.5065).

Social status and occupation of blood donors with occult HBV infection is also shown in Table 4.9. Out of 14 blood donors with OBI identified, traders/business men have 5 blood donors with OBI followed by technical worker/farmers with 4 OBI representing 37.5% and 28.6% respectively. Students affected with OBI stood at 3 (21.4%) of the 14 OBI identified while applicants and civil servants had one (7.1%) representative each. No statistically significant difference was observed.

Table 4.9: Demographic Characteristics of Blood Donors with Occult HBV Infection in the Study Population

Blood Donors Demographics	No. of OBI Donors (n= 14)	Frequency (%)	Chi-square (p-value)
Gender			
Male	13	92.9%	*0.1807*
Female	1	7.1%	*(0.9136)*
Marital Status			
Married	8	57.1%	*0.3187*
Single	6	42.9%	*(0.6507)*
Separated	0	0%	
Academic Status			
Primary	2	14.3%	*0.007013*
Secondary	6	42.9%	*(0.9998)*
Tertiary	6	42.9%	
Age Group (Years)			
18-25	3	21.4%	
26-40	9	64.3%	*0.2189*
41-60	2	14.3%	*(0.5065)*
Occupation/Profession			
Applicants	1	7.1%	
Students	3	21.4%	
Business/Trading	5	35.7%	*0.00505*
Civil Servants	1	7.1%	*>0.9999*
Artisans	4	28.6%	

Statistically significant (p< 0.05)

Key:

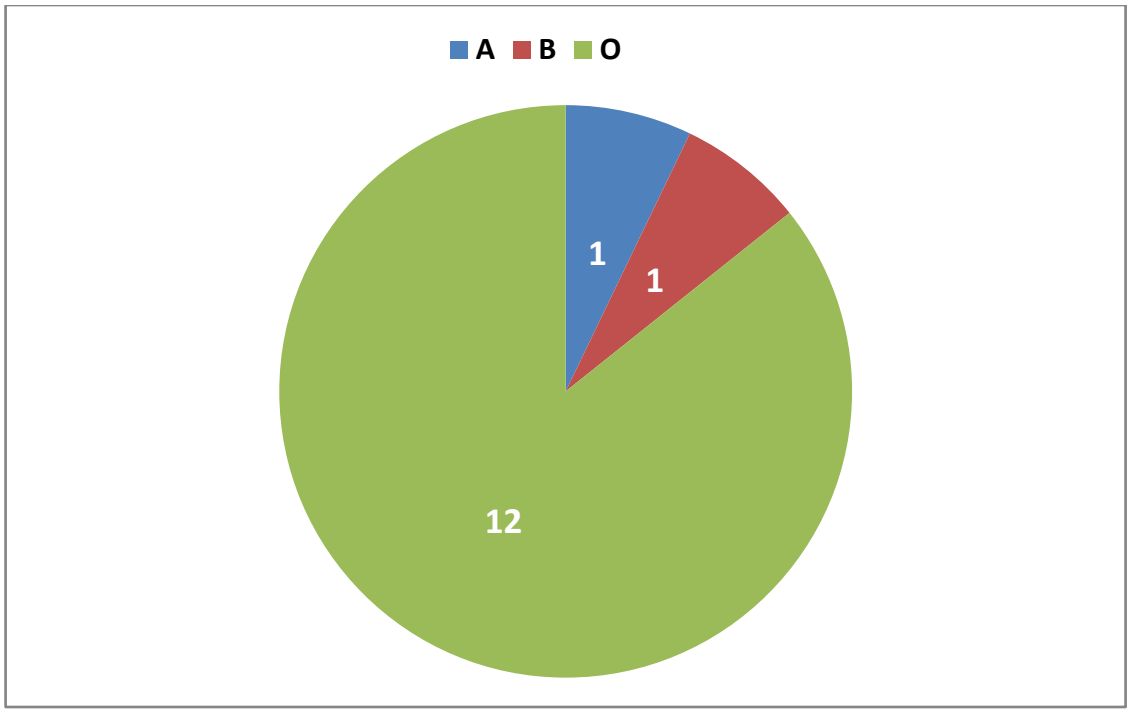
n: Number of OBI Blood Donors**, %:** Percentage**, >:** Greater than, <: Less than

Figure 4.5 presented the ABO Blood group distribution of blood donors with occult HBV infection. It is evident from the figure 4.5 that blood group O was the most prevalent among blood donors with occult HBV infection with 12 out of 14 OBI donors identified representing 85.7% while blood group A and B had 1 OBI blood donor each. This represents 7.1% prevalence. There is no OBI associated with blood donors with AB blood group.

Classification of OBI blood donors isolated in respect to their status is presented in Figure 4.6. The finding showed that 9 out of 14 blood donors with OBI belonged to family replacement donors representing 64.3% while 5 blood donors with OBI were voluntary non-remunerated donors representing 35.7%%. No blood donor with OBI was identified as commercial paid donor.

Figure 4.7 presented the prevalence of OBI among blood donors with respect to time of donation. The result showed that 9 out of 14 blood donors with OBI are Repeat (old) blood donors representing 64.3% prevalence while the remaining 5 blood donors were first time donors representing 35.9% of the population. There is no statistically significant difference between the old (Repeat) blood donors and first timers with regard to having occult HBV infection.

HBV genotype among blood donors with occult and overt HBV Infection is presented in Table 4.10. The data showed that all 10 HBV isolates that were sequenced belong to HBV Genotype E representing 100%. Also 5 HBV isolates each are from blood donors with occult and overt infection respectively. There was no evidence of coinfection with 2 or more HBV Genotypes.

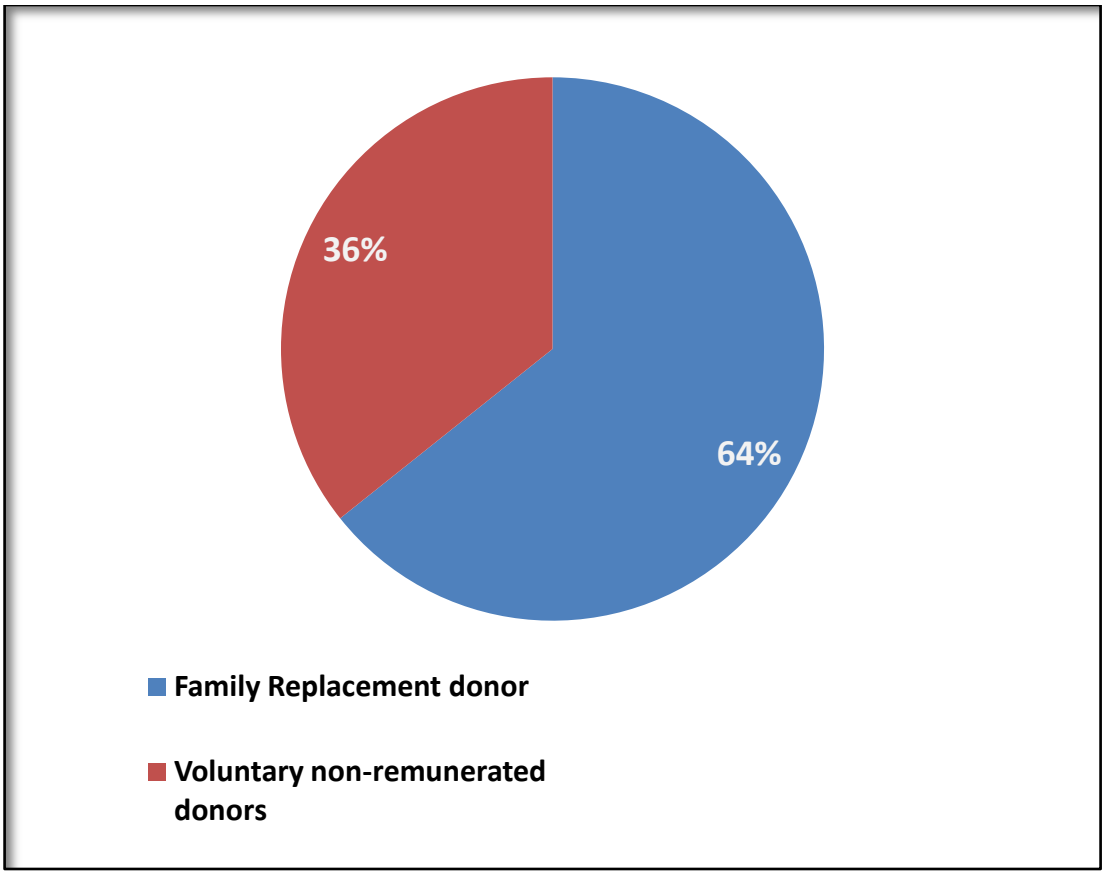


Statistically significant (p< 0.05) Chi-square: 0.1807 p-value: 0.9136

Figure 4.5: ABO Blood Group Distribution of Blood Donors with Occult HBV Infection in the Study Population

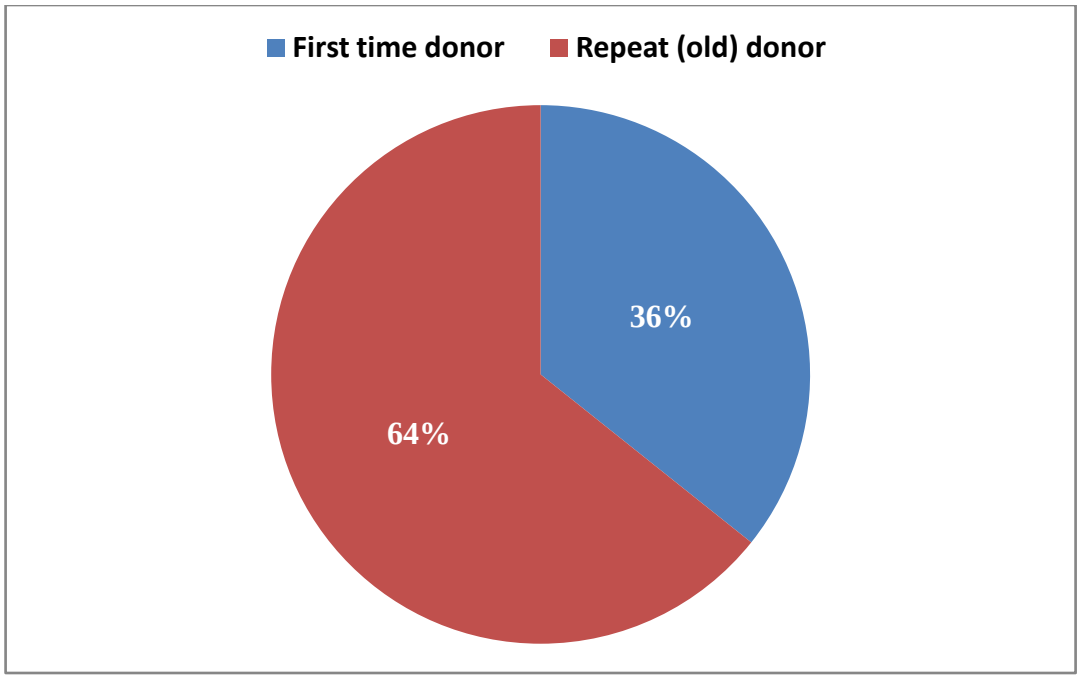
Key:

A: Blood group A, **B:** Blood group B, **O:** Blood group O



Statistically significant (p< 0.05) Chi-square: 0.2029 p-value: 0.9996

Figure 4.6: Classification of Blood Donors with OBI in Respect to Their Donation Status in the Study Population



Statistically significant (p< 0.05) Chi-square: 0.2029 p-value: 0.9996

Figure 4.7: Classification of Blood Donors with OBI in Respect to Time of Donation in the Study Population

Table 4.10: Pattern of HBV Genotypes among Blood Donors with Occult and Overt HBV Infection in the Study Population

HBV Genotypes	No. (%) OBI Genotype	No. (%) of HBsAg positive Genotype	Chi-Square (p-value)
A	0 (0%)	0 (0%)	*0.06 (0.79)*
B	0 (0%)	0 (0%)	
C	0 (0%)	0 (0%)	
D	0 (0%)	0 (0%)	
E	5 (100%)	5 (100%)	
F	0 (0%)	0 (0%)	
G	0 (0%)	0 (0%)	
H	0 (0%)	0 (0%)	
Coinfection	0 (0%)	0 (0%)	
Total	**5 (100%)**	**5 (100%)**	

Statistically significant (p<0.05)

Key:

Hepatitis B Virus has 8 Genotypes represented as A-H, **OBI:** Occult HBV Infection, **%:** Percentage

Gene Sequencing and Phylogenetic Analysis Results

The DNA sequences obtained from this study were analyzed using BioEdit 9.7 and Codon-code Aligner 4.0 software. Sequence analysis and comparison were conducted by using molecular programs deposited in the web site of the National Centre for Biotechnology Information (NCBI). The sequences were compared with the same region of HBV sequences from different genotypes found in the genotyping reference set available on the NCBI website. The phylogenetic tree was constructed according to previous methods (Utama *et al.,* 2009).

Ten (66.7%) out of 15 HBV isolates were successfully sequenced with HBV Genotypes identified. Five isolates each were for occult and overt HBV. The pre s/s gene (the surface antigen) was sequenced. The HBV gene sequence result showed that all our HBV isolates (occult and overt) belonged to Genotype E. Four (80%) out of 5 OBI isolates sequenced had a gene sequence of close relatedness with HBV isolates from Sudan. Gene sequences were deposited at Gen Bank. The accession numbers of HBV isolates as deposited at NCBI were MG562502- MG562503. The nucleotide sequence of these isolates is shown in Appendix 16.

Four (80%) out of five HIV isolates were successfully sequenced. The V3 gene of HIV isolates was sequenced. The sequencing result showed that all HIV isolates from this study were HIV-1. The figures below (Figure 4.8 – Figure 4.12) showed phylogenetic trees of occult HBV isolates in relation to other isolates matched with them to show their relatedness based on gene sequences. Figures 4.13 – Figure 4.17 present the phylogenetic trees of overt HBV isolates in relation to other isolates matched with them indicating their relatedness based on gene sequences.

The sequence emanated from the isolate produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The sequence of HB1 showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the isolates within the Hepatitis viruses and revealed a closely relatedness to Hepatitis B Virus isolate SDAC_059 (gb: KF170780.1) than other Hepatitis B viruses (Figure 4. 8).

The sequence of HB2 isolate showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the isolates within the Hepatitis viruses and revealed a closely relatedness to Hepatitis B virus strain AYNF241 (gb: KU984100.1) than other Hepatitis B viruses (Figure 4. 9).

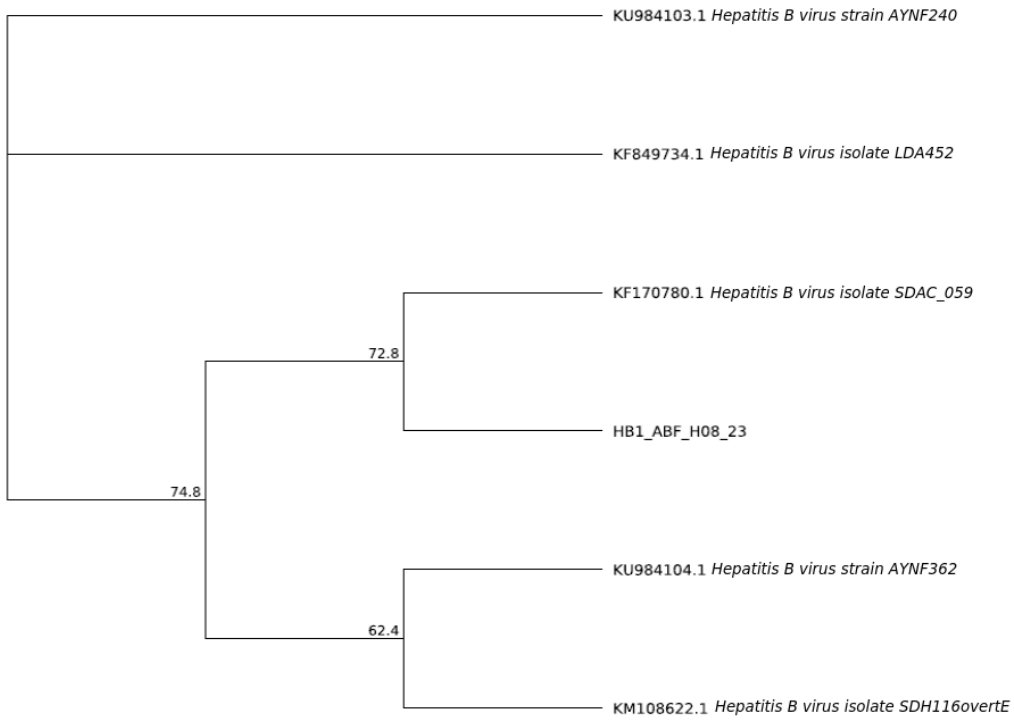


Figure 4.8: Phylogenetic Tree showing Relationship between HB1 Isolated from Blood Donor with OBI and other Hepatitis B viruses.

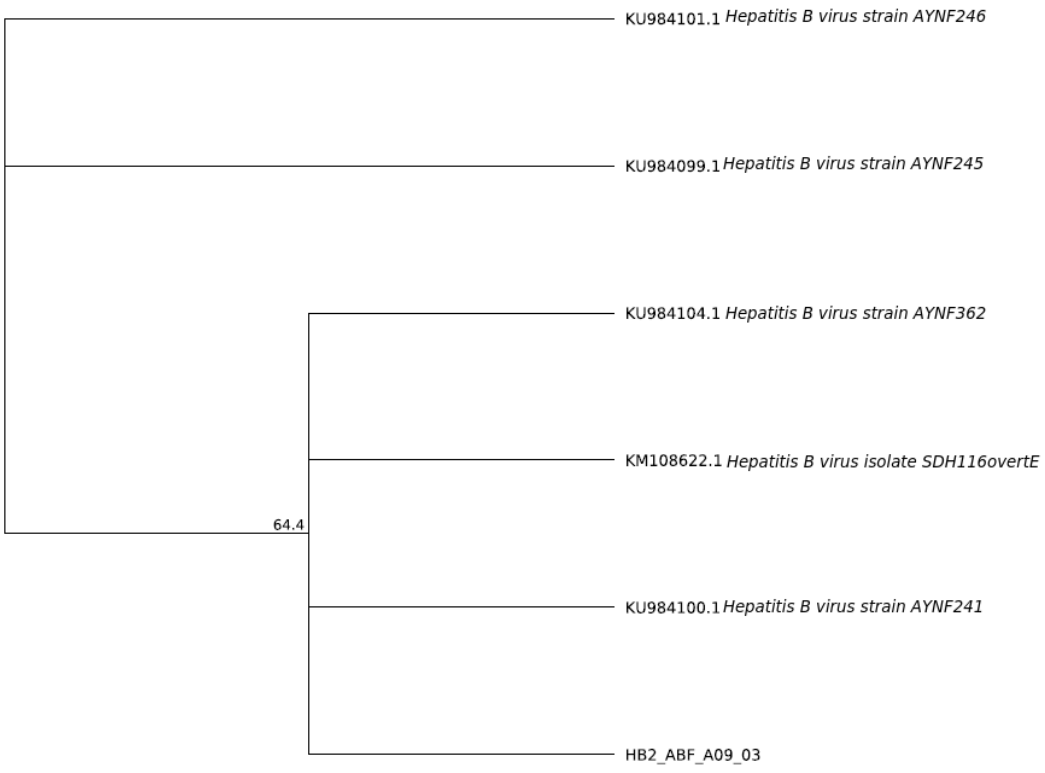


Figure 4.9: Phylogenetic Tree showing Relationship between HB2 Isolated from Blood Donor with OBI and other Hepatitis B viruses.

The sequence of HB3 isolate from a blood donor with occult HBV infection showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the isolates within the Hepatitis viruses and revealed a closely relatedness to Hepatitis B virus strain SDAC_059 (gb: KF170780.1) than other Hepatitis B viruses (Figure 4.10).

The obtained sequence from the isolate produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The sequence of HB4 showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the isolates within the Hepatitis viruses and revealed a closely relatedness to Hepatitis B Virus isolate SDH 116 overt E (gb: KM108662.1) than other Hepatitis B viruses (Figure 4.11).

The obtained sequence from the isolate produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The sequence of HB5 showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the isolates within the Hepatitis viruses and revealed a closely relatedness to Hepatitis B Virus isolate SDAC_059 (gb: KF170780.1) than other Hepatitis B viruses (Figure 4.12).

The sequence of HB6 isolate from a blood donor showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the isolates within the Hepatitis viruses and revealed a closely relatedness to Hepatitis B virus partial pre s/s (gb: AJ604932.1) than other Hepatitis B viruses (Figure 4.13).

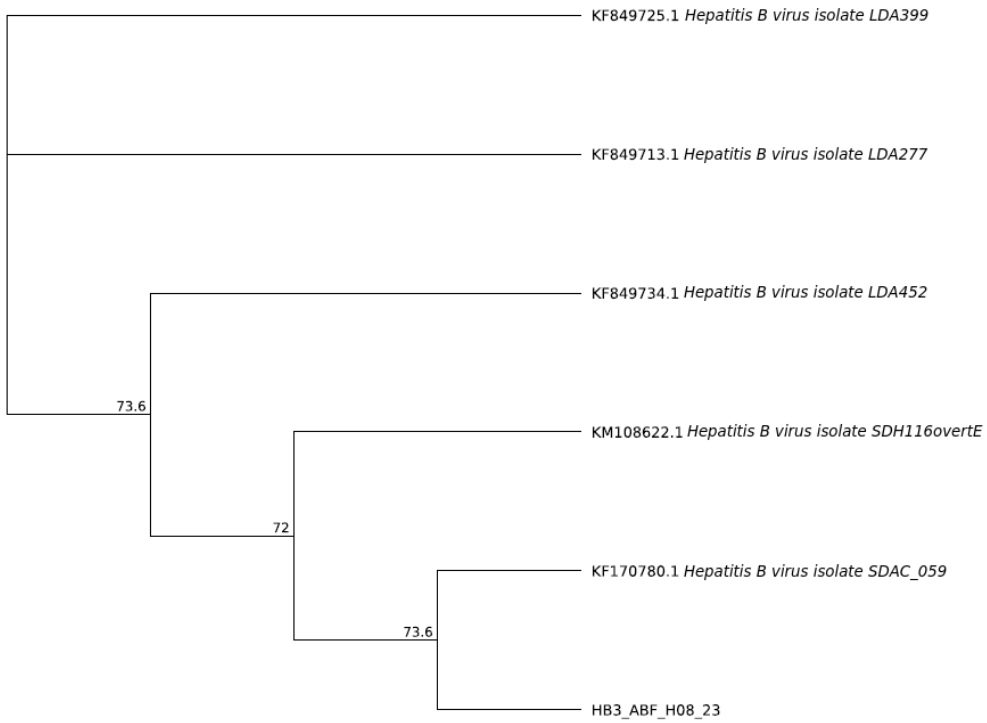


Figure 4.10: Phylogenetic Tree showing Relationship between HB3 Isolated from Blood Donor with OBI and other Hepatitis B viruses.

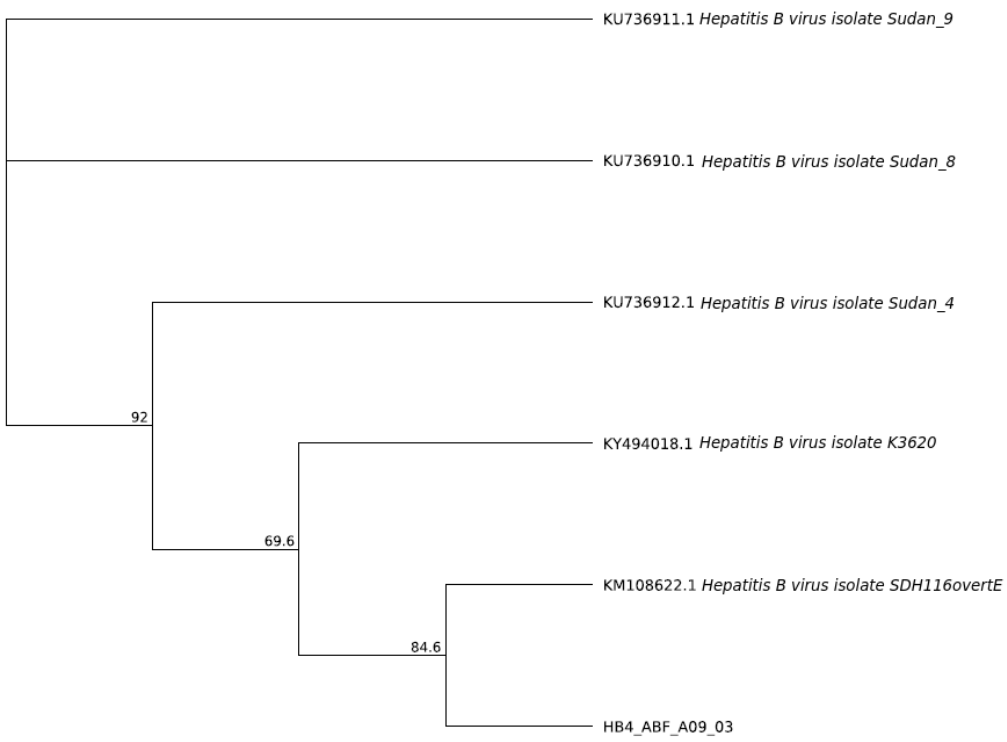


Figure 4.11: Phylogenetic Tree showing Relationship between HB4 from Blood Donor with OBI and other Hepatitis B viruses.

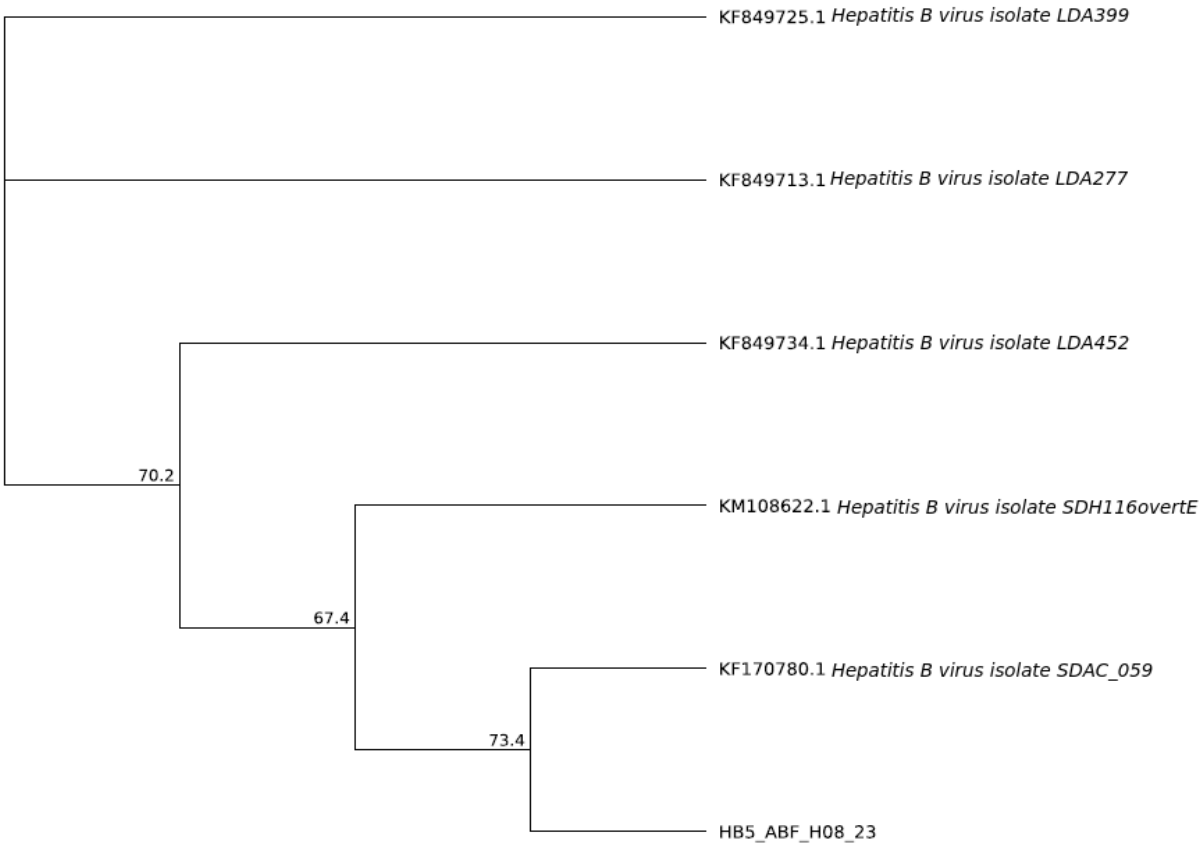


Figure 4.12: Phylogenetic Tree showing Relationship between HB5 from Blood Donor with OBI and other Hepatitis B viruses.

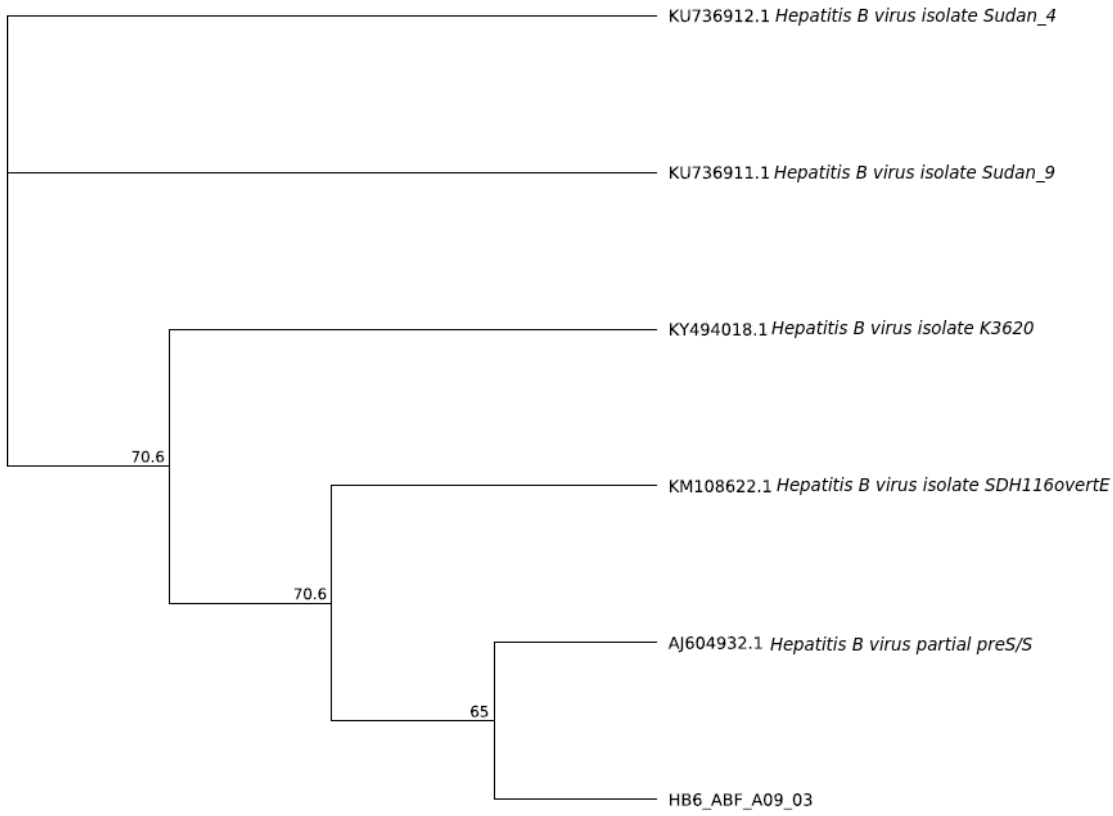


Figure 4.13: Phylogenetic Tree showing Relationship between HB6 Isolated from Blood Donor with overt HBV infection and other Hepatitis B viruses.

The obtained sequence from the isolate produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The sequence of HB7 showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the isolates within the Hepatitis viruses and revealed a closely relatedness to Hepatitis B virus isolate SDH 116 overt E (gb: KM108662.1) than other Hepatitis B viruses (Figure 4.14).

The obtained sequence from the isolate produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The sequence of HB8 showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the isolates within the Hepatitis viruses and revealed a closely relatedness to Hepatitis B Virus isolate SDAC_059 (gb: KF170780.1) than other Hepatitis B viruses (Figure 4.15).

The sequence of HB9 isolate from a blood donor showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the isolates within the Hepatitis viruses and revealed a closely relatedness to Hepatitis B virus strain AYNF 362 (gb: KU984104.1) than other Hepatitis B viruses (Figure 4.16).

The obtained sequence from the isolate produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The sequence of HB10 showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the isolates within the Hepatitis viruses and revealed a closely relatedness to Hepatitis B Virus strain AYNF241 (gb: KU984100.1) than other Hepatitis B viruses (Figure 4.17).

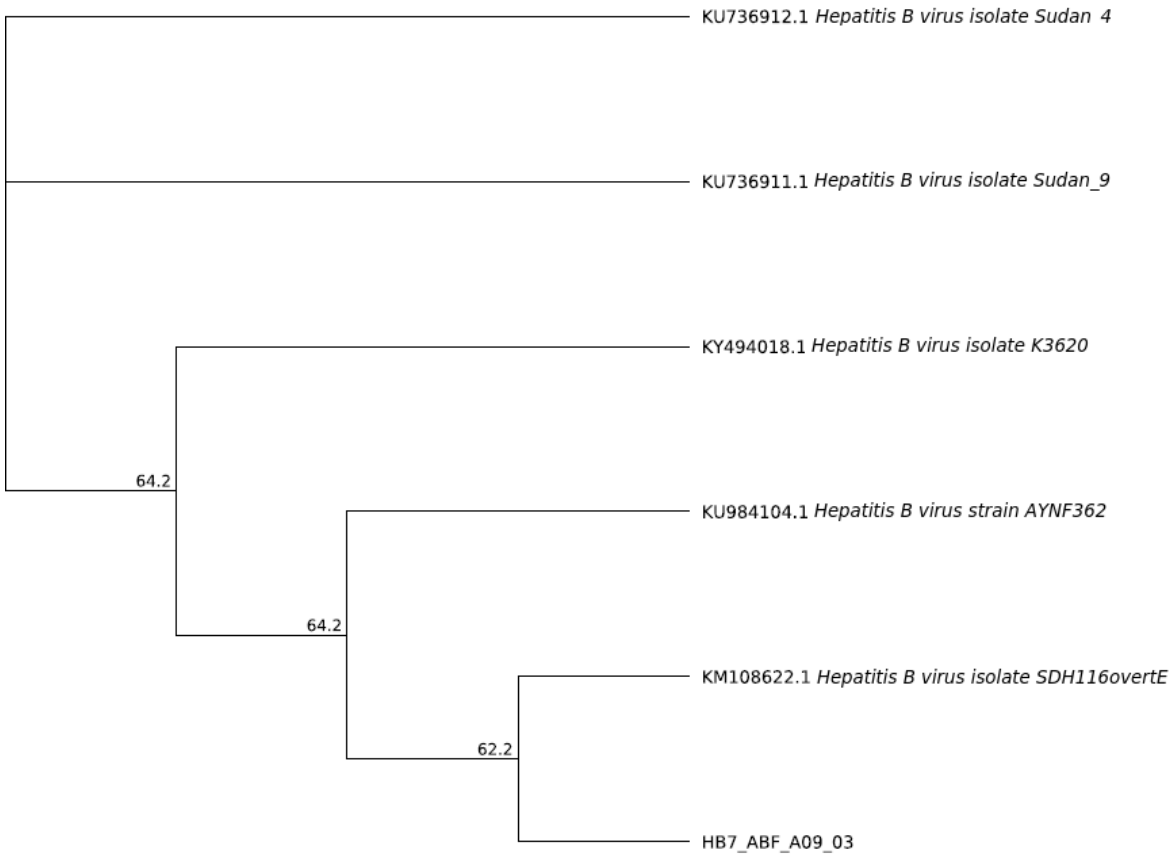


Figure 4.14: Phylogenetic Tree showing Relationship between HB7 Isolated from Blood Donor with Overt HBV Infection and other Hepatitis B viruses.

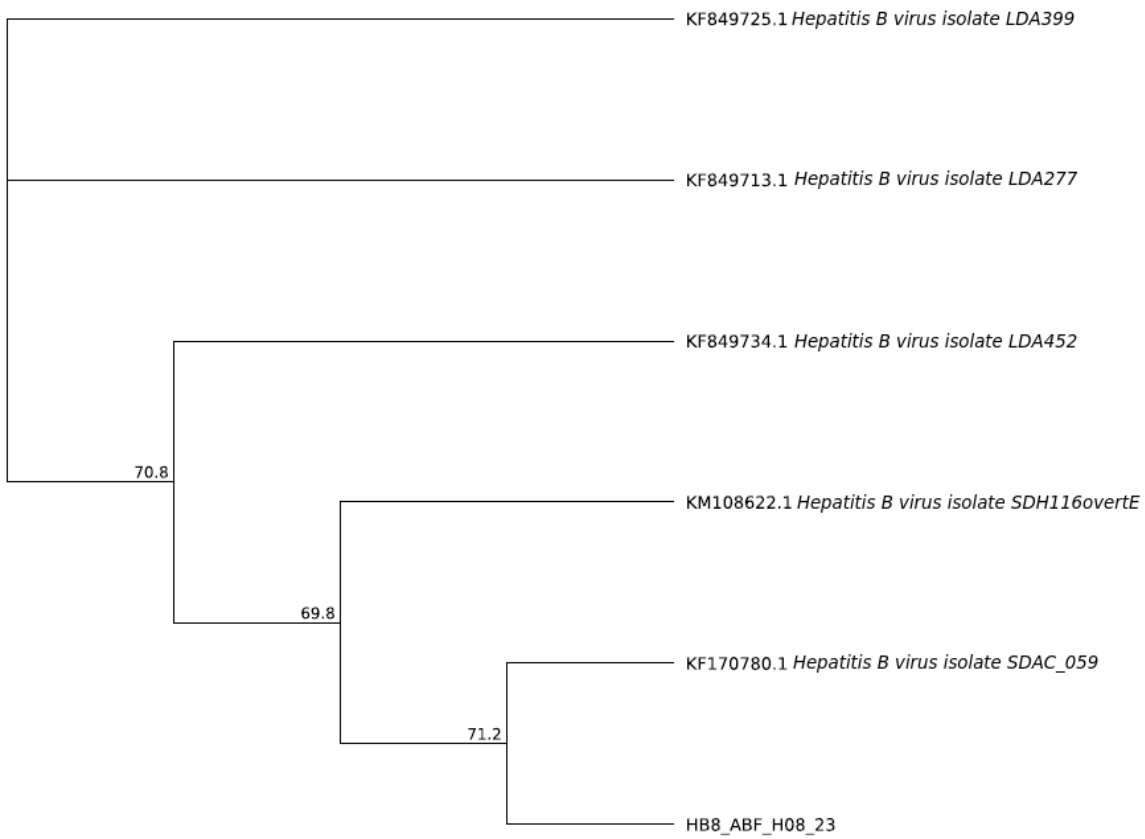


Figure 4.15: Phylogenetic Tree showing Relationship between HB8 Isolated from Blood Donor with Overt HBV Infection and other Hepatitis B viruses

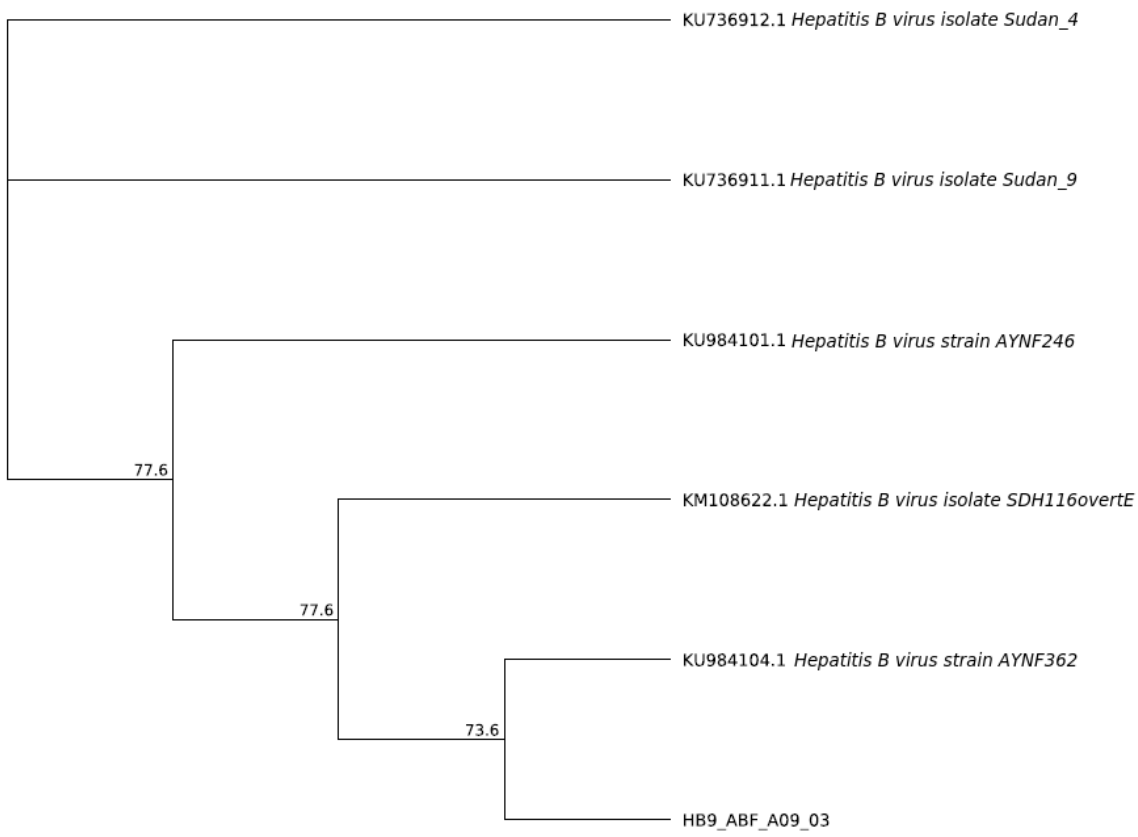


Figure 4.16: Phylogenetic Tree showing Relationship between HB9 Isolated from Blood Donor with Overt HBV Infection and other Hepatitis B viruses.

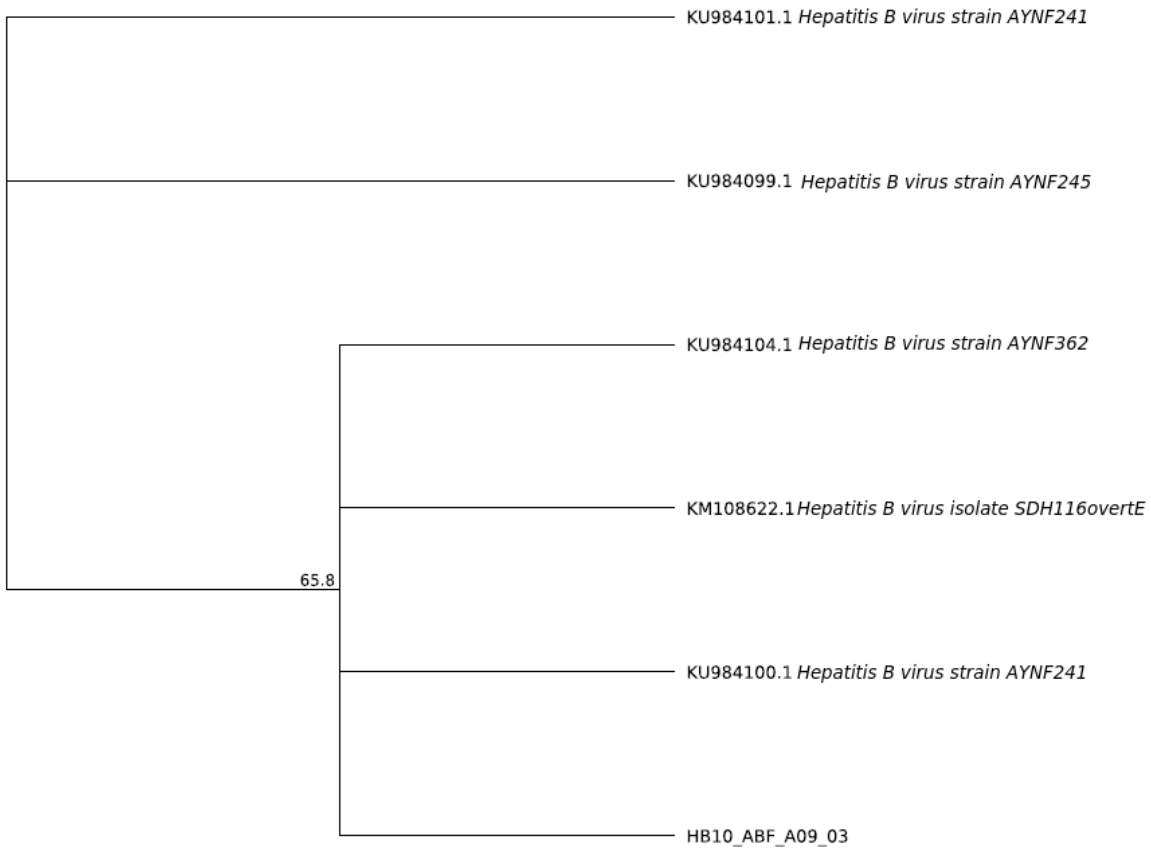


Figure 4.17: Phylogenetic Tree showing Relationship between HB10 Isolated from Blood Donor with Overt HBV Infection and other Hepatitis B viruses.

The obtained V3 sequence from the isolates produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The V3 of the isolate P1 showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the V3 of the isolate P1 within the HIV viruses and revealed a closely relatedness to the V3 of HIV-1 isolate P3599 (gb: DQ32363.1) than the V3 of other HIV viruses (Figure 4.18).

The V3 of the isolate P2 showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the V3 of the isolate P2 within the HIV viruses and revealed a closely relatedness to the V3 of HIV-1 isolate NGIB 04_009 (gb: KF437610.1) than the V3 of other HIV viruses (Figure4.19).The obtained V3 sequence from the isolates produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database.

The V3 of the isolate P3 showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the V3 of the isolate P1 within the HIV viruses and revealed a closely relatedness to the V3 of HIV-1 isolate MFU54_D7 (gb: HQ236565.1) than the V3 of other HIV viruses (Figure 4.20).The obtained V3 sequence from the isolates produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database.

The V3 of the isolate P4 showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the V3 of the isolate P4 within the HIV viruses and revealed a closely relatedness to the V3 of HIV-1 isolate 025P34778/02 (gb: AY304381.1) than the V3 of other HIV viruses (Figure 4.21).

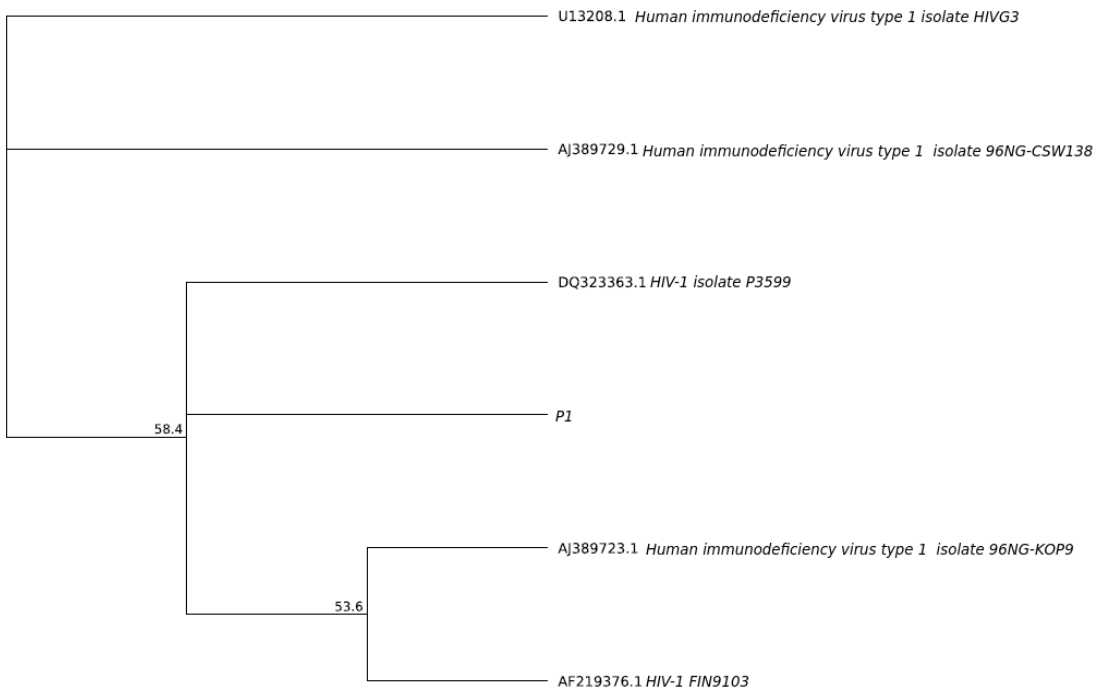


Figure 4.18: Phylogenetic Tree showing Relationship between the V3 of the isolate P1 from a Blood Donor and the V3 of other HIV isolates.

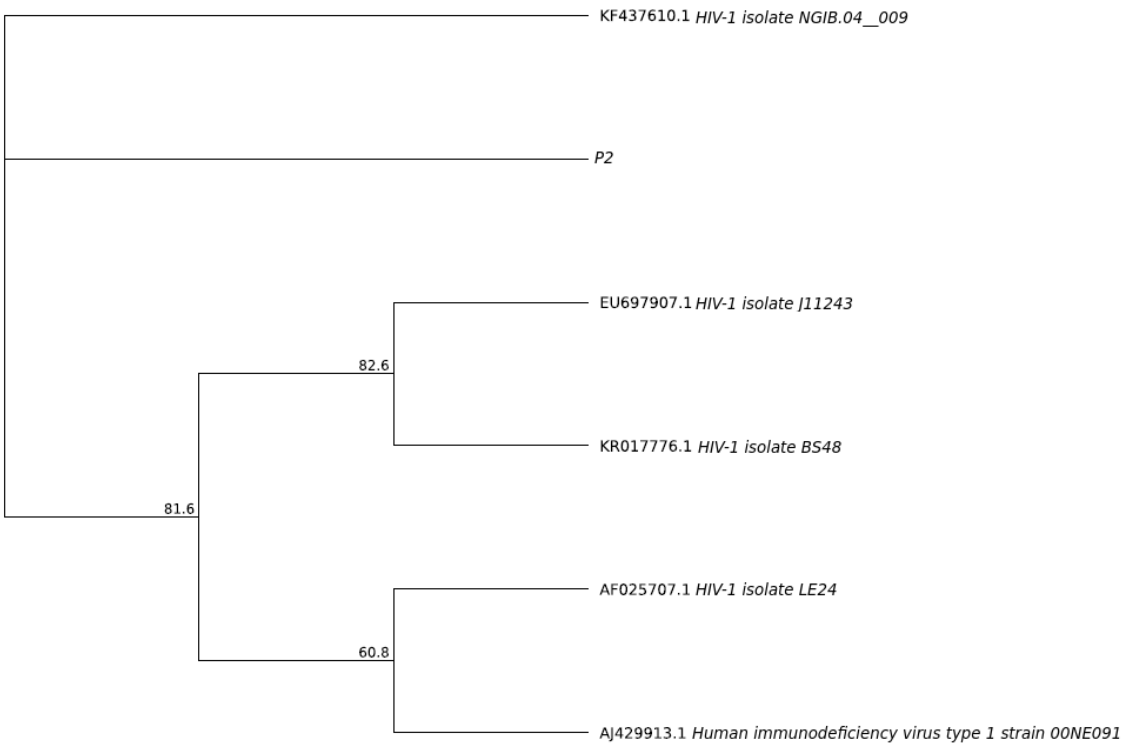


Figure 4.19: Phylogenetic Tree showing Relationship between the V3 of the isolate P2 from a Blood Donor and the V3 of other HIV isolates.

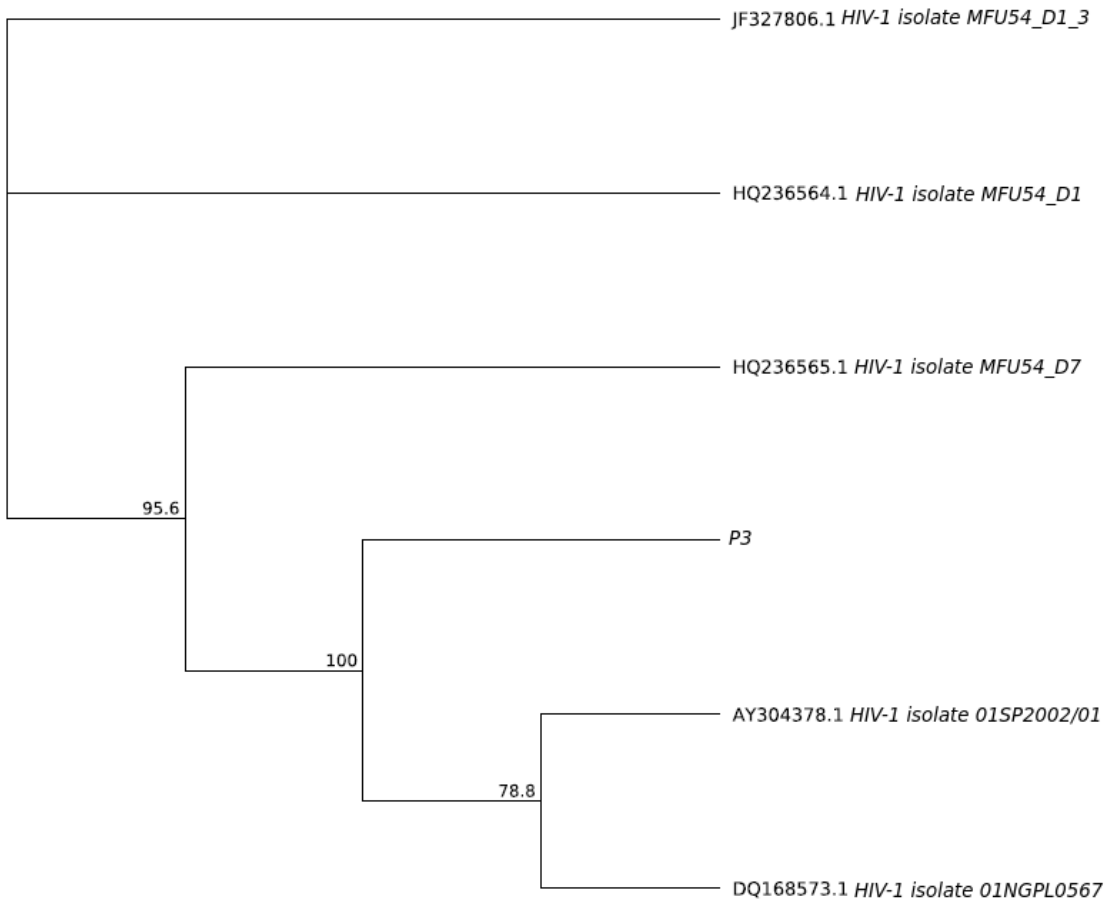


Figure 4.20: Phylogenetic Tree showing Relationship between the V3 of the isolate P3 from a Blood Donor and the V3 of other HIV isolates.

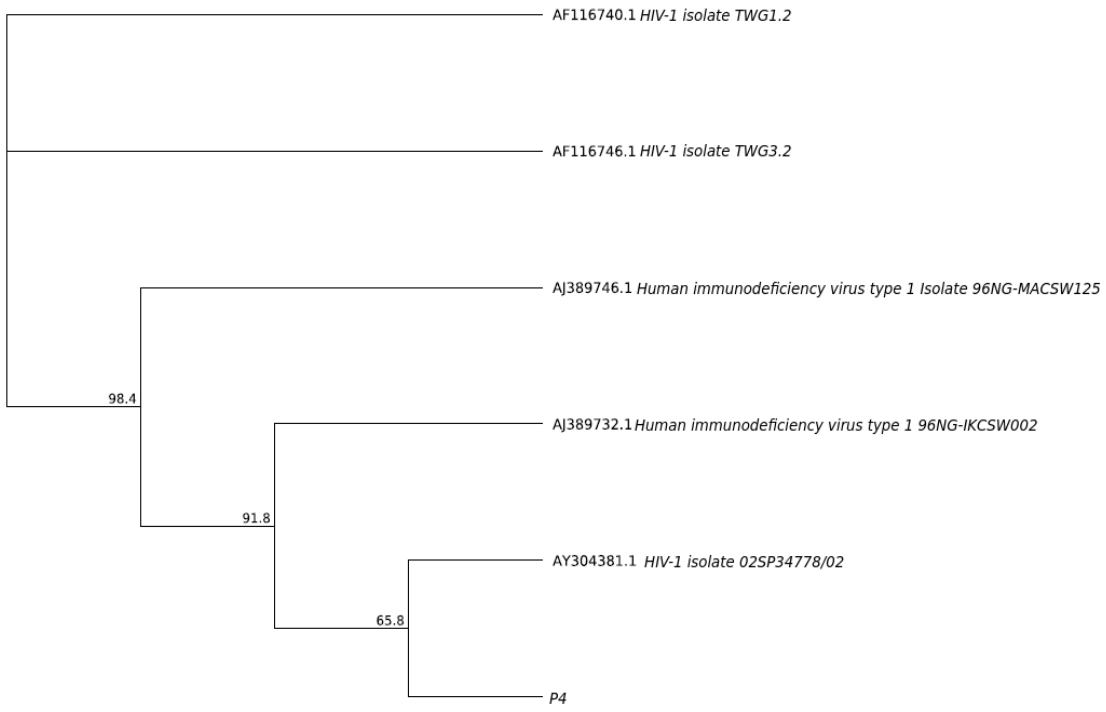


Figure 4.21: Phylogenetic Tree showing Relationship between the V3 of the isolate P4 from a Blood Donor and the V3 of other HIV isolates.

Table 4.11 presented HBV DNA viral load among blood donors with occult HBV infection compared with individuals with overt HBV infection. The result showed that most of the OBI isolates tested were unquantifiable indicating that the viral load was below 20 IU/mL, the LLOD of COBAS Roche Real Time PCR used. However, the average HBV DNA for OBI blood donors that were quantifiable stood at 93±35 IU/mL with a range of < 20-128 IU/mL. It was also observed that the average HBV DNA (viral load) for blood donors positive for HBsAg was 33176108±574579 IU/mL with a range of 924- 99520111IU/mL, indicating that blood donors with overt infection have higher viral load than donors with occult HBV infection. Table 4.12 showed transfusion transmissible viral infections risk assessment at the blood bank of these two Teaching Hospitals. Of the 198 blood units screened and certified fit for transfusion, it was observed that 34 representing 17.2% were positive for HBV DNA, 4 (2.0%) was positive for HCV RNA and 8 blood units representing 4.0% was positive for HIV RNA. This is statistical significant $p<0.0001$. Pattern of Alanine Aminotransferase (ALT) level among blood donors with occult HBV infection, overt HBV infection, HCV and HIV positive blood donors is presented in Table 4.13. Out of the 61 samples tested for Alanine Aminotransferase, 1 sample had a significant high level above the expected normal range representing 1.6% population. All the OBI blood donors as well as HIV and HCV positive samples have insignificant level of ALT (normal value) in their blood samples. The only positive sample identified with significant level of ALT was HBsAg positive blood donor representing 8.3% of HBsAg positive samples tested.

Table 4.14 presented the Alpha Feto-protein levels among the blood donor participants. Out of the 61 samples tested for Alpha Feto Protein, 3 samples have a significant high level above the expected normal range representing 4.9% population. All the OBI blood donors as well as HBV and HCV positive samples have insignificant levels (normal values) of AFP in their blood. The 3 samples with significant level (high values) of AFP in their blood samples were HIV positive blood donors representing 37.5% of HIV positive samples tested. Table 4.15 compares Alanine aminotransferase (ALT) and Alpha feto protein (AFP) among three groups of blood donors: occult, overt and HBV seronegative blood donors using analysis of variance (ANOVA). The values obtained were normal as no statistical significant difference was observed.

Table 4.11: Mean Comparison of HBV DNA Load of Blood Donors with Occult and Overt HBV Infection in the Study Population

HBV DNA Status of Blood Donors	No. of Samples Tested	Average (Mean) in IU/mL X±SD	Range in IU/mL	Student-t Test (p-value)
Occult Blood Donors	3	93±35	<20- 128	*57.9 (<0.0001)**
Overt Blood Donors	3	33,176,108±574579	924- 99520111	

****Statistically significant (p<0.05)***

Key:

IU/mL: International unit per milliter, **<:** Less than, X±SD: Mean Standard Deviation

Table 4.12: Transfusion Transmissible Viral Infections Risk at UATH, Abuja and NAUTH, Nnewi Blood Banks

Viral Agents	No. of Blood Units Screened and Certified safe for Transfusion	No. of Positive Samples (PCR)	% Frequency	Chi-Square (p-value)
HBV	198	34	17.2	*53.92 (<0.0001)**
HCV	198	4	2.0	
HIV	198	8	4.0	

**** Statistically significant (p< 0.05)***

Key:

HBV: Hepatitis B Virus, **HCV:** Hepatitis C Virus, **HIV:** Human Immunodeficiency Virus**PCR:** Polymerase Chain Reaction, **<:** Less than

Table 4.13: Alanine Aminotransferase Level among Blood Donors with Occult and Overt HBV Infection in the Study Population

Blood Donors Status	No. of Samples Tested	No. (%) Samples with Significant ALT Value	Mean±SD value of ALT in IU/L	Range in IU/L
OBI	14	0 (0)	4.9±3.3	1.4- 11.8
Overt	20	1 (5)	5.8±2.4	3.2- 12.2
HCV Positive	04	0 (0)	4.5±2.9	3.7- 5.6
HIV Positive	08	0 (0)	4.1±1.2	3.0- 5.4
HBV, HCV & HIV Negative	15	0 (0)	3.5±0.87	2.9- 5.3
Total	**61**	**1 (1.6)**	**4.6±3.1**	**1.4 - 12.2**

Significant ALT value >12.0 IU/L

Key:

ALT: Alanine Aminotransferase, **IU/L:** International Unit per Litre, **OBI:** Occult HBV Infection, **Overt:** HBsAg Positive, **>:** Greater than

Table 4.14: Alpha-Feto Protein Level of Blood Donors with Occult and Overt HBV Infection in the Study Population

Status of Blood Donors	No. of Samples Tested	No. (%) Samples with Significant AFP Value	Mean±SD (Average) value of AFP in ng/ml	Range in ng/ml
OBI	14	0 (0)	4.2±1.1	2.2-7.3
Overt	20	0 (0)	3.7±0.9	2.3- 4.3
HCV Positive	04	0 (0)	3.4±0.85	3.2- 4.4
HIV Positive	08	3 (37.5)	6.9±2.7	3.7- 10.3
HBV, HCV & HIV Negative	15	0 (0)	3.7±0.9	3.2- 4.2
Total	**61**	**3 (4.9)**	**4.4±1.2**	**2.2- 10.3**

Significant AFP value >8.5ng/ml

Key:

AFP: Alpha Feto Protein, **ng/ml:** nanogram per milliter**OBI:** Occult HBV Infection, **Overt:** HBsAg Positive, >: Greater than

Table 4.15: Alanine Aminotransferase and Alpha Feto-Protein Levels among Blood Donors with Negative, Occult and Overt HBV Infection in the Study Population

Parameters (Significant value)	OBI n=14 X±SD	OVERT n=20 X±SD	HBV negative n=15 X±SD	ANOVA (p-value)
ALT (>12 IU/L)	4.9±3.3	5.8±2.4	3.5±0.87	*1.622 (0.209)*
AFP (>8.5ng/ml)	4.2±1.1	3.7±0.9	3.7±0.9	*0.999 (0.376)*

Statistically Significant (p< 0.05)

Key:

OBI: Occult HBV blood donors, **Overt:** HBV positive blood donors, **n:** Number of samples, **ANOVA:** Analysis of variance, **X±SD:** Mean Standard Deviation, **ALT:**Alanine Aminotransferase, **AFP:**Alpha Feto-Protein, **>:** Greater than

Table 4.16 compared the means of (RBC) red blood cells, (HCT) hematocrit, (HGB) hemoglobin, (WBC) white blood cells, CD4 T-lymphocytes and platelets among OBI blood donors and seronegative blood donors. We found no statistically significant difference across all blood cells examined between the two groups. The mean blood values of HCT, HGB, WBC, RBC, Platelets count and CD4 Cell count fall within the normal acceptable range.

Table 4.17 showed the haematological values and CD4 cell count of blood donors with occult HBV Infection. Out of 14 blood donors with OBI 1, 6, 3, 1 and 2 have low values of CD4, HGB, WBC, Platelets count and RBC respectively. This represents 7.1%. 42.9%, 21.4%, 7.1% and 14.3% respectively. None of the OBI blood donors had low HCT value.

Table 4.18 showed the relationship of HBV serologic Markers to HBV DNA (Viral Load) among some blood donors with occult and overt HBV infection. From the Table 4.21, it was observed that the viral load of blood donors with overt HBV infection was greater than those of OBI. However, one of the OBI blood donors had a very high HBV DNA level of 31379 copies per ml. Also the blood donor sample positive for HBeAg marker has the highest HBV DNA (Viral load) among other blood donors with anti-HBc and anti-HBs markers.

Table 4.19 presented the characteristics of the blood donor who had occult HBV infection with very high viral load. The significant characteristics observed were as follow; the donor lacked knowledge of HBV/HBV infection and has not been vaccinated with HBV vaccines. It was also seen that this OBI blood donor had low blood value of Haemoglobin, 9.3g/dl and CD4 cell count of 511/uL. Moreover, this blood donor is a young man of 24 years old, a student and a repeat (old) blood donor with anti-HBs positivity to mention but a few. One (1) out of 14 OBI blood donors representing 7.1% isolated in this study with high viral load is quite significant and remarkable.

Table 4.16: Mean Comparison of CD4 Cells and some Haematological Parameters of OBI and Seronegative Blood Donor Participants in the Study Population

Blood Cells (Reference values)	**OBI Blood Donors n=14 X±SD**	**Seronegative Blood Donors n= 18 X±SD**	**Student-t Test**	**p-value**
RBC ($4.3\text{-}6.0 \times 10^9$/L)	4.8 ± 0.6	5.1 ± 0.6	1.37	*0.25*
HCT (35-54%)	42.2 ± 5.0	41.5 ± 3.5	0.18	*0.67*
HBG (12-18.0g/dl)	12.7 ± 1.7	13.4 ± 1.3	1.41	*0.24*
WBC ($4.0\text{-}10.0 \times 10^9$/L)	4.9 ± 1.1	4.5 ± 0.9	1.13	*0.29*
CD4 (500-1500/ul)	773.6 ± 269.5	891.1 ± 283.4	1.41	*0.24*
PLT ($150\text{-}450 \times 10^{12}$/L)	196.5 ± 50.6	215.3 ± 35.2	1.53	*0.22*

Statistical significant (p<0.05)

Key:

RBC: Red Blood Cell; **HCT:** Haematocrit; **HGB:** Haemoglobin; **WBC:** White Blood cell; **CD4:** CD4 Cells; **PLT:** Platelets,**n=** Number of samples, **X±SD:** Mean Standard Deviation **g/dl:** Gram per deciliter, **ul:** Microliter

Table 4.17: Blood Values of CD4 and some Haematological Parameters of Blood Donors (n=14) with Occult HBV Infection in the Study Population

Haematological Parameters (Reference values)	No. of Samples Tested with Low values	% of Samples with Low values	X±SD Value n= 14	Range
CD4 (500-1500/ul)	1	7.1	774±269.5	263-1359/ul
HCT (35-54%)	0	0	42±5.0	36.8-54.7%
HGB (12-18g/dl)	6	42.9	12.4±1.6	9.3-15.8g/dl
RBC ($4.3\text{-}6.0 \times 10^{12}$/L)	2	14.3	4.74±0.5	4.19-6.10× 10^{12}/L
WBC $4.0\text{-}10.0 \times 10^{9/L}$)	3	21.4	4.9±1.1	$2.9 - 6.8 \times 10^{9}$/L
PLT ($150\text{-}450 \times 10^{9}$/L)	1	7.1	197±50.5	$140\text{-}279 \times 10^{9}$/L

Key:

RBC: Red Blood Cell; **HCT:** Haematocrit; **HGB:** Haemoglobin; **WBC:** White Blood cell; **CD4:** CD4 Cells; **PLT:** Platelets, **Low values:** Values below normal reference values, **ul:** Microliter, **g/dl:** Gram per deciliter

Table 4.18: Relationship of HBV Markers to HBV DNA (Viral Load) among Some Blood Donors with Occult and Overt HBV Infection at UATH, Abuja and NAUTH, Nnewi

Sample ID	HBV Status	HBV Markers Detected	HBV DNA (Viral Load)
UATH 067	OBI	Seronegative	92IU/mL
UATH 026	OBI	Anti-HBs	31,379 IU/mL*
UATH 102	OBI	Anti-HBc, & Anti-HBs	128IU/mL
UATH 047	OBI	Anti-HBc	60IU/mL
UATH 029	OVERT	HBsAg, Anti-HBc & Anti-HBe	7,290IU/mL
NAUTH 094	OVERT	HBsAg, Anti-HBc & Anti-HBe	924IU/mL
UATH 009	OVERT	HBsAg, HBeAg Anti-HBc & Anti-HBe	99,520,111 IU/mL

****OBI Blood Donor with very high HBV DNA (viral load)***

Key:

UATH: University of Abuja Teaching Hospital, **NAUTH:** Nnamdi Azikiwe University Teaching Hospital, **OBI:** Occult hepatitis B virus infection, **Overt:** Confirmed HBV Infection, **HBV DNA:** Hepatitis B virus Deoxyribonucleic Acid

Table 4.19: Characteristics of OBI Blood Donor with High HBV DNA (Viral Load) in the Study Population

Characters	**Observations**
Sociodemographic Data	24 years, Male, Single, Student
ABO Blood Group	O+
Donor Status	Repeat donor/Family replacement
HBV Risk Factors	Lack of knowledge of HBV, No HBV vaccination, Domestic accident and visiting commercial barber.
HBV Markers Pattern	HBsAg- HBsAb+ HBcAb- HBeAg- HBeAb- HBcAb IgM-
HBV DNA (Viral Load)	31379 IU/mL
ALT Value	2.9IU/L (Normal <12.0 IU/L)
AFP Level	6.6ng/ml (Normal <8.5ng/ml)
HBV Genotype	Genotype E
Haematological Parameters	WBC:6.0 $\times 10^9$/L,PLT:157 $\times 10^9$/L, HCT: 36.6%, HGB:9.3g/dl RBC: 4.93 $\times 10^{12}$/L
CD4 Cell Count	511/uL

Key:

O+: Blood group O Rhesus positive, **+:** Positive,**-:** Negative, **IU/L:** International unit per Litre, **ng/ml:** nanogram per millitre,**<:** less than, **mL:** Milliter, **ul:** Microliter

Plate 4.1 presents Agarose Gel Electrophoresis Showing Amplified HBV Gene of Blood Donors with Occult HBV Infection. From this plate, Lanes 1,2,3,4,5,6,7, 10, 11 13 14, 15 showed successful amplification while lanes 8, 9, 12 and 16 indicated unsuccessful amplification. The plate showed a positive conventional PCR result from hepatitis B surface negative core positive plasma samples of healthy blood donors. A 600bp DNA product was amplified using primers specific for hepatitis B virus S-gene. All lanes showed a PCR product of the expected size. Lane M represents a 500bp (ladder) molecular marker.

Plate 4.2 presents Agarose Gel Electrophoresis Showing Amplified HBV Gene of Seronegative Blood Donors. Lane M represents a 500bp Ladder and lane 21 showed the only amplified env gene of HBV. The result of Agarose Electrophoersis for detection of PCR amplified product showed that 1 (3.1%) out of 32 samples was positive for HBV DNA.

Plate 4.3 represents Agarose Gel Electrophoresis showing the Amplified HCV Bands.

L represents 500bp ladder while lanes 2, 3, 11, and 12 represent the amplified HCV bands. Out of 14 samples of PCR amplified product, 4 samples representing 28.6% were positive for HCV RNA.

Agarose Gel Electrophoresis showing the V3 Bands of the HIV isolated from Blood Donors is presented in Plate 4.4. M represents the 500bp molecular ladder, while lanes 1, 2, 9,10,11,12, 14 and 22 represent the amplified V3 region of HIV. The plate showed that 8 (36.4%) out of 22 PCR amplified products were positive for HIV RNA using Agarose electrophoresis for detection.

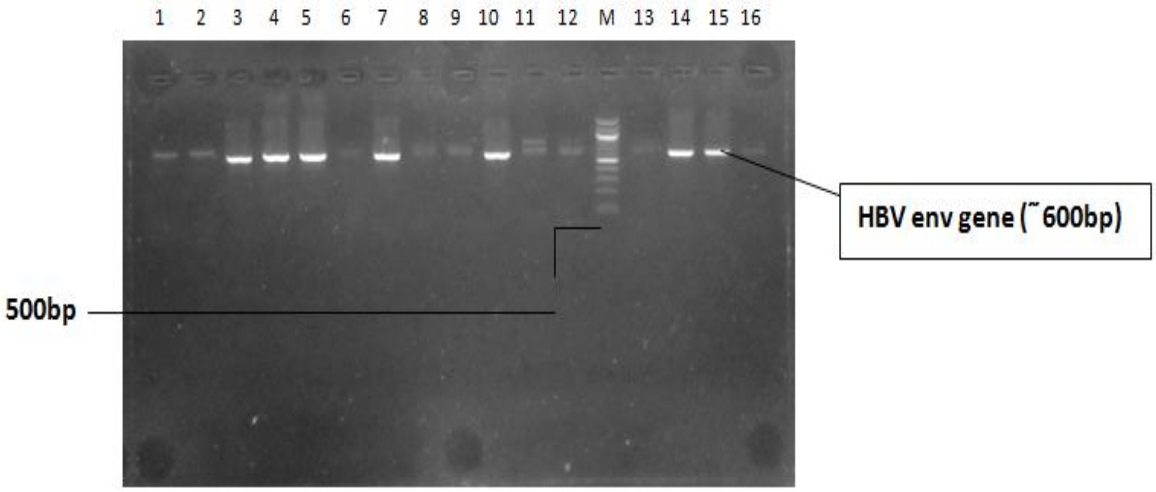


Plate 4.1: Agarose Gel Electrophoresis Showing Amplified HBV Gene of Blood Donors with Occult HBV Infection in the Study Population

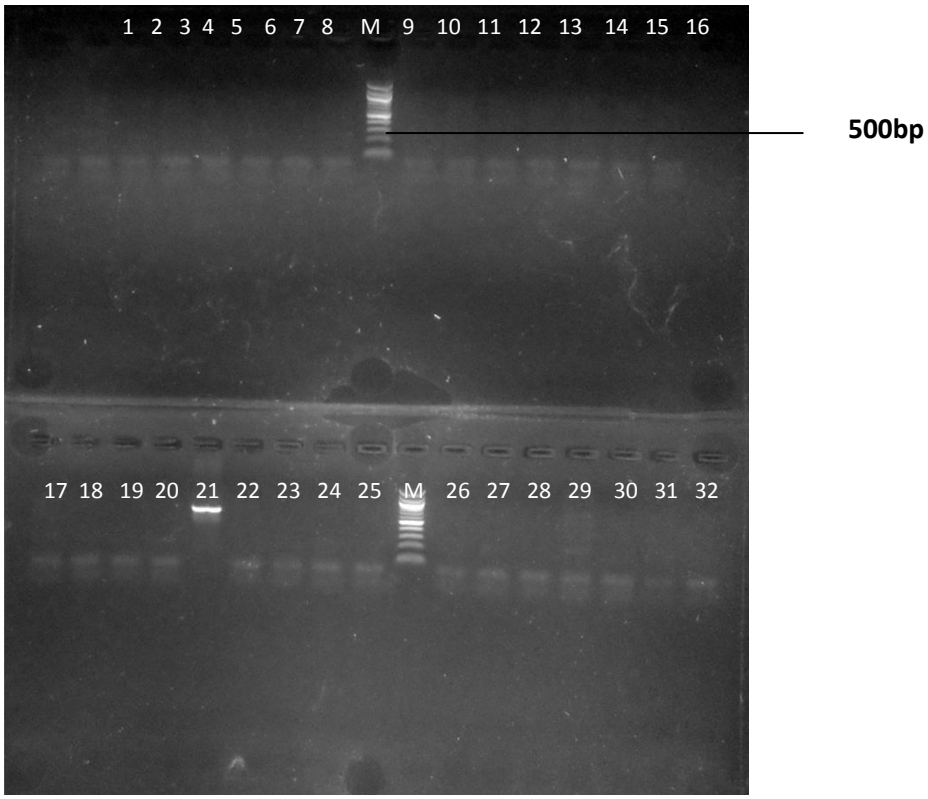


Plate 4.2: Agarose Gel Electrophoresis Showing Amplified HBV Gene of Seronegative Blood Donors (negative for HBV serologic markers) in the Study Population

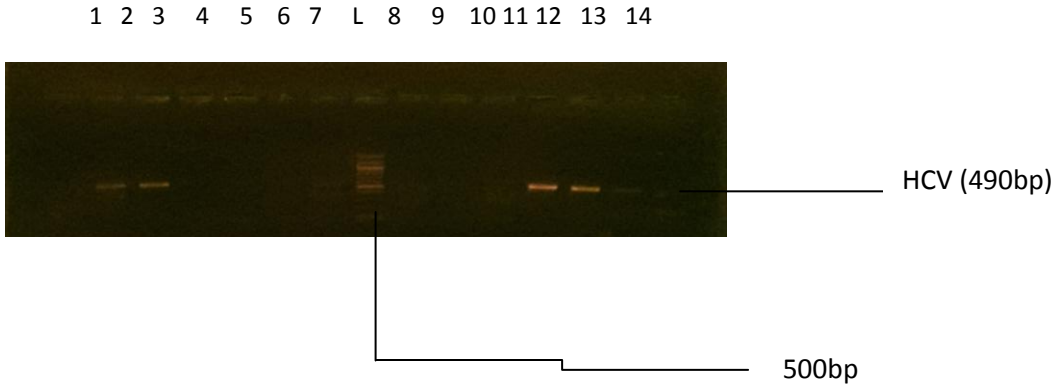


Plate 4.3: Agarose Gel Electrophoresis Showing the Amplified HCV Bands among blood donors in the Study Population

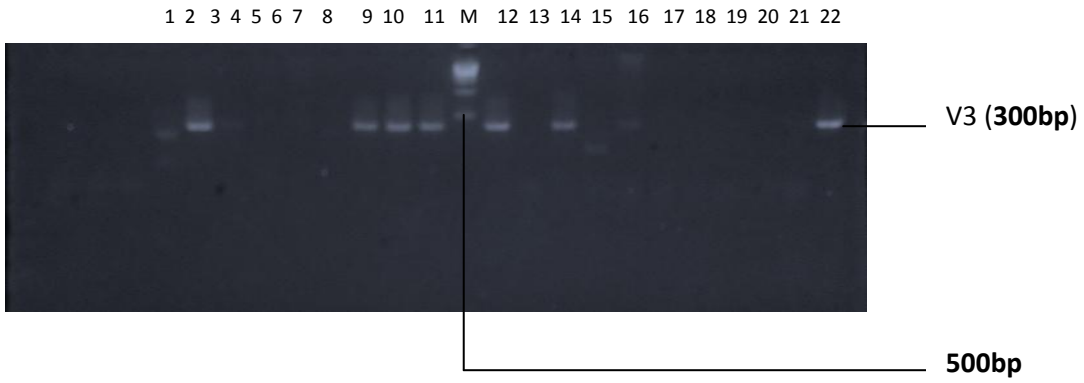


Plate 4.4: Agarose Gel Electrophoresis Showing the V3 Bands of the Human Immunodeficiency Virus from Blood Donors in the Study Population

CHAPTER 5

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

Laboratory Diagnosis of Hepatitis B Virus (HBV) infection is routinely based on serological assay of Hepatitis B surface antigen (HBsAg) (Badur and Akgun 2001). Occult hepatitis B virus infection is generally defined as the detection of HBV -DNA in the serum or liver tissues of subjects who have negative test for HBsAg. Occult Hepatitis B virus infection (OBI) has been reported recently in Nigeria (Oluyinka *et al.*, 2015; Olotu *et al.*, 2016). There is relatively high prevalence of OBI in areas where HBV infections are endemic worldwide, and OBI represents a major threat to blood safety (Torbenson and Thomas, 2002). Therefore, performing HBsAg test alone for blood donors does not completely eliminate the risk of HBV transmission to blood recipients. Studies have shown that OBI is associated with the presence of anti-HBc and/ or anti-HBs serologic markers (Oluyinka *et al.*, 2015; Olotu *et al.*, 2016).

In this study, we investigated the prevalence of OBI among blood donors at two Teaching Hospitals in Nigeria by Nucleic Acid Amplification Testing (NAT) and characterized the serological as well as molecular and genetic aspects of those OBI strains in different Molecular Diagnostic laboratory settings in Nigeria and South Africa. The result of this study shows an overall prevalence of occult HBV infection of 14%, with 22% and 6% prevalence rates at UATH, Abuja and NAUTH Nnewi respectively. This is statistically significant $p<0.05$. A study on Occult Hepatitis B virus infection (OBI) in Nigeria has reported a prevalence rate of 8% among 100 blood donors in Abakaliki, South-Eastern Nigeria (Nna *et al.*, 2014). The OBI prevalence of 14% reported in current study is higher than Nna *et al.*, (2014) finding but this might not be surprising for a sub-Saharan country endemic to HBV where the prevalence of anti-HBc exceeds over 50% of the blood donor population (Allain and Candotti 2009; Olotu *et al.*, 2016). However, findings from this study is in agreement with the study of Oluyinka *et al*, (2015) that reported 17% prevalence among 429 blood donors in South Western Nigeria. In another study, Opaleye *et al.*,

(2014a) found a very high prevalence of 36 % among 429 blood donors. These prevalence rates observed from different areas is not surprising as there may be differences in the prevalence of OBI among blood donors from one part of the country to another, reflecting differences in the prevalence of overt HBV infection which exist from one part of the country to the other (Jombo *et al.*, 2005; Forbi *et al.*, 2010). Occult Hepatitis B Virus infection (OBI) is unexpectedly high in Nigeria taking into account that the seroprevalence of HBV infection in Nigeria is from 9%-39% (Emechebe *et al.*, 2009; Ugwuja and Ugwu, 2010).

Other studies on OBI in Nigeria have been in other subject populations such as Ola *et al.*, (2009) who found OBI in 2 of 28 chronic hepatitis C patients in Ibadan, Opaleye *et al.*, (2014b) also found a prevalence of 11.2 % of OBI in HIV positive patients using archived specimens from Ikole Ekiti in Nigeria. However, these subjects were not likely to qualify as blood donors and as such no risk of their blood being transfused. The prevalence of 14% recorded in this study was higher than the 1.7 % found in Ghana by Zahn *et al.*, (2008). In Brazil, Silva *et al.*, (2004) found 3.3 % but used a PCR assay with a Low Limit of Detection (LLOD) of about 200 IU/mL, which is less sensitive than the real-time PCR assay used in this study with LLOD of about 20 IU/mL. This could have resulted in a lower detection of OBI. The prevalence found in this study is much higher than what has been found in the USA and some other western countries where only 0.1–2.4 % of HBsAg negative, anti-HBc positive blood donors were found to have HBV DNA (Hollinger and Sood, 2010). This is not surprising as 5 % of the population have come in contact with HBV in those regions unlike in Nigeria where over 70 % of the population have at some time in their lives been exposed to or infected with HBV (Kire, 1996; Uneke *et al.*, 2005; Mbawuaga *et al.*, 2014; Olotu *et al.*, 2016). Prevalence of 14% recorded in this study was much lower than 38 % that was reported by Yotsuyanagi *et al.*, (2001) in Japan although the sample size was just 50 blood donors which is small compared to this study with sample size of 100 seronegative blood donors that was assayed for HBV DNA. This may also be the reason why Jafarzahdeh *et al.*, (2007) found 28.6 % prevalence in Iran as they only assayed 14 HBsAg negative, anti-HBc positive samples for HBV DNA and found four samples positive. The prevalence of OBI varies to a great extent in different countries,

depending on a number of factors that includes HBV endemicity, liver disease, HBV screening method, sample size and primers employed for NAT (Allain, 2017). In Northeast China, the prevalence of OBI was observed up to 10.6% among 359 HBsAg-negative healthy individuals (Fang *et al.,* 2009). In other studies, only 0.1% of OBI was detected among 10,727 seronegative blood donors from Taiwan (Li *et al.*, 2008) and 3% in an Italian migrant population (Tramuto *et al.,* 2012). It has been reported in Laos, a HBV endemic region, Jutavijittum *et al.*, (2014) high OBI prevalence of 10.9% among blood donors who were HBsAg-negative, anti-HBc and/or anti-HBs-positive. In North Africa, a study conducted among 1026 Egyptian blood donor samples revealed that 8% were reactive to anti-HBc and 0.5% was positive for HBV-DNA (Antar *et al.*, 2010). The data on OBI prevalence is limited in sub-Saharan Africa. Nevertheless, studies in patients with HIV infection from Ivory Coast and Sudan have shown that OBI prevalence was 10% and 15%, respectively (N'Dri-Yoman *et al.*, 2010; Mudawi *et al.*, 2014).

This study demonstrated that the OBI samples had significantly lower HBV-DNA copies compared to HBsAg positive blood samples. The average viral load for OBI samples tested was 93 IU/mL while for overt HBV blood samples is over 33,000,000 (33 Million) IU/mL. This is in concordance with other studies in Nigeria (Oluyinka *et al.*, 2015; Olotu *et al*., 2016). Both studies obtained HBV DNA load less than 100 IU/mL among blood donors with occult HBV infection. The low level of viral load inferred in this current study showed almost all OBI cases are infected with replication competent HBV, revealing a strong suppression of replication activity and gene expression, resulting in a reduced viral load (Raimondo *et al.*, 2008b). However, 1 (7.1%) out of 14 OBI blood donors tested for HBV DNA (viral load) has high value of 31379 IU/mL. This high value has not been reported among blood donors with occult HBV infection in Nigeria. In a study by Diarra *et al.*, (2018) in Burkina Faso, more than two-thirds of subjects with HBV DNA (40/56) and HBsAg negative had a viral load > 200 IU/mL (200 to 13.6 10^6 IU/mL). Another study reported a viral load between undetectable and 3,670 IU/mL in "OBI" cases among blood donors in Southeast Asia (Candotti *et al.*, 2012). A possible reason for this high HBV DNA load could be that the virus was released from the hepatocytes to blood circulation at the point of bleeding the donor for screening hence the high viral load recorded. This could also

be attributed to escape mutations that can lead to a change in the immunologic epitope thus inhibiting HBsAg secretion (Bremer *et al.*, 2009).

Another reason for this high viral load could be reactivation of latent virus. Reactivation of HBV is an abrupt increase of HBV replication in a patient with inactive or resolved hepatitis B (Hoofnagle, 2009). Studies have shown that reactivation can occur spontaneously (Hoofnagle, 2009; Roche and Samuel, 2011). In addition, individuals who undergo chemotherapy have a higher risk of viral reactivation than people who are not on medication (Mastroianni *et. al.*, 2011). This is because chemotherapy could lead to immunosuppression with resultant viral multiplication. It is also being reported that intermittent viremia can occur in occult HBV infection, and periodic testing of HBV DNA will improve detection of occult HBV infection (Kannangai *et al.*, 2007). Surprisingly, it was observed that this blood donor with high viral load has a CD4 cell count of 511/ul which is on a low side and a mark of immunosuppression. This blood donor was also positive for anti-HBs. It has been shown by Minuk *et al.*, (2005) that individual with anti-HBs ≥100 U/L is probably noninfectious. Possibly, this blood donor under review has anti-HBs titre < 100 U/L. This signifies that the blood of this donor could be highly infectious if transfused to another individual. We were unable to determine the titre of Anti-HBs marker in this blood donor sample. This calls for further study. In 2008, the statements from the Taormina expert meeting on occult hepatitis B virus infection had clarified the definition of OBI in establishing a threshold value of serum HBV DNA < 200 IU/mL (Raimondo *et al.*, 2008b). Furthermore, it also clarified the confusion between a cleared infection of HBV and a "false OBI". Thus, cases with serum HBV DNA levels comparable to those usually detected in the different phases of serologically evident (overt) HBV infection have to be considered as "false OBI" and are usually due to infection by HBV variants (Raimondo *et al.*, 2008b). These become in fact chronic hepatitis B cases. Whatever may be the case whether occult or "false OBI" this blood donor under review and other individuals with high viral load and surface antigen negative poses a risk to blood transfusion as most blood transfusion centres in Nigeria screen blood donors for HBsAg only and bleed donors based on its negativity.

It was also observed in this study that all OBI blood donors as well as those with overt HBV infection belong to HBV Genotype E. This finding is in accord with the report of Oluyinka *et al.*, (2015) that found HBV Genotype E being most prevalent in South West, Nigeria. It also collaborates with the finding of Diarra *et al.*, (2018) in a study in Burkina Faso. They reported that HBV Genotype E was most prevalent in OBI cases in their study.

This current study contradicted previous studies and indicated that the HBV subgenotype A3 and the recombination between HBV genotypes A and E were observed frequently in West Africa (Kurbanov *et al.*, 2005; Makuwa *et al.*, 2006). Iqbal *et al.*, (2016) reported that Genotype D is the most prevalent genotype in Pakistan using type specific primers and multiplex PCR. HBV genotype E is the most prevalent in Nigeria (Odemuyiwa *et al.*, 2001). HBV genotype E is endemic in West Africa and exhibited low genetic diversity (Mulders *et al.*, 2004). In this study, it could be genetically distinguished between the HBV genotypes E from other HBV genotypes by phylogenetic analysis of the *PreS/S* and *PreC/C* regions. Genotyping and sub-genotyping of HBV is important when one is dealing with mixed genotypes and should be identified based on the analysis of full length sequences. However, full length HBV sequences are not always available so that phylogenetic analyses of the *PreS/S* regions are widely accepted for routine HBV genotyping. Based on the phylogenetic analyses of the *PreS/S* regions, it was concluded that all the OBI isolates from this study belong to HBV genotype E. The phylogenetic analysis as seen in phylogenetic trees developed showed that most of our HBV isolates have high relationship with HBV isolates from Sudan (Yousif *et al.*, 2013). In addition, the HIV isolates from sequence analysis have a close relatedness with isolates from Senegal, Cameroon and Italy and all isolates belong to HIV-1 (Hamel *et al.*, 2007). This could be as result of intra border transfer of these viruses from one country to another. Moreover, the HBV isolates from UATH Abuja and NAUTH Nnewi are similar. This could be as a result of people traveling from state to another part of Nigeria and in the process spread this virus from one person to another as HBV can be transmitted through various modes. This include through sex, needle prick injuries and visiting commercial barbing saloon to mention but a few (Lavanya *et al.*, 2012).

Understanding the molecular and immunological mechanisms in determining an OBI has to be investigated explicitly. A hypothesis proposed for the development of OBI was that mutations in the *PreS/S* regions may alter HBsAg antigenicity thereby inhibiting anti-HBs production. A single mutation at the ‘‘a” determinant (amino acids 124–147, e.g., G145R) of HBsAg can lead to a change in the immunologic epitope thus inhibiting HBsAg secretion. The amino acid substitutions in the RT domain of the *P* gene contribute to low copies of HBV-DNA and HBsAg synthesis that may have an associative effect on an occult infection (Samal *et al.*, 2012). We were unable to determine mutation in the HBV sequences obtained. In a study by Oluyinka *et al.*, (2015), all of their OBI strains had the G779 nucleotide substitution that resulted in amino acid change at L217R in the overlapping RT domain of the *P* gene and this may account for the occult nature of the HBV infection in our study. Another study also observed that the L217R substitution within the RT domain was predominant in OBI individuals (Delfino *et al.*, 2012). In addition, mutations in the major hydrophilic region (MHR) also influence the antigenicity and can impair virion secretion consequently leading to HBsAg detection failure in OBI individuals (Huang *et al*., 2012). However, the L209V substitution, which corresponds to the L217R substitution, is not located at the ‘‘a” determinant or in the MHR of the *S* gene. It is therefore assumed that the L217R mutation may possibly be associated with OBI status in investigated samples. However, the association of this L217R substitution with OBI has to be validated from further studies as they stated (Delfino *et al.*, 2012). In addition, they also observed that the substitutions A128V and R169G (located in the MHR of the *S* gene) occurred only in OBI samples and these maybe the escape mutations during OBI development. Furthermore, in agreement with a previous study from Ghana (Zahn *et al.*, 2008), the *PreC* stop codon W28* mutant was observed at position 1896 in four of the investigated OBI strains. The mutation in the stop codon was shown to associate with the HBeAg status (Kim *et al.*, 2012). Interestingly, this mutation has also proposed to be associated with the OBI status (Pollocino *et al.*, 2007; Besharat *et al.*, 2014). However, more studies are needed to ascertain the role of W28 (G1896A) mutation in the development of occult hepatitis B infection.

The prevalence and pattern of HBV serologic markers associated with blood donors with OBI in this study showed that most OBI isolates are seropositive. Of the 14 OBI isolated in this study, 12 were seropositive representing 84.7% of OBI population while only 2 (14.3%) are seronegative. This finding corroborates with study of Lin *et al.*, (2016) that reported a prevalence of 88.8% seropositive OBI and 11.2% of OBI are seronegative. The pattern of seropositivity showed that anti-HBs marker has the highest prevalence among all the serologic markers detected. Seven (7) out of 12 seropositive OBI blood donors identified were anti-HBs seropositive representing 58.3% while 3 (25%) were anti-HBc seropositive. Two OBI blood donors have a combination of anti-HBs and anti-HBc seropositivity representing 16.7% prevalence. None of OBI blood donors has HBeAg and anti-HBe markers. This pattern disagreed with what Oluyinka *et al.*, (2015) obtained. Their study recorded 62% seropositivity of anti-HBc, 35% of anti-HBs and 3% of HBeAg. However, our study corroborated with theirs because none of OBI blood donor was positive for more than two serologic markers. High prevalence of anti-HBs marker recorded in this study correlates with the findings of Allain, (2017).This signifies the usefulness of testing for HBV markers in screening blood donors before donation. The presence of anti-HBs or/and anti-HBc in any blood unit is a suspect that the blood unit could be infected with HBV and hence should be confirmed using PCR.

The result from this study showed that 2 (2.9%) out of 70 seronegative blood donors (negative for all HBV serologic markers) are positive for OBI. This finding is discordant with the result of Minuk*et al.*, (2005) that recorded 8% prevalence of OBI among seronegative blood donors in a North American community-based population. What this means is most of our OBI blood donors were positive for HBV serologic markers. The blood donors that were seronegative (negative for all HBV markers) have a high probability that the donors are not infected with HBV. The blood donors positive for anti-HBc, anti-HBs and HBV-DNA but negative for HBsAg represent the viral persistence after recovery with a low viral load as seen in this study as also observed in previous reports (Brojer *et al.*, 2006; Oluyinka *et al.*, 2015). A plausible explanation for this observation is that anti-HBs antibody is poorly neutralized due to loss of recognition, allowing these mutant viruses to escape neutralization even when antibody is present at protective levels (Candotti *et al.*,

2008; Levicnik-Stezinar *et al.*, 2008). The OBI individuals' positive only for HBV-DNA without any detectable HBV antibodies (seronegative OBI) might be as a result of long lasting persistence of HBV cccDNA or the possibility of integration of the HBV-DNA into the host genome (Brechot *et al.*, 2001). This study showed that there were blood donors positive for anti-HBc but negative for both HBsAg and HBV DNA. Possibly these blood donors must have been exposed to HBV. In agreement with a previous study validating the inclusion of anti-HBc testing in Egyptian blood donors negative for HBsAg (Antar *et al.*, 2010), it was presumed that the individuals who were negative for both HBsAg and HBV-DNA but positive for anti-HBc might have been infected or exposed with HBV. But the HBV-DNA viral loads were below detection level (Urbani *et al.*, 2010). Such individuals in future may experience viral multiplication as result of HBV reactivation leading to overt HBV infection. Studies have shown that HBV reactivation could occur spontaneously or as a result of immunosuppression or intake of immunosuppressive drugs (Hoofnagle, 2009; Roche and Samuel, 2011). Therefore, anti-HBc should be considered as an additional serological marker for screening of HBV infection in blood donors (Urbani *et al.*, 2010).

The anti-HBc positive donors remained positive for HBV-DNA to a greater extent and the donors who are positive with anti-HBc but negative with HBV-DNA should also be excluded for blood transfusion to avoid the risk of HBV transmission. This is in agreement with a report that revealed anti-HBc positivity alone contributes to OBI with low viral replication (Prati *et al.*, 2006). The viral DNA may persist either as cccDNA or may integrate into the host genome in hepatocytes, thus HBV is undetectable in the serum (Brechot *et al.*, 2001). However, viral DNA can possibly be detected in liver tissue of healthy individuals positive for anti-HBc (Marusawa *et al.*, 2000; Ponde *et al.*, 2010). Individuals who were positive for anti-HBC alone had low OBI occurrence (1.7%) in a study reported from South Korea. The lower OBI occurrence in anti-HBC alone may be because of low viral load (Kang *et al.*, 2010). In a study by Olotu *et al.*, (2016) it was found in HBsAg negative blood donors an anti-HBc prevalence of 70.5 %. This means over 70 % of our adult population have been infected with HBV at some point in their lives. Previous studies by Uneke *et al.*, (2005) and Kire (1996) reported that 72.5 % of Nigerians showed evidence of exposure to HBV infection. Mbawuga *et al.*, (2014) also reported that

85.9% of their studied population in Benue state, Nigeria showed a serological evidence of exposure to HBV infection. This means that the burden of HBV infection in Nigeria has not changed significantly over the last 20 years (1996- 2016) especially in adults. This is not surprising as these individuals were born before 2004 when hepatitis B vaccine actually became widely available as part of the universal immunization schedule for infants in Nigeria (Sadoh and Eregie 2008). Other workers such as Japhet *et al.*, (2011) found a prevalence of 5.4 % for IgM anti-HBc only positive blood donors but did not look for total anti-HBc. Salawu *et al.*, (2011) also found about 4.4 % of anti-HBc in HBsAg negative blood donors. This is in accordance with this study that found 5.9% of HBsAg negative donors have anti-HBc marker. Anti-HBs prevalence has been studied in Nigeria. Salawu *et al.*, (2011) in a study in Ile-Ife among 457 blood donors negative for hepatitis B surface antigen (HBsAg) found a prevalence rate of anti-HBs was 12.7 %, Japhet *et al.*, (2011) found a rate of 15.2 % among 92 donors also studied in Ile-Ife while Oluyinka *et. al.*, (2015) found 35 % of those with OBI had anti-HBs. In this study, 64.3% prevalence of anti-HBs marker was found among blood donors with OBI. Also out of 204 blood donors negative for HBsAg marker by rapid test device, anti-HBs prevalence stood at 10.8% (Table 4.3). This was quite similar to what Salawu *et al.*, (2011) reported.

Serological diagnosis of hepatitis B virus identifies virally-encoded antigens and their corresponding antibodies in serum. Three clinical useful antigen-antibody systems havebeen identified for hepatitis B: hepatitis B surface antigen (HBsAg) and antibody to HBsAg (anti-HBs), antibody (anti-HBc IgM and anti-HBc IgG) to hepatitis B core antigen (HBcAg), hepatitis B e antigen (HBeAg) and antibody to HBeAg (anti-HBe) (Servoss and Friedman 2006). Hepatitis B surface antigen (HBsAg) is the first serological marker to appear during the course of HBV infection. Hepatitis B surface antigen (HBsAg) is a protein on the surface of hepatitis B virus. It can be detected in high levels in serum during acute or chronic hepatitis B virus infection. The presence of HBsAg indicates that the person is infectious. The body normally produces antibodies to HBsAg as part of the normal immune response to infection. Detection of HBsAg allowed for the first time screening of inapparently infected blood donors for a dangerous pathogen. The simultaneous detection of antibodies against HBsAg (anti-HBs) and HBcAg (anti-HBc) can

be useful to ascertain the evolution of disease. The presence of IgM antibody to hepatitis B core antigen (IgM anti-HBc) is diagnostic of acute or recently acquired HBVinfection. Antibody to HBsAg (anti-HBs) is produced after a resolved infection and is the only HBV antibody marker present after immunization. Hepatitis B surface antibody (anti-HBs) presence in the serum sample is generally interpreted as recovery and immunity from hepatitis B virus infection (Levinson, 2014). Anti-HBs also develop in a person who has been successfully vaccinated against hepatitis B. The presence of HBsAg and total anti-HBc, with a negative test for IgM anti-HBc, indicates chronic HBV infection. IgM antibody to hepatitis B core antigen (IgM anti-HBc) positivity indicates recent infection with hepatitis B virus (<6 months). Its presence indicates acute infection. Total hepatitis B core antibody (anti-HBc) appears at the onset of symptoms in acute hepatitis B and persists for life. The presence of anti-HBc indicates previous or ongoing infection with hepatitis B virus in an undefined time frame. The presence of anti-HBc alone might indicate a false-positive result or acute, resolved, or chronic infection (Servoss and Friedman 2006). In the light of this, there is need for prospective study to be done on blood donors with anti-HBc alone without HBV DNA to find whether HBV DNA could be detected in their blood/liver biopsy and duration of detection.

The ELISA results of blood donors tested for HBsAg, HIV and anti-HCV in this study population showed that out of 202 samples that tested negative for HBsAg by rapid test device, 28 samples representing 13.9% were positive , HIV ELISA result yielded 27 (13.7%) positive out of 197 samples tested. Anti-HCV ELISA showed that out of 185 samples tested, 15 samples representing 8.1% were positive. These values were quite high compared with 1% positivity for HBsAg and 0% positivity for HCV reported by Olotu *et al.*, (2016) after retesting with ELISA format. Oluyinka *et al.*, (2015) also reported that all samples tested negative by rapid test device were negative by ELISA technique. The reason for this difference could be that this study used 4th generation ELISA test kits which is more sensitive than the 3rd generation kit they used. The ELISA HIV 4th generation kit for example contains antibody and antigen (p24) making it more sensitive in detection of small concentration of HIV in the blood specimen (Constatine *et al.*, 2005). However, this study collaborates with the study of Erhabor *et al.*, (2014) that reported 9% of samples that

initially tested negative with HBsAg rapid kits, were positive with ELISA technique among blood donors in University Teaching Hospital Sokoto, Nigeria. This indicated that ELISA technique is more sensitive and superior than rapid test for screening of blood donors for HBsAg. Failure of the rapid kits to detect the presence of markers of infectious viral (HIV, HBV and HCV) diseases may be due to inadequate coating of the antigen, nature of antigens used and genetic heterogeneity of the virus (Yokosuka and Arai, 2006; Torane and Shastri, 2008). In this study we observed high false negative results with the rapid diagnostic kit compared to ELISA. Our finding is in agreement with previous report of Salawu *et al.,* (2011) which stated that there is risk of donor blood samples containing HBV being transfused to patients due to suboptimal testing using HBsAg rapid kits only. The results obtained from ELISA testing for HBV, HIV and HCV show that ELISA format is more sensitive than rapid test kit (Levinson, 2014) but PCR is also highly sensitive and specific in the detection of DNA/RNA of infectious viral agents. This also signifies that the rapid test kits being used for HBsAg screening in the blood banks where the subjects were recruited are not adequate for screening in blood transfusion services and would allow transfusion of HBsAg positive blood in at least 10 out of every 100 blood donations.The inadequacy in sensitivity and variation in performance between different locations of some rapid test kits used in resource poor settings for HBsAg screening has been shown by Bjoerkvoll *et al.*, (2010). In their cross-sectional epidemiological study they compared the accuracy of rapid test immunochromatographic kits in the detection of HBsAg, anti-HBc and anti-HCV against ELISA, in two populations of 1200 potential blood donors in rural Cambodia and Vietnam. For HBsAg specifically, they found the rapid test kits to be high in specificity (99.8–99.9 %) but lower in sensitivity (86.5 %). They also found a difference in its sensitivity between both countries. In Cambodia the sensitivity was 93.5 % and in Vietnam 81.8 %.These collaborates with this study that showed that ELISA is more sensitive than rapid test in detection of HBV infection.

In addition, results from this study also showed evidence of coinfection of transfusion transmissible viral infectious agents (HBV, HCV and HIV) among blood donors. Of the 198 blood donors screened with ELISA 4th generation kits (HBsAg, Anti-HCV and HIV with Ag+Ab), 12 blood donors have coinfection of transfusion transmissible viral infections representing 6.1% prevalence of multiple infections (Figure 4.4). One (1) out of the 12

samples has coinfection of these three viruses (HIV, HBV & HCV) representing 8.3% prevalence. This finding disagrees with the result of Akpulu *et al.*, (2017) that recorded 26 (1.4%) out of 1875 blood donors with co-infection of transfusion transmissible infectious agents in Zaira, Nigeria. The reason for this difference could be attributed to high sample size they used compared to this study that was 198 samples. However, our result correlates with their finding in relation to HIV/HCV coinfection having the highest prevalence. The reason for this multiple infection could be attributed to the fact that these viral infections have common routes of transmission such as sexual contacts, vertical transmission from mother to child and occupational hazard/ accidents such as needle prick (Levinson, 2014). However, there was no coinfection with blood donors with occult HBV infection.

Sociodemographic analysis of data obtained from this study showed that most blood donors with occult hepatitis B virus infection are within age 18-40 years old with median and mean age as 32 years and 31.4 years respectively. This finding correlates with the work of Olotu *et al.*, (2016); Oluyinka *et al.*, (2015) that reported that OBI isolates falls within the age group of 31-39. Zheng *et al.*, (2011) also reported 28 years as median age of OBI isolates in China. This age bracket is the most sexual active age and most people may likely be infected with HBV and other transfusion transmissible viral agents (HIV and HCV). These viral agents have common route of transmission among which is sex (Levinson, 2014). This study did not find any statistical significant association between occult HBV and the variables tested such as age, sex, academic status, marital status and occupation/profession. However, 13 out of 14 individuals with OBI in this study were found to be males. Only one female blood donors has OBI. This finding is similar to what Olotu *et al.*, (2016) found. All their OBI isolates were males and mostly below 36 years. This also tallies with Zheng *et al.*, (2011) findings that reported that male to female ratio of OBI identified stood at 3.4:1 (17:5). Moreover, Allain, (2017) in a review article on global epidemiology of occult HBV infection recorded that most people infected with OBI were male.

Hepatitis B virus risk factors assessment among blood donors was performed. This study also showed that most blood donors recruited had not been vaccinated with HBV vaccines. Of the 212 donors recruited, 184 (86.8%) have not been vaccinated with HBV vaccines. This actually reflected in the high prevalence of occult and overt HBV infection recorded.

This study showed that 12 (85.7%) out 14 OBI blood donors detected have not been vaccinated with HBV vaccines (Table 4.8). The remaining two blood donors that claimed they were vaccinated do not have anti-HBs marker, the protective antibody that signifies HBV vaccination. This indicated that they were not protected. It could also mean that the vaccine administered was not potent to boost their immunity or they did not take the complete dosage. The potency of the vaccine could be affected by many factors among which storage conditions is just one of them. The findings from this study showed that many blood donors were not protected and hence susceptible to HBV infection as they have not received HBV vaccination. In addition, many of the blood donors recruited have at least one risk factor to HBV infection. Out of 14 OBI blood donors identified in this study, it was observed that 10 lacked knowledge of HBV, 12 have no HBV vaccination, 8 have occupational/domestic accident and 11 blood donors with OBI visit commercial barber/manicurer/pedicurer to mention but few among the risk factors. This represents 71.4%, 85.7%, 57.1% and 78.6% respectively. This study observed no statistical significant difference among blood donors with occult and overt HBV infection with regard to HBV risk factors assessed (Table 4:8). This means anyone can be infected with hepatitis B virus irrespective of risk factors. In a similar study by Lavanya *et al*., (2012) on prevalence of HBV markers among blood donors, they observed that the high prevalence of HBV markers among blood donors in India were found to be instigated with risk factors like alcoholism, smoking, tattooing, ear piercing, visiting barber's shop and family history of jaundice.

The ABO blood group among blood donors with occult HBV infection was studied. The result showed that blood group O was the most prevalent among blood donors with Occult HBV Infection with 12 out 14 OBI identified representing 85.7% belonged to blood group O while blood group A and B have one OBI blood donor each. This represented 7.1% prevalence. There is no OBI associated with blood donors with AB blood group. In a similar study by Lavanya *et al.*, (2012) they found a contrasting result of blood group B being more prevalent followed by blood group O although not statistically significant. Nine out of 14 blood donors with OBI in this study were family replacement donors representing 64.3% while 5 donors with OBI were Voluntary non-remunerated donors representing 35.7%. No blood donor with OBI was identified as commercial paid donor. This could be so because most of the blood donors recruited in this study belonged to family replacement

blood donors. Repeat (old) blood donors have more prevalent rate of occult HBV infection than first time blood donors. The data showed that 9 out of 14 blood donors with OBI are Repeat (old) blood donors representing 64.3% while the remaining 5 donors are first time donors representing 35.7% of the population. This is not statistically significant at $p>0.05$. What this means is any blood donor could be infected with HBV infection irrespective of blood donor status they belong. It also depends on exposure to risk factors and the immune status of the individual. However, the reasons behind this finding could be that repeat donors had been exposed to HBV risk factors more than first timers hence higher prevalence was recorded in this group. Therefore there is need to recruit first time blood donors who have not been exposed frequently to HBV infection and other transfusion transmissible viral infections. Whosoever is recruited for blood donation should be adequately screened for these transfusion transmissible viral agents using highly sensitive and specific test kits to prevent transmission of these viruses to blood recipients and its attendant consequences.

The result of Alanine Aminotransferase (ALT) from this study showed that 61 samples of healthy blood donors tested for Alanine Aminotransferase; only 1 sample has a significant high level above the expected normal range representing 1.6% prevalence of the population. All the OBI blood donors as well as HBV and HCV positive samples have insignificant level of ALT (normal value). The only sample identified with significant high level of ALT was HBsAg positive blood donor representing 8.3% of HBsAg positive samples tested. This result correlates with the finding of Lin *et al.*, (2016) that reported only one blood donor with OBI having elevated level of ALT. In addition, Zheng *et al.*, (2011) reported from Chinese blood donors with OBI normal ALT values. This result is possible and not surprising because these viral infections were detected in apparently healthy blood donors. These infectious agents (HBV, HCV and HIV) have not attacked the liver cells to extent to cause liver damage with resultant elevated liver enzymes hence normal liver enzyme (ALT) recorded in this study. This confirms the report of Gitlin (1997) and WHO (2009a) that Alanine aminotransferase (ALT) is markedly elevated in acute HBV infection moderately increased in chronic infection and remain normal in past and occult infection. The blood donor with high ALT showed a slight elevation, an indication that the HBV has started to attack the hepatocytes and this blood donor has an overt HBV infection. The HBV viral

load could be high compared to those of individuals with occult HBV infection (Levinson, 2014).

The Alpha Feto- Protein (AFP) level among blood donors tested showed that of the 61 samples tested for Alfa-Feto Protein, 3 samples had a significant high level above the expected normal range representing 4.9% population. All the OBI blood donors as well as HBV and HCV positive samples have insignificant level of AFP in their blood. The 3 positive samples identified are HIV positive blood donors representing 37.5% of HIV positive samples tested. Alpha –Feto protein (AFP) is a glycoprotein produced during fetal development of the hepatocytes. Elevated levels of AFP are found in patients with primary hepatoma and yolk sac-derived germ tumors (Burtis *et al.*, 2006). AFP is the most useful marker for the diagnosis and management of hepatocellular carcinoma caused by viral hepatitis and HIV infection (Levinson, 2014). Limited study has been done on the relationship between Alpha-feto-protein and transfusion transmissible infectious viral agents in Nigeria. The result obtained from this study indicated normal values of AFP among blood donors with occult HBV infection as well as blood donors infected with hepatitis C virus. This shows that the viral load of these individuals could be at low level to cause damage to hepatocytes. The three samples with significant high level of AFP were samples positive for HIV. The HIV viral load could be high as we were unable to perform HIV viral load among blood donors positive for HIV RNA.Patients with chronic HCV and steatosis have a higher AFP levels than those without steatosis. In chronic HCV with steatosis, elevated AFP levels correlated positively with Hospital associated infection (HAI) and negative significant correlation with albumin level (Mousa *et al.*, 2012). Studies have shown that AFP and ALT is elevated in patients with chronic HCV infection (Goldstein *et al.*, 1999; Gupta *et al.*, 2003). This increase could be as result of chronicity of the infection with high viral load. In this study both ALT and AFP levels were normal in blood donors with occult HBV infection. A plausible explanation for this is the viral load in OBI is low and the viral particles are not multiplying or latent even in these apparently healthy blood donors that were tested positive for HBV, HIV and HCV. Although there are no significant changes in liver enzyme (ALT) and Alpha Feto protein levels of healthy blood donors with occult and overt HBV infection, these subjects need to be monitored and adequately managed to prevent risk of liver cirrhosis and Hepatocellular carcinoma. Further studies

need to be done to determine the levels of ALT and AFP in known chronic liver disease (CLD) patients caused by hepatitis viruses particularly hepatitis B virus.

Haematological values and CD4 cell count analysis showed that blood samples of some blood donors screened and certified fit for donation in this study including blood donors with occult HBV infection have low values of some haematological indices. Out of 14 OBI blood donors, 1, 6, 3, 1 and 2 have low values of CD4 count, Hemoglobin, WBC, Platelets count and RBC representing 7.1%, 42.9%, 21.4%, 7.1% and 14.3% prevalence respectively. None of the OBI blood donors has low HCT. This could be a reflection of other blood donors without transfusion transmissible viral infections. The low values of these blood cells indicate that some blood units transfused to recipients are not rich enough in Haemoglobin content to solve the intended problem of anemia it was designed for. The study observed that some blood donors including those with occult HBV infection have leucopenia and low values of CD4 cell count. The possible causes of leucopenia and low CD4 cell counts among blood donor participants could not be investigated. This could be due to chronic infections that could be transmitted via blood transfusion (Acheson, 2011). Further studies need to be done to identify the possible reasons for low white blood cell count and low values of CD4 cell count among blood donor participants. This finding corroborated with the study of Ajugwo *et al*., (2015) at Elele, Rivers States, Nigeria, that found no significant difference in haematological indices (HCT, HGB, WBC Total and ESR) between symptomatic and asymptomatic patients infected with Hepatitis B. They concluded that patients with HBV infection have the tendency to develop anaemia if not properly managed. A comparative analysis of some haematological parameters and CD4 cell count of blood donors with OBI and HBsAg negative samples in this study indicated no statistical significant difference between these 2 groups of blood donors. The mean haematological values of HCT, HGB, WBC, RBC, platelets count and CD4 Cell count fall within the normal acceptable range (Table 4.16). This result is discordant with the result of Francisca *et al.*, (2017) that reported a statistical significant difference in Haemoglobin, CD4 cells and monocytes between individuals with overt HBV infection and subjects negative for HBsAg. The blood donors in this study are apparently healthy individuals though infected with viral agents which have not destroyed blood cells unlike in their study

where some of infected individuals have envelop antigen (HBeAg), a marker for viral infectivity and replication.

In another study by Akinbo *et al.*, (2015) on haematological indices and CD4 count of apparently healthy population in Owo, Ondo State, Nigeria, they found the mean blood values of WBC, RBC, HGB, HCT and Platelets are higher in males than females. The reference range of males include 4.2-9.7, 4.0-5.3, 11.6-16.3, 35-48, 152-288 and 403-1900 for WBC, RBC, HB, HCT, platelets and CD4 respectively. Their finding correlated with our study that showed the average (mean) value for OBI blood donors and HBsAg negative blood donors for HCT for instance stood at 42.2 and 41.5 respectively. This is not statistically significant $p>0.05$. Although there was no statistical significant difference in haematological parameters among blood donors with occult HBV infection and blood donors seronegative for HBV, the study has established that large number of healthy blood donors have low WBC, Pack cell volume and hemoglobin levels. It is recommended that screening of all haematological profile using auto-hematology analyzer be adopted as screening test for blood donors as this will give more detail haematological parameters of blood donors compared with only Packed Cell Volume done to assess donor's suitability for blood donation.

The risk and implications of transfusion of infected blood units to recipients cannot be overemphasized. Transfusion Transmissible Infections (TTIs) are a major problem associated with blood transfusion. Accurate estimates of risk of TTIs are essential for monitoring the safety of blood supply and evaluating the efficacy of currently employed screening procedures (Sharma *et al.*, 2014). An unsafe blood transfusion is very costly both from human and economic point of view. Morbidity and mortality resulting from the transfusion of infected blood have far reaching public health consequences, not only for the recipients themselves but also for their families, their communities and the wider society (WHO, 2002; WHO, 2007b). The economic cost of the failure to control transfusion transmissible viral infections includes increase requirement for medical care, higher level of dependency, loss of productive labor force and placing heavy burden on already overstretched health and social services on national Economy (WHO, 2002).

The risk assessment of transfusion of transmissible viral infections such as HBV, HCV and HIV from this study was high. Of the 212 blood donors recruited in this study, 8 were confirmed positive for HBV and 6 were confirmed positive for HCV by rapid test kits and were excluded from the study. The remaining 198 blood units have been confirmed suitable for transfusion and possibly these blood units may have been transfused to their recipients at these two Teaching Hospitals under study. The findings from this study had shown that 14 donors out 198 have OBI, 20 blood donor samples that were ELISA positive for HBsAg were positive for HBV DNA, 8 HIV ELISA positive samples were positive for HIV RNA and 4 HCV ELISA positive samples were positive for HCV RNA. In summary, of the 198 blood units that have been screened, certified safe and possibly transfused to their patients in these two Teaching Hospitals in Nigeria, 46 blood units representing 23.2% are infected with transfusion transmissible viral infections. What this means is 23 out 100 blood units used in this study population have risk of transmission of infectious viral agent such as HBV, HCV and HIV to their recipients. Of these 46 infected blood units, HBV infected blood units is 34 representing 73.9% of infected blood units being transfused to patients followed by HIV which have 8 blood units with HIV RNA representing 17.4% and HCV infected blood with HCV RNA is 4 units representing 8.7% of infected blood units. The percentage risk of transfusion of viral agents, HBV, HIV and HCV in relation to the 198 blood units certified fit for transfusion in our study population is 17.1%, 4% and 2% respectively and is statistically significant $p<0.05$. The implication of transfusion of infected blood units is the resultant increase in the prevalence of overt infection of these viral agents in the population with its attendance public health consequences. The residual risk obtained from this study correlates with the study by Candotti and Allain, (2009) which reported that the estimated residual risk of HBV transfusion transmission remains significantly higher than the risk of either HIV-1 or HCV. Whether residual risk estimates translate into true rate of infection is largely unknown since estimates are generally based on the simplification that all HBV DNA-containing donations are infectious (Candotti and Allain, 2009). They concluded by saying that all blood units containing HBV DNA have been shown to be infectious in immunocompromised individuals, such as organ- or bone marrow-transplant recipients. HBV transmission was previously reported from OBI donors who had circulating HBV DNA at a low level (Manzini *et al.*, 2007; Bouike *et al.*, 2011).

These authors emphasized that the lack of a clear relationship between infectivity and viral load in blood components may be related to immune factors affecting the susceptibility to infection in recipients. In addition, HBV infectivity is related to the amount of plasma transfused and the viral load in the product (Candotti and Allain, 2009).

Studies have shown that the risk of transmitting occult hepatitis B infection is dependent on presence of copies of HBV DNA in the plasma and the volume of the plasma transfused (Allain *et al.*, 2013; Allain, 2017). The higher the viral load in the transfused blood the higher the chance of infectivity (Allain *et al.*, 2013). The presence of high anti-HBs in the donors' blood also influences the rate of infectivity (Seed and Kiely, 2013). Immune status of the recipient and donors also play an important role in determining infectivity (Yuen *et al.*, 2011). Recent infectivity data indicated transmission rate of 3.8% (Seed and Kiely, 2013) but the rate is higher in unvaccinated recipients of occult hepatitis B blood or blood products. Blood products from donors with occult hepatitis B carry a high risk of HBV transmission by transfusion. This may justify safety measures such as anti-HBc and HBV nucleic acid test screening depending on epidemiology. Anti-HBc may be used in < 2% to 4% prevalence, while, HBV-NAT in high endemic areas (Allain *et al.*, 2013). Occult hepatitis B virus infections have significant clinical importance since they can become reactivated when the immune system is suppressed and can be transmitted through blood or blood product transfusion, organ transplant, and sexual intercourse. It may also enhance the progression of liver fibrosis and, subsequently, hepatocellular carcinoma.

In summary, availability of safe blood for transfusion is a must for the recipients and community as well and can be achieved by vigorous and cautious screening of donors / or donated blood units with highly sensitive and specific laboratory tests that can detect OBI and other transfusion transmissible viral infections (WHO, 2015). The discussion of the results from this study as could be seen in this chapter shows that in many respects findings are consistent with existing knowledge and in some aspects new findings have emerged from this study. Also to a great extent, the outcome of the study is consistent with research objectives, research questions and the hypotheses of the study. This simply indicates that the aim and objectives of the study have been achieved and research questions answered. These agreed with the suggestion that occult hepatitis B virus infection among blood donors

and transfusion transmissible viral infections can be prevented or reduced if blood donors are screened with NAT, HBV serologic markers and ELISA 4th generation kits for HBsAg, Anti-HCV and HIV 1 & 2 with antibody and p24 antigen.

5.2 CONCLUSION

Results obtained from this study strongly justify that NAT remains a prerequisite for blood donor screening for avoidance of HBV transmission and other transfusion transmissible viral infections risk in UATH Abuja and NAUTH Nnewi in particular and Nigeria in general. Moreover, findings from this study strongly indicate that the use of Enzyme Linked Immuno-Sorbent Assay (ELISA) for HBsAg, HCV and HIV will increase blood transfusion safety. In addition, inclusion of HBV serologic markers like anti-HBc, anti-HBc IgM and anti-HBs will detect most of Occult Hepatitis B virus Infection and Window period infections among blood donors.

Hepatitis B virus infection still continues to be a menace to the society because the prevalence of the disease is still very high in the general population. This study has established that there is transfusion transmissible viral infections risk at University of Abuja Teaching Hospital and Nnamdi Azikiwe University Teaching Hospital Nnewi, with 14% prevalence of occult HBV infection among blood donors recorded at our study population. There is urgent need for renewed intensification of prevention programmes aimed at changing high-risk behaviors among Nigerian youths and vigilant donor selection. The introduction of more sensitive tests such as Nucleic Acid Amplification Testing (NAT) for HBV, HCV and HIV that detect infection earlier (reduce the window period) will further decrease risks and thereby be a useful tool in the quest to approach near to zero risk of transfusion transmissible viral infectious agents. The study has also shown that almost all seronegative samples for HBV, HCV and HIV by 4th generation ELISA format and HBV markers were negative by NAT. It means 4th generation ELISA format can be used in screening of blood donors as it is more sensitive than rapid test device. Moreover, most of the OBI isolates were seropositive with anti-HBc and anti-HBs seropositivity in high prevalence. The inclusion of HBV markers particularly anti-HBc and anti-HBs in blood donor screening will reduce HBV transmission risk. All this will be cost intensive; hence the study has come up with a testing algorithm for screening blood donors which is cost

effective. First and foremost, all blood donors should be screened with ELISA 4TH generation for HIV, HBV HCV and Syphilis antibodies. If negative, screen for HBV markers using HBV 5 panel assay. If all HBV markers are negative, the blood donor is certified fit for donation.

However, if anti-HBc or/and anti-HBs is positive the sample should be subjected to DNA amplification to detect HBV DNA. This will minimize the risk of acquiring HBV burden in transfused individuals. The use of the above testing algorithm for screening blood donors though will lead to rejection of many blood donors or donated blood units, however, will go a long way in reducing HBV transmission during blood transfusion. Moreover, it will be cost effective and cost efficient for our society where the cost of DNA testing for all blood units is high. There is a relatively high burden of occult HBV infection in our environment and the use of HBsAg alone for screening either in blood transfusion or transplantation services does not eliminate the risk of HBV transmission. The present study also showed that HBV is highly endemic in Nigeria, with a predominance of HBV genotype E in both group of population of blood donors with occult and overt infection at UATH Abuja and NAUTH, Nnewi. Apart from occult HBV infection, there is a relatively high burden of HIV and HCV infection in our environment and the reason could be that these viral agents have similar transmission routes and risk factors.

5.3 RECOMMENDATIONS

It is tradition in any research endeavor that when problems are identified, solutions are proffered. Proffering solutions take the shape of recommendations. Recommendations are directed at providing the leeway to solving the research problem. It is on this basis that the following recommendations are hereby put forward;

It is recommended that assays including rapid test kits used for HBsAg, HIV and HCV screening should be validated before routine use locally comparing their performance with 4th generation ELISA Kits and possibly PCR technologies. Only ELISA kit or rapid test kits with comparable performance with regard to sensitivity or specificity should be used for screening blood donors in blood transfusion. To have a near zero transfusion transmissible infection rate, blood donors should be screened with 4th generation ELISA for HIV, HCV

and HBsAg as ELISA technique has proven to be more sensitive than rapid test devices. In addition, Nucleic Acid Tests (NATs) should be introduced for routine screening of blood donors in blood transfusion as it is highly sensitive and specific. Anti-HBc and anti-HBs markers should also be include as screening tests for blood donors as using HBsAg only is inadequate. These viral markers indicate signs of previous contact with HBV. Though it may lead to more rejection of blood donors but it will reduce the transmission of HBV during blood transfusion. Moreover, it may attract additional expenses to patients/relatives. It should be advocated that Government take up the cost as health is a public service to humanity. In the light of the above, Government of Nigeria should develop at least one functional blood transfusion service in all states including Abuja, the Federal Capital Territory (FCT) with modern equipment for ELISA and molecular assays for testing blood donors. The present National Blood Transfusion Service (NBTS) in six geo-political zones is grossly insufficient and inadequate. Each NBTS should aim at recruiting voluntary non-remunerated first timer blood donors from institutions of higher learning/traders in markets. Family replacement and repeat (old) blood donors are more infected with OBI and other transfusion transmissible infections and reliance on replacement donors might lead to insufficient blood units in blood banks. Blood donors with OBI should be monitored and possibly treated to prevent transmission of hepatitis B virus to other people in the community. Knowledge of HBV and Hepatitis B virus infection is not much despite the study population is more of literate individuals. There is need for awareness of HBV infection in the population particularly on mode of transmission, prevention and risk factors associated with HBV infection. This can be achieved by mass mobilization campaign using media and in various local languages of the country. This study had shown that most blood donor participants have not been vaccinated or protected from HBV. This might be a reflection of the general population. Therefore the populace should be vaccinated with recombinant HBV vaccines. This will go a long way in protecting people that are susceptible as well as reducing the prevalence rate in the population. Finally, manufacturer of Hepatitis B virus rapid test device (HBsAg Strips/Cassettes) should consider including mutants forms of HBV that could make the testing kit more sensitive and specific particularly in detection of occult hepatitis B virus infection.

Limitations of the Study

At this point, it is important to mention some limitations of the study as this project is not without constraints. Apart from huge finances involved in the study which most was supplied by the principal investigator, other drawbacks include;

- Incompleteness of research questionnaire by some blood donors. It was observed that some blood donors particularly from University of Abuja Teaching Hospital Blood bank did not fill the aspect that relates to having multiple sexual partners, injection of illegal drugs, alcohol abuse and also previous sexually transmitted infection.
- Blood specimens were insufficient to perform some assays in some blood donors.
- The delay in approval of research proposal by Health Research Ethics Committee of Nnamdi Azikiwe University Teaching Hospital, Nnewi led to delay in commencement of sample collection at this study site. Sample collection however commenced at NAUTH, Nnewi on 17th October, 2016 and ended on 30th October, 2016.
- Inability to perform amplification on all samples collected and Gene sequencing of four HCV isolates positive by PCR because of limited fund. However, all the challenges encountered during the course of this project were surmounted and at the end, the study was concluded successfully as the aim and specific objectives of the study were achieved.

5.4 FUTURE RESEARCH BASED ON THE FINDINGS FROM THIS STUDY

Further communication/research on occult hepatitis B virus infection and other transfusion transmissible viral infections among blood donors should focus on:

- Molecular and immunological mechanisms underlying occult HBV Infection.
- Transfusion and infectivity of blood units with occult HBV infection and other transfusion transmissible viral (HCV and HIV) infectious agents.
- Whole Gene sequencing of occult HBV to identify mutation on the genes.
- Use of the mutants form in development of HBV testing kit to unable laboratory to detect occult HBV infection through rapid test devices or ELISA technique.

- Detailed investigations on blood donor samples that were positive for HBsAg, Anti-HCV and HIV by rapid test device/ELISA but were negative for viral DNA/RNA by PCR.
- Use of multiplex PCR in detection of transfusion transmissible viral infections and its cost benefit and cost effectiveness compare with testing individual donor samples with NAT for each infectious agent.
- Detection of infectious agents like Malaria parasites, Cytomegalovirus etc. among blood donors that could be transmitted via blood and their implications in blood transfusion.
- Investigation of blood donors with low CD4 cell count and WBC count to detect and identify if they are infected with chronic microbial infections that could be transmitted via blood transfusion.

5.5 CONTRIBUTIONS TO KNOWLEDGE

The uniqueness of this study rest on the facts that it has closed several gaps from previous researchers on occult Hepatitis B virus infection and other transfusion transmissible viral infections among blood donors and hereby made the following contributions to knowledge:

1. This study has determined the actual prevalence of occult HBV infection and HBV genotype among blood donors at University of Abuja Teaching Hospital, Gwagwalada and Nnamdi Azikiwe University Teaching Hospital, Nnewi.
2. The study has identified that the use of HBsAg marker only for screening blood donors for HBV infection is inadequate as it cannot detect occult HBV infection and infection at window period.
3. There is transfusion transmissible viral infections in the study population apart from OBI and hence, the use of 4^{th} generation ELISA system for HBV, HCV, HIV and NAT will increase blood safety as it reduces transfusion transmissible viral infections risk to a near zero position.
4. The study has identified that HBV serologic markers such as anti-HBc total, anti-HBc IgM, and anti-HBs can be used as surrogate markers for detection and identification of OBI among blood donors.

5. Finally, the study was able to identify that any blood donor that is seronegative for HBV, HCV, and HIV by ELISA 4th generation kit and seronegative for HBV markers (anti-HBc, anti-HBc IgM, anti-HBs, HBeAg and anti-HBe) has a high probability of being negative for viral DNA/RNA by PCR hence, free from Transfusion Transmissible viral infections. Likewise, any blood donor that is positive for HBV, HCV and HIV by ELISA 4th generation kit and HBV markers poses a transfusion risk and should be screened by NAT. This testing algorithm discovered by this study for screening blood donors if utilized will minimize transfusion transmissible viral infections among blood donors in our study population in particular and Nigeria in general.

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APPENDICES

Appendix 1: Informed Consent Form

My name is Ahaneku Osuji, a PhD student of Department of Medical Laboratory Science, Nnamdi Azikiwe University Awka in Anambra State, Nigeria. I am conducting a research on a topic captioned, **"Occult Hepatitis B Virus Infection and other Transfusion Transmissible Viral Infections among Blood Donors at Two Teaching Hospitals in Nigeria."**

Benefits and Risks of the study: The study is designed to benefit the society as it will mitigate and prevent transfusion of blood borne pathogens to blood recipients as well as add new knowledge. In addition the study will be beneficial to blood donors who participate as it will detect occult HBV infection and commence early management to prevent damage to liver which could ultimately result to Liver Cancer. There is no anticipated risk to blood donors who will participate in this study. The participants will be required to answer some questions which might pertain to their health and social life. When any participant feels some discomfort at responding to some questions, please feel free to skip them. However, it is important you fill the questionnaire completely as it will enhance the interpretation of the results. Your participation will not alter your personality and also entails giving small amount of your blood sample for laboratory testing and analysis. Sample collection will not be harmful to the participants.

Voluntary Nature of Participation: Participation in the study is voluntary. If you choose to participate you are free to withdraw from the study at any stage without penalty. But my hope is that you will participate fully since your views are important to the success of the study. The cost of all tests done in this study shall be borne by the principal investigator. Blood donors are not expected to pay for any laboratory test performed in the course of the study. No money or incentives will be given to blood donors for participation in the study.

Confidentiality: All information gathered as well as the outcome of the study will be

treated with utmost confidentiality and will not be used against you in any form. Data presented from this information will not in any way reveal individual participation as participants' names/addresses shall not be used or appear in research questionnaire form.

Usefulness of Research Result/ Findings

The result/findings from this study will be made available to the management of the teaching hospitals where the study was conducted if need be. This is to improve service delivery to the people that use these teaching hospitals as well as to prevent transfusion transmissible infectious agents in the community. In addition, research results will be used for publication in both national and international scientific/medical journals. This shall provide some required information on the prevalence of occult hepatitis B virus infection among blood donors and transfusion transmissible infection risks at our study population. This will complement other similar studies imperative for assessing and monitoring the effectiveness and efficiency of strategies for blood safety and policy as well as regular review of strategies and policies by local hospital blood banks or National Blood Transfusion Service (NBTS) for providing adequate, safe and quality blood/blood products for clinical use.

Response: I have read and understood the above or had someone read and explain the entire study to me. The nature and benefit of the study is clear and hereby consent to participate in it. Thank you for agreeing to participate in this research.

Signature of Participant---------- Date----------

Signature of Interviewer---------- Date----------

Donor Number----------

Name of Blood Bank---------- Study ID Code----------

In case of any concerns, complains and enquires contact the Principal investigator,

Mr. Ahaneku Iherue Osuji

Department of Medical Laboratory Science, Nnamdi Azikiwe University Awka, Nnewi, Campus, Nnewi, Anambra State.

Contact Phone: 08033507775; E-mail: iherueosuji@yahoo.co.uk; ahanekuos@gmail.com

Appendix 2: UATH Health Research Ethics Committee Approval

UNIVERSITY OF ABUJA TEACHING HOSPITAL

P.M.B. 228, ABUJA - F.C.T. NIGERIA
07040045614, 09-2905535, 09-2904040
www. uath. ng.org.

FCT/UATH/HREC/PR/ 514

Our Ref:_____ 15/4/16

Your Ref:_____ Date: _____

Ahaneku Iherue Osuji
Lab Scientist
UATH
F C T - Abuja

Letter of Approval

Proposed Title: Occult hepatitis B virus infection among blood donors and its implications for blood transfusion.

Proposed Site: UATH

Sponsor: Principal Investigator

Your submission to the committee on UATH Health Research Ethics Committee on the above named protocol refers.

The Committee reviewed the following documents:

- A completed UATH HREC Application form
- Informed Consent Form
- Research Proposal
- Questionnaire/Proforma

The committee has considered the ethical merit of your submission and approved the protocol. The approval is for one year and will lapse on 12/4/17. It can be renewed on request. Modification of any part of the research methodology will require an approval.

Accept assurances of our highest regards.

Prof. B Ekele
Chairman UATH HREC

Appendix 3: NAUTH Health Research Ethics Committee Approval

NNAMDI AZIKIWE UNIVERSITY TEACHING HOSPITAL

P.M.B. 5025, NNEWI, ANAMBRA STATE, NIGERIA

Chairman
Board of Management

Mrs. Chinyelu Ogoamaka Nwofor
B.Ed, M.Ed, MHP&M, AHA, FCAI
Director of Administration/
Secretary to the Board

Professor Anthony O. Igwegbe
MBBS, FWACS, FICS, FISS
Chief Medical Director/
Chief Executive

Dr. E. A. E. Afiadigwe
B.Sc (Hons) Nig. MBBS (NAU), FWACS, FICS
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Our Ref: NAUTH/CS/66/VOL.9/40

Your Ref: _____

Date: 20th September, 2016

Ahaneku Iherue Osuji
Department of Medical Laboratory Science,
Faculty of Health Sciences and Technology,
Nnamdi Azikiwe University,
Nnewi Campus

ETHICS COMMITTEE APPROVAL

RE: OCCULT HEPATITIS B VIRUS INFECTION AMONG BLOOD DONORS AND ITS IMPLICATIONS FOR BLOOD TRANSFUSION

We write to inform you that after due consideration of your research proposal, approval is hereby conveyed for you to commence the study.

The principal investigator is required to send a progress report to the Ethics Committee at the expiration of three (3) months after ethical clearance to enable the Committee carry out her oversight function.

Please note that this approval is subject to revocation if you fail to obtain proper authorization from your study site/unit.

.....
Dr. Joy Ebenebe
Chairman, NAUTH Ethics Committee

.....
Udemezue N.O (Mrs)
Sec., NAUTH Ethics Committee

Appendix 4: Research Questionnaire

Section A: Bio-Data/Scio-Demographic Data

1. Identification Number----------
2. Donor's Blood Group----------
3. Blood donor status: 1st time Donor----------Repeat (Old) Donor---------
   Commercial remunerated----------Voluntary non-remunerated----------
   Family replacement donor----------
4. Nationality----------
5. Ethnic group----------
6. Gender: Male----------Female----------
7. Marital status: Single---------- Married---------- Divorced----------
   Separated----------Widower---------- Widowed----------
8. Age----------
9. Occupation----------
10. Residential location: Urban setting---------- Rural setting----------
11. Educational Qualification: Primary--------- Secondary---------- Tertiary---------
    Others----------(Please specify)----------

Section B: History of HBV Infection/Risk Factors Assessment

12. History of needle prick injury: Yes---------- No---------- If yes, did you receive post exposure prophylaxis (PEP): Yes---------- No----------
13. Do you know about Hepatitis B Virus: Yes---------- No----------
14. Have you known your HBV Status: Yes---------- No----------
15. If yes, what is the result? Positive---------- Negative----------
16. Have you been vaccinated against Hepatitis B virus? Yes---------- No----------
    If yes: When---------- Where----------
17. How many inoculations did you received? ----------
18. Have you been recently diagnosed of having Hepatitis? YES---------- NO--------
    a. If yes, when? Last month--------- Last week---------- Yesterday----
       Others (Please specify):----------
19. Do you have any family member with liver disease?: YES-------- NO----------

20. Have you received Hepatitis B Immunoglobulin before? YES--------NO---------

21. If yes for what purpose? Specify----------

22. Have you received blood transfusion/s or blood products before? YES------NO--

23. If yes how many times in the last 12 months? Specify----------
---

24. Have you had dialysis or surgery before? YES----------NO----------

25. If yes how many times in the last 12 months? Specify (Kind of Surgery)----------
---

26. Do you have multiple sexual partners? YES----------NO----------

27. If yes how many----------

28. Have you suffered from any form of sexually transmitted infections before? YES---
------NO---------- If yes, which of the diseases? Specify----------
---

29. Do you use to inject yourself with illicit/illegal drugs? YES--------NO----------

30. If yes, how often? Specify----------

31. Have you ever had accidental injury with sharp object before (needle stick, razor)?
YES----------NO----------

32. If yes from where? Healthcare facility----------Domestic----------
Others---------- (Specify) ----------

33. Have you had any organ transplantation before? Yes----------NO----------

34. If yes which organ and when? Specify----------

35. Which of the following is applicable to you?
Alcohol abuse---------- ------ Tribal Markings----------
Medicinal Scarification----------Visiting Barbing saloons----------
Visiting commercial Manicurers/Pedicurers---------- Body Tattooing----

Appendix 5: Interpretation of Results of ABO Blood Grouping

Tube Grouping

Tube 1 Anti-A	Tube 2 Anti-B	Tube 3 A cells	Tube 4 B cells	Tube 5 Control	Group
+	-	-	+	-	**A**
-	+	+	-	-	**B**
+	+	-	-	-	**AB**
-	-	+	+	-	**O**

Cell /Tile Grouping

Anti-A	Anti-B	Group
+	-	**A**
-	+	**B**
+	+	**AB**
-	-	**O**

Key:

+: Positive

-: Negative

Preparation of 5% Pooled A and B cell Suspension

1. Pool known A Cells and B cells on separate test tube.
2. Transfer about 0.5 ml of these pooled cells into tubes and add about 5ml of physiological saline.
3. Centrifuge at about 1000g for 2-3 minutes. Discard supernatant fluid, resuspend the sedimented red cells in a further 5ml saline and centrifuge at 1000g for 2 minutes. Discard the supernatant fluid.
4. Make 5% red cell suspension by mixing 1 drop of sedimented cells in 20 drops of saline using transfer Pasteur pipette.

Appendix 6: Assay Procedure for Detection of HBsAg Using Fortress Diagnostics

4TH Generation ELISA Kit

1. Add 50uL of positive control, negative control and specimen in respective wells.
2. Add 50uL of HRP Conjugate to each well except the blank and mix by tapping the plate gently.
3. Cover the plate and incubate at 37◦C for 1 hour.
4. Wash the plate 5 times with wash buffer.
5. Dispense 50uL of Chromogen A and 50uL of Chromogen B in all the wells and mix by tapping.
6. Incubate the plate at 37◦C for 15 minutes in the dark.
7. Add 50uL of stop Solution into each well and mix gently. Intensive yellow color develops in Positive control and HBsAg positive sample wells.
8. Measure the absorbance within 5 minutes after stopping the reaction at 450nm and 630nm as differential filter using Stat Fax 2100 Plate Reader.
9. Calculate the Cut-off value and evaluate the results.

The absorbance was measured using double filters at 450 and 630 nm respectively in an automated microplate reader (Model: Stat Fax 2100, Awareness Technology Inc., USA). The cut-off for each batch was calculated using the mean optical densities of the negative control in accordance with the manufacturer's instruction. This cut-off value was then used to calculate the activity index for each sample by dividing the OD of each sample with the cut-off value. Samples with the activity index values higher or equal to those of positive control were considered positive, while those with values below were reported as negative.

Appendix 7: Assay Procedure for Detection of HIV (Ag/Ab) Using Fortress

Diagnostics 4TH Generation ELISA Kit

1. Add 20uL of biotinylated anti-HIV p24 antibodies into each well except in the blank.
2. Add 100uL of positive control, negative control and specimen in respective wells.
3. Cover the plate and incubate at 37◦C for 60 minutes.
4. Wash the plate 5 times with buffer.
5. Add 100uL of HRP Conjugate to each well except the blank and mix by tapping the plate gently.
6. Cover the plate and incubate at 37◦C for 30 minutes.
7. Wash the plate 5 times with buffer.
8. Dispense 50uL of Chromogen A and 50ul of Chromogen B in all the wells and mix by tapping.
9. Incubate the plate at 37◦C for 15 minutes in the dark.
10. Add 50UL of stop Solution into each well and mix gently. Intensive yellow color develops in Positive control and HCV positive sample wells.
11. Measure the absorbance within 5 minutes after stopping the reaction at 450nm and 630nm as differential filter using Stat Fax 2100 Plate Reader.
12. Calculate the Cut-off value and evaluate the results.

Appendix 8: Assay Procedure for Detection of Anti-HCV Using Fortress Diagnostics 4TH Generation ELISA Kit

Assay Procedure

1. Add 100uL of specimen Diluent to all the wells.
2. Add 10uL of positive control, negative control and specimen in respective wells.
3. Cover the plate and incubate at 37◦C for 30 minutes.
4. Wash the plate 5 times with buffer.
5. Add 100uL of HRP Conjugate to each well except the blank and mix by tapping the plate gently.
6. Cover the plate and incubate at 37◦C for 30 minutes.
7. Wash the plate 5 times with buffer.
8. Dispense 50uL of Chromogen A and 50ul of Chromogen B in all the wells and mix by tapping.
9. Incubate the plate at 37◦C for 15 minutes in the dark.
10. Add 50uL of stop Solution into each well and mix gently. Intensive yellow color develops in Positive control and HCV positive sample wells.
11. Measure the absorbance within 5 minutes after stopping the reaction at 450nm and 630nm as differential filter using Stat Fax 2100 Plate Reader.
12. Calculate the Cut-off value and evaluate the results.

Appendix 9: Assay Procedure for Detection of Anti-HBc Total Using Fortress Diagnostics 4TH Generation ELISA Kit

Assay Procedure

1. Add 100uL of specimen Diluent to all the wells.
2. Add 20uL of positive control, negative control and specimen in respective wells.
3. Cover the plate and incubate at 37◦C for 30 minutes.
4. Wash the plate 5 times with buffer.
5. Add 100uL of HRP Conjugate to each well except the blank and mix by tapping the plate gently.
6. Cover the plate and incubate at 37◦C for 30 minutes.
7. Wash the plate 5 times with buffer.
8. Dispense 50uL of Chromogen A and 50ul of Chromogen B in all the wells and mix by tapping.
9. Incubate the plate at 37◦C for 15 minutes in the dark.
10. Add 50uL of stop Solution into each well and mix gently. Intensive yellow color develops in Positive control and anti-HBc positive sample wells.
11. Measure the absorbance within 5 minutes after stopping the reaction at 450nm and 630nm as differential filter using Stat Fax 2100 Plate Reader.
13. Calculate the Cut-off value and evaluate the results.

Appendix 10: Assay Procedure for Detection of Anti-HBc IgM Using Fortress Diagnostics 4TH Generation ELISA Kit

Assay Procedure

1. Add 100uL of specimen Diluent to all the wells.
2. Add 20uL of positive control, negative control and specimen in respective wells.
3. Cover the plate and incubate at 37◦C for 30 minutes.
4. Wash the plate 5 times with buffer.
5. Add 100uL of HRP Conjugate to each well except the blank and mix by tapping the plate gently.
6. Cover the plate and incubate at 37◦C for 30 minutes.
7. Wash the plate 5 times with buffer.
8. Dispense 50uL of Chromogen A and 50ul of Chromogen B in all the wells and mix by tapping.
9. Incubate the plate at 37◦C for 15 minutes in the dark.
10. Add 50uL of stop Solution into each well and mix gently. Intensive yellow color develops in Positive control and anti-HBc IgM positive sample wells.
11. Measure the absorbance within 5 minutes after stopping the reaction at 450nm and 630nm as differential filter using Stat Fax 2100 Plate Reader.
12. Calculate the Cut-off value and evaluate the results.

Appendix 11: Assay Procedure for Detection of Anti-HBS Using Fortress Diagnostics 4TH Generation ELISA Kit

Assay Procedure

1. Add 100uL of specimen Diluent to all the wells.
2. Add 20uL of positive control, negative control and specimen in respective wells.
3. Cover the plate and incubate at 37◦C for 30 minutes.
4. Wash the plate 5 times with buffer.
5. Add 100uL of HRP Conjugate to each well except the blank and mix by tapping the plate gently.
6. Cover the plate and incubate at 37◦C for 30 minutes.
7. Wash the plate 5 times with buffer.
8. Dispense 50uL of Chromogen A and 50ul of Chromogen B in all the wells and mix by tapping.
9. Incubate the plate at 37◦C for 15 minutes in the dark.
10. Add 50uL of stop Solution into each well and mix gently. Intensive yellow color develops in Positive control and anti-HBc positive sample wells.
11. Measure the absorbance within 5 minutes after stopping the reaction at 450nm and 630nm as differential filter using Stat Fax 2100 Plate Reader.
12. Calculate the Cut-off value and evaluate the results.

Appendix 12: Procedure of Preparation of 2% Agarose Gel and Electrophoresis of PCR Amplified Product

Preparation of 2% Agarose Gel

- Weigh 2g of agarose powder
- Add 100ml of running buffer (TBE or TAE)
- Heat for 3-5mins in a microwave to dissolve the powder
- Allow to cool to 56°C
- Add 2ul ethidium bromide
- Cast the gel solution in gel mould in which the gel comb has been appropriately inserted.
- Allow the agarose gel to polymerize for 45mins at room temperature

Electrophoresis of PCR Amplified Product

- Load 10ul of 100bp molecular marker mixed in loading dye in the first well
- Load 10ul test samples+ 2ul loading dye in other wells of agarose
- Run at 90-120V for 25-50mins
- View under UV transillumination

Appendix 13: Assay Procedure for Determination of Alanine Aminotransferase (ALT) Using Randox Reagent Kit and STAT FAX 1904 Spectrophotometer

Pipette into test tubes 50ul of serum samples and 50ul of distilled water in blank tube.

1. Add 250ul of Reagent 1 (Buffer) into all the test tubes
2. Mix, incubate for exactly 30 minutes at 37°C.
3. Add 250ul of Reagent 2 (2, 4-dinitophenylhydrazine) into all the tubes.
4. Mix and incubate in the dark for exactly 20 minutes at 20- 25°C.
5. Add 2.5ml of 0.4N Sodium Hydroxide solution into all the tubes.
6. Mix, read the absorbance of the samples against the reagent blank after 5 minutes using Stat Fax Spectrophotometer.
7. Calculate and obtain the activity of ALT in the specimen from the chart provided by kit manufacturer below.

CALCULATION

Obtain the activity of ALT in the serum from the table:

Absorbance	U/L	Absorbance	U/L
0.025	4	0.275	48
0.050	8	0.300	52
0.075	12	0.325	57
0.100	17	0.350	62
0.125	21	0.375	67
0.150	25	0.400	72
0.175	29	0.425	77
0.200	34	0.450	83
0.225	39	0.475	88
0.250	43	0.500	94

Appendix 14: Procedure of Determination of Haematological Indices Using Sysmex Auto-Haematology Analyzer

Operating Procedure

Start-Up

1. Check for fluid in the pneumatic trap chamber on the left side of the main unit.
   a. If fluid is present, unscrew the chamber counterclockwise, empty and dry.
   b. Replace the chamber taking care to make a good seal. Vacuum errors occur if not sealed tightly.
2. Empty the waste tank.
3. Check paper supply, Stromatolyser and Cell pack.
4. Switch on the Automatic Voltage Regulator and allow to stabilize.
5. Switch on UPS and allow to stabilize.
6. Switch on the KX-21N main unit, and allow the instrument to perform its automatic microprocessor tests, motor check, auto-rinses and a background count.
7. A background check is performed on the third rinse. Two additional rinses occur if the background count was unacceptable. Auto-rinse cycles are identified by the sample ID# of zero.
   a. When the background counts of all parameters are acceptable, the instrument is "Ready".
   b. If the counts are unacceptable "Background Error" displays and the alarm sounds briefly. Repeat the Autorinse.
      1) Press **[SELECT]**.
      2) Press **[5]** Autorinse.

KX-21N Acceptable Background Counts	
Parameter	**Count**
WBC	0.3 x 10^3/ µL or less
RBC	0.02 x 10^6/ µL or less
HGB	0.1 g/dl or less
PLT	10 x 10^3/ µL or less

Patient Sample Processing:

1. **Whole Blood Mode (50 µL sample volume)**
   a. The LCD should display "WB" and "READY".
      1) To change the mode, press **[MODE]**.
      2) Press **[◂ or ▸]** to select the "Whole Blood" mode.
      3) Press **[ENTER]**.
   b. Press **[SAMPLE No.]**.
   c. Using the numeric keys, enter the patient ID number (up to 15 digits), and then press **[ENTER]**. Do not use "0" for a patient ID number, as the results are not judged against any criteria, and will not print.
   d. Mix the specimen tube well, remove the stopper and hold the tube up to the sample probe.
   e. Press the Start switch.
   f. When the KX-21N beeps twice, remove the sample from the sample probe. Do not wipe the sample probe. Results print on the thermal printer after 60 seconds.

Daily Shutdown: Takes approximately 5 minutes to perform.

The Shutdown program cleans the transducer chambers and the diluted sample line. Perform Shutdown at the end of daily operation or at least once every 24 hours.

1. Press **[SHUTDOWN]**. The message “Aspirate CELL CLEAN. It will take approx. 5 minutes” displays.
2. Place the Cell clean up to the sample probe. Press the Start switch. Remove the tube of bleach when the unit beeps.
3. Once the Shutdown program finishes, “Turn off the power” appears.
4. Power off the KX-21N.

Appendix 15: Procedure for Determination of CD4 Cell Counts of Blood Donors Using Partec Cyflow Counter

Operations Procedures

A **Start Up**:

1. Fill the sheaths fluid bottle to 800ml mark and expel air from in line filter before corking tightly.
2. Discard the fluid in waste bottle and rinse it with 10% hypochlorite solution and cork tightly.
3. Check cable and electrical connection to the Cyflow counter and ensure it is properly connected.
4. Switch on Automatic Voltage Regulator and allow to stabilize.
5. Switch on UPS and allow to stabilize.
6. Switch on the Cyflow counter and allow it to boot.

This allows Cyflow Counter to calibrate and initialise to the previously used settings. A display shows empty histogram showing two cursor lines. Allow the laser about 15mins to warm up.The Cyflow counter is ready for acquisition. Verify the settings and compare it with the previous day's settings and if it falls within the acceptable values then run the fluid in the order below by inserting the sample tube into the sample port until you recognise a click.

- 1.6ml of cleaning fluid.
- 1.6ml of sheaths fluid.
- 850ul of count check beads.

Some adjustment may be made on the instrument setting to ensure that the peaks or signal of the count check beads is at 100 mark on the x-axis. If it is okay then allow the count check beads to run and count to obtain the result. Check that the gating of the signal of the count check the beads is okay to obtain the result. Calculate the result obtained from the counter as follows to convert the result to cell per ml and also to eliminate the dilution factor set on the Counter.

Results in cells per ml = results cells/ul * 1000 .

42.

Compare the result obtained with the value of the count check beads. The value will be accepted if it fails ±10% to the value. After the run then a 1.6ml of sheath fluid is plugged to run. This rinses the count check beads. It is important to note that the intra sample contamination is very low due to the availability of the biosafety valve system. Samples prepared can be analysed after if count check beads result is okay.

Sample Preparation:

A. **CD4 T lymphocyte cell count**:

The sample is prepared as follows

Into a Rohren test tube add:

- 20µL of CD4 PE mAb
- 20µL of well mixed EDTA whole blood collected within 6 hours.
- Mixed and incubate in the dark for 15mins at room temperature.
- Add 800µ of CD4 non-lyse buffer.
- Mix and read on the Cyflow counter.

Shut Down:

To shut down the system demands the cleaning procedure as follows.

- Run with 1.0 ml cleaning fluid twice.
- Run with 1.0 ml of decontamination fluid once .
- Run with 1.0 ml of sheaths fluid once.
- Plug a tube on the sample port to protect.

Shut down the counter, UPS, AVR and finally mains supply.

Appendix 16: Assay Procedure for Determination of Alpha Feto Protein (ALP) Level in Blood Specimens of Blood Donors

Secure the desired number of coated wells in the microwell holder.

1. Dispense 20ul of the standard, specimens and controls into appropriate wells.
2. Dispense 100ul of zero buffer into each well.
3. Thoroughly mix for 10 seconds. It is important to have a complete mixing in this step.
4. Incubate at room temperature (18-22°C) for 30 minutes.
5. Remove the incubation mixture by flicking plate content into a waste container.
6. Rinse the flick microtiter wells 5 times with washing Buffer (1X).
7. Strike the wells sharply onto absorbent paper or paper towel to remove residual buffer droplets.
8. Dispense 150ul of Enzyme Conjugate reagent into each well. Gently mix for 5 seconds.
9. Incubate at room temperature for 30 minutes.
10. Remove the incubation mixture by flicking plate content into a waste container.
11. Rinse and flick the microtiter wells for 5 times with washing buffer.
12. Strike the wells sharply onto absorbent paper to remove residual buffer droplets.
13. Dispense 100ul TMB substrate into each well. Gently mix for 5 seconds.
14. Incubate at room temperature for 20 minutes.
15. Stop the reaction by adding 100ul of stop solution to each well.
16. Gently mix for 30 seconds to ensure that the blue color changes to yellow color completely.
17. Read optical density at 450nm with differential filter of 630nm with a microplate Reader within 15 minutes of stopping the reaction.

Calculation of Results

Construct a standard curve by plotting the absorbance obtained from each reference standard against the concentration in ng/ml on a graph paper, with the absorbance value on the vertical or Y-axis and concentration on the horizontal or X-axis. Use the mean absorbance value for each specimen to determine the corresponding concentration of AFP in ng/ml from the standard curve.

Expected Values and Sensitivity

In high risk patients, AFP values between 100 and 350ng/ml suggest a diagnosis of Hepatocellular Carcinoma, and a level over 350ng/ml usually indicates the disease. Approximately 97% of the healthy subjects have AFP levels less than 8.5ng/ml. It is recommended that each laboratory establish its own normal range. The minimum detectable concentration of AFP by this assay is estimated to be 2.0ng/ml

Appendix 17: Nucleotide Sequence of HBV Isolates MG562502- MG562503

H1A

>TCACTCATCTCGTCATCTTCTCGAGGATTGGGGACCTTGCACCGAACATGGAA
AACATCACATCAGGATTCCTAGGACCCCTGCTCGTGTTACAGGCGGGGTTTTTC
TTGTTGACAAAAATCCTCACAATACCGCAGAGTCTAGACTCGTGGTGGACTTCT
CTCAATTTTCTAGGGGGAGCTCCCGTGTGTCTTGGCCAAAATTCGCAGTCCCCA
ATCTCCAGTCACTCACCAACCTGA

H6A

>TCATCTCGTCATCTTCTCGAGGATTGGGGACCCTGCACCGAACATGGAAAGCA
CAACATCAGGATTCCTAGGACCACTGCTCGTGTTACAGGCGGGGTTTTTCTTGT
TGACAAAAATCCTCACAATACCGCAGAGTCTAGACTCGTGGTGGACTTCTCTCA
ATTTTCTAGGGGGAGCTCCCGTGTGTCTTGGCCAAAATTCGCAGTCCCCAATCT
CCAGTCACTCACCAACCTGA

Sequence_ID	Organism	isolate
H1A	Hepatitis B virus strain AYNF362	H1A
H6A	Hepatitis B virus isolate SDAC_059	H6A

Appendix 18: Testing Research Hypotheses/Assumptions

Hypothesis 1

Null *(H0)*:Blood donors' sample that tested negative for HBV Serologic markers (Seronegative) is not negative for HBV DNA.

Alternate (HA): Blood donors' sample that tested negative for HBV Serologic markers (Seronegative) is negative for HBV DNA**.**

Data for this hypothesis testing was culled from Table 4.2

HBV DNA	**HBV Markers Negative**	**HBV Markers Positive**	**TOTAL**
POSITIVE	2	12	14
NEGATIVE	68	18	86
TOTAL	70	30	100

Contingency Table

CELL	**O**	**E**	**O-E**	**$(O-E)^2$**	**$(O- E)^2/E$**
1	2	10	-8	64	6.40
2	68	60	8	64	1.07
3	12	4	8	64	16.00
4	18	26	-8	64	2.46
TOTAL					25.93= X^2

Calculation of Expected= Column Total X RowTotal /Grand Total

E1= 14 X 70/100= 9.8 APPROX =10

E2 = 86 X 70/ 100=60.2 APPROX= 60

E3= 30 X14/100= 4.2 APPROX= 4

E4 = 30 X 86/100 = 25.8 APPROX= 26

Analysis: Calculated value, 25.93 is greater than the theoretical book value of 5.99 at three degree of freedom (df) .Therefore the null hypothesis is rejected and the alternate hypothesis that states:Blood donors' samples negative for HBV Serologic markers (Seronegative) is negative for HBV DNA is accepted. This means that the observed difference is statistically significant at 5% level ($p<0.05$).

Hypothesis 2

Null (H0): Blood donor sample that tested positive for anti-HBc marker is not positive HBV DNA

Alternate (HA): Blood donor sample that tested positive for anti-HBc marker is positive HBV DNA

Data for this Hypothesis testing was culled from Table 4.2 and Table 4.4

HBV DNA	**Anti-HBc-**	**Anti-HBc+**	**TOTAL**
POSITIVE	9	5	14
NEGATIVE	80	6	86
TOTAL	89	11	100

Contingency Table

CELL	O	E	O-E	$(O-E)^2$	$(O- E)^2/E$
1	**9**	**12**	**-3**	**9**	**0.75**
2	**80**	**77**	**3**	**9**	**0.11**
3	**5**	**2**	**3**	**9**	**4.50**
4	**6**	**3**	**-3**	**9**	**3.00**
TOTAL					**8.36=** X^2

E1= 89 x 14/100= 12.46

E2= 89 x 86/100= 76.54

E3= 11 X 14/100= 1.54

E4= 11 X 86/100= 9.46

Analysis

Calculated value 8.36 is greater than book value which is 5.99 at three degree of freedom. Therefore the null hypothesis is being rejected. The alternate hypothesis that states that: Blood donor sample negative for HBsAg but positive for anti-HBc marker is positive for HBV DNA is accepted. Hence, the observed difference is statistically significant at 5 percent level ($p<0.05$).

Hypothesis 3

Null (H0): Blood donors' samples that tested positive by HBsAg ELISA technique are not positive for HBV DNA.

Alternate (HA): Blood donors' samples that tested positive by HBsAg ELISA technique are positive for HBV DNA.

Data for analysis of this Hypothesis was culled from Table 4:2, Table 4.5 and Table 4.12

HBV DNA	HBsAg ELISA Negative	HBsAg ELISA Positive	TOTAL
POSITIVE	14	20	34
NEGATIVE	86	8	94
TOTAL	100	28	128

E1= 34X100/128= 26.56

E2= 94 X 100/128= 73.44

E3= 34X 28/128= 7.44

E4= 94 X28/128= 20.56

Contingency Table

CELL	O	E	O-E	$(O-E)^2$	$(O- E)^2/E$
1	14	27	-13	169	6.26
2	86	73	-13	169	2.32
3	20	7	13	169	24.14
4	8	21	-13	169	*8.05*
TOTAL					40.77= X^2

Analysis: Calculated Value 40.77 is greater than book value, 5.99; therefore the null hypothesis is rejected. The alternate hypothesis that states: Blood donor samples tested negative for HBsAg by rapid test but positive by ELISA technique are positive for HBV DNA.

Hypothesis 4

Null (H0): Blood donors' samples that tested positive for HBsAb are not positive for HBV DNA.

Alternate (HA): Blood donors' samples that tested positive for HBsAb are positive for HBV DNA.

Data for this Hypothesis Testing was culled from Table 4.2 and Table 4.4

HBV DNA	**HBsAb-**	**HBsAb+**	**TOTAL**
POSITIVE	5	9	14
NEGATIVE	76	10	86
TOTAL	81	19	100

E1= 81 X 14/100= 11.34

E2= 81 X 86/100= 69.66

E3= 19 X 14/100= 2.66

E4= 19 X 86/100= 16.34

Contingency Table

CELL	O	E	O-E	$(O-E)^2$	$(O- E)^2/E$
1	5	11	-6	36	3.27
2	76	70	6	36	0.51
3	9	3	6	36	12.00
4	10	15	-6	36	2.40
					18.18

Analysis: The calculated value 18.18 is greater than the book value of 5.99 at three degree of freedom. Therefore, the null hypothesis is rejected.The alternate hypothesis that states; Blood donors' samples negative for HBsAg but positive for HBsAb are positive for HBV DNA is accepted. The observed difference is statistically significant at 5% level ($p<0.05$).

Appendix 19: Some Results of Roche COBAS Real Time PCR

your company name — your department

AMPLILINK 3.3 Report: Sample Result Detail

Order

Order Number: UA/17/HBV/019
Order Date/Time: 06/13/2017 08:53:23
Ordered by: UATHLAB
Sample ID: UA/17/HBV/019
Diluted: No
Doctor:
Hospital:
OrderCom2:

Patient

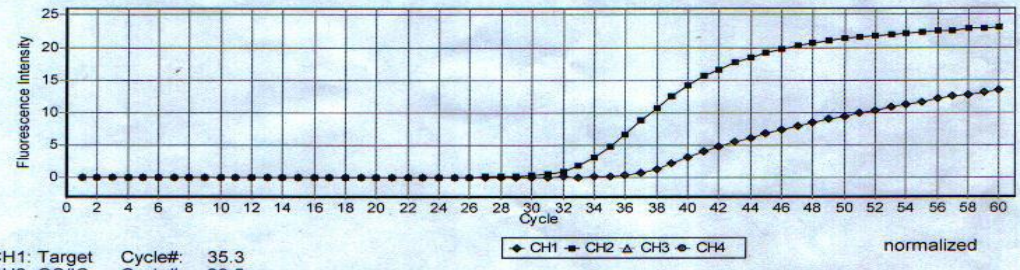
Patient ID: UATH 047
Patient Name:
Date of Birth:
Sex:
PatientCom1:
PatientCom2:

Test	Result	Flag Remark	Timestamp
HB2CAP48	60 IU/mL		06/13/2017 13:24:22

Workflow

Process Steps	Name	System ID	Position	Timestamp	Clip#
Primary Pipetting	HB2CAP48	Manual	n/a	n/a	n/a
AmpliPrep Preparation	HB2CAP48	UATH-CAP1 (394923	0001 - 22	06/13/2017 10:55:12	$SC2A05E7D
TaqMan48 Amplification	HB2CAP48	UATH-CTM48 (00463	TCA 002 - 05	06/13/2017 13:24:12	$800205
TaqMan48 Detection	HB2CAP48	UATH-CTM48 (00463	TCA 002 - 05	06/13/2017 13:24:22	$800205

Measurement Details



normalized

CH1: Target Cycle#: 35.3
CH2: QS/IC Cycle#: 29.5

Reagent Kits

Reagent Kit	Lot Number	Cassette Serial Nr.	Expiration Date	Calibration
PCR Kit	X0541400000	5354	04/30/2018	A: -0.0003 B: 0.3150 C: 3.6100

Reviewing Information

Accepted by: UATHLAB
Accepted Date/Time: 06/13/2017 13:33:59

1 / 1 — UATHLAB — 06/13/2017
AMPLILINK 3.3.7 (Build 1201)

your company name | your department

AMPLILINK 3.3 Report: Sample Result Detail

Order

Order Number: UA/17/HBV/021
Order Date/Time: 06/13/2017 08:53:23
Ordered by: UATHLAB
Sample ID: UA/17/HBV/021
Diluted: No
Doctor:
Hospital:
OrderCom2:

Patient

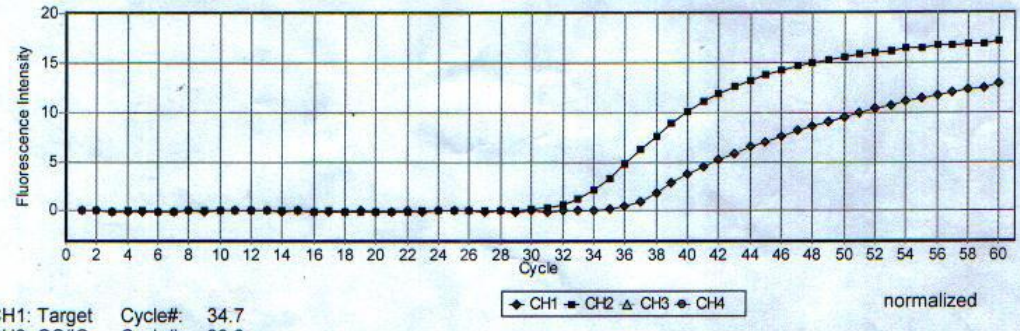
Patient ID: NAUTH 102
Patient Name:
Date of Birth:
Sex:
PatientCom1:
PatientCom2:

Test	Result	Flag Remark	Timestamp
HB2CAP48	128 IU/mL		06/13/2017 13:24:22

Workflow

Process Steps	Name	System ID	Position	Timestamp	Clip#
Primary Pipetting	HB2CAP48	Manual	n/a	n/a	n/a
AmpliPrep Preparation	HB2CAP48	UATH-CAP1 (394923	0001 - 24	06/13/2017 11:02:14	$SC2A05E7B
TaqMan48 Amplification	HB2CAP48	UATH-CTM48 (00463	TCA 002 - 24	06/13/2017 13:24:12	$800224
TaqMan48 Detection	HB2CAP48	UATH-CTM48 (00463	TCA 002 - 24	06/13/2017 13:24:22	$800224

Measurement Details



CH1: Target Cycle#: 34.7
CH2: QS/IC Cycle#: 30.0

Reagent Kits

Reagent Kit	Lot Number	Cassette Serial Nr.	Expiration Date	Calibration
PCR Kit	X0541400000	5354	04/30/2018	A: -0.0003 B: 0.3150 C: 3.6100

Reviewing Information

Accepted by: UATHLAB
Accepted Date/Time: 06/13/2017 13:33:59

your company name | your department

AMPLILINK 3.3 Report: Sample Result Detail

Order

Order Number: UA/17/HBV/018
Order Date/Time: 06/13/2017 08:53:23
Ordered by: UATHLAB
Sample ID: UA/17/HBV/018
Diluted: No
Doctor:
Hospital:
OrderCom2:

Patient

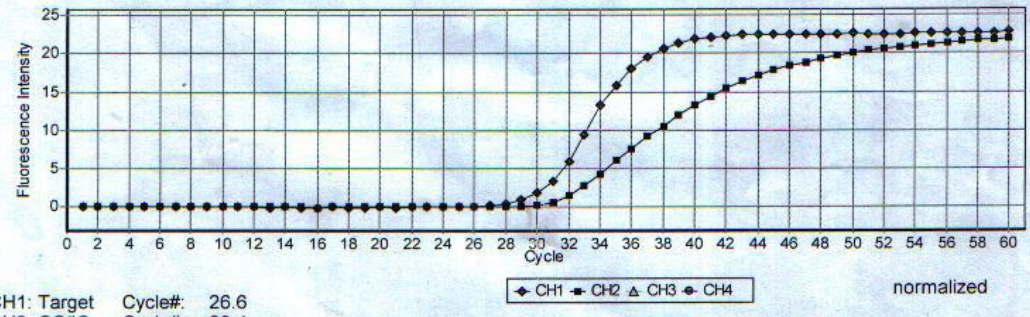
Patient ID: UATH 026
Patient Name:
Date of Birth:
Sex:
PatientCom1:
PatientCom2:

Test	Result	Flag Remark	Timestamp
HB2CAP48	31379 IU/mL		06/13/2017 13:24:22

Workflow

Process Steps	Name	System ID	Position	Timestamp	Clip#
Primary Pipetting	HB2CAP48	Manual	n/a	n/a	n/a
AmpliPrep Preparation	HB2CAP48	UATH-CAP1 (394923	0001 - 21	06/13/2017 10:51:36	$SC2A05E71
TaqMan48 Amplification	HB2CAP48	UATH-CTM48 (00463	TCA 002 - 20	06/13/2017 13:24:12	$800220
TaqMan48 Detection	HB2CAP48	UATH-CTM48 (00463	TCA 002 - 20	06/13/2017 13:24:22	$800220

Measurement Details



CH1: Target Cycle#: 26.6
CH2: QS/IC Cycle#: 29.4

Reagent Kits

Reagent Kit	Lot Number	Cassette Serial Nr.	Expiration Date	Calibration
PCR Kit	X0541400000	5354	04/30/2018	A: -0.0003 B: 0.3150 C: 3.6100

Detail Flags

TM42: SPK_CORR-1

Reviewing Information

Accepted by: UATHLAB
Accepted Date/Time: 06/13/2017 13:33:59

your company name

your department

AMPLILINK 3.3 Report: Sample Result Detail

Order

Order Number: UA/17/HBV/017
Order Date/Time: 06/13/2017 08:53:23
Ordered by: UATHLAB
Sample ID: UA/17/HBV/017
Diluted: No
Doctor:
Hospital:
OrderCom2:

Patient

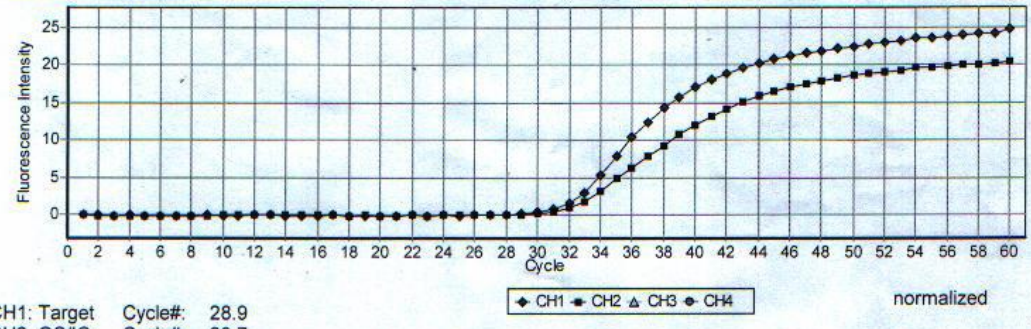
Patient ID: UATH 029
Patient Name:
Date of Birth:
Sex:
PatientCom1:
PatientCom2:

Test	Result	Flag Remark	Timestamp
HB2CAP48	7290 IU/mL		06/13/2017 13:24:22

Workflow

Process Steps	Name	System ID	Position	Timestamp	Clip#
Primary Pipetting	HB2CAP48	Manual	n/a	n/a	n/a
AmpliPrep Preparation	HB2CAP48	UATH-CAP1 (394923	0001 - 20	06/13/2017 10:48:21	$SC2A05E78
TaqMan48 Amplification	HB2CAP48	UATH-CTM48 (00463	TCA 002 - 23	06/13/2017 13:24:12	$800223
TaqMan48 Detection	HB2CAP48	UATH-CTM48 (00463	TCA 002 - 23	06/13/2017 13:24:22	$800223

Measurement Details



CH1: Target Cycle#: 28.9
CH2: QS/IC Cycle#: 29.7

Reagent Kits

Reagent Kit	Lot Number	Cassette Serial Nr.	Expiration Date	Calibration
PCR Kit	X0541400000	5354	04/30/2018	A: -0.0003 B: 0.3150 C: 3.6100

Detail Flags

TM42: SPK_CORR-1

Reviewing Information

Accepted by: UATHLAB

Accepted Date/Time: 06/13/2017 13:33:59

your company name | your department

AMPLILINK 3.3 Report: Sample Result Detail

Order

Order Number: UA/17/HBV/020
Order Date/Time: 06/13/2017 08:53:23
Ordered by: UATHLAB
Sample ID: UA/17/HBV/020
Diluted: No
Doctor:
Hospital:
OrderCom2:

Patient

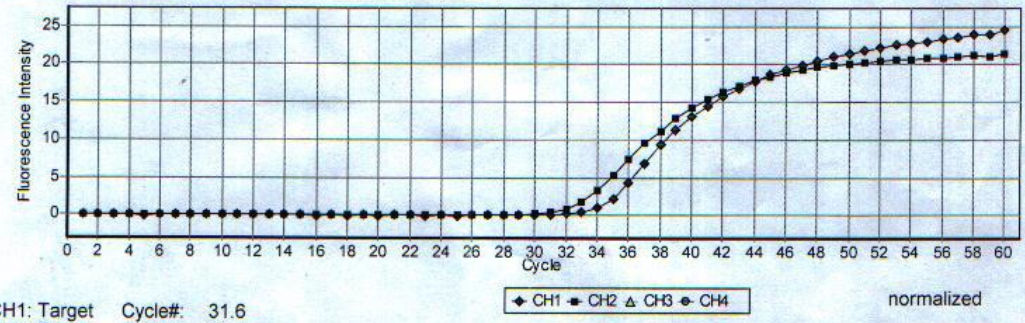
Patient ID: UAUTH 094
Patient Name:
Date of Birth:
Sex:
PatientCom1:
PatientCom2:

Test	Result	Flag Remark	Timestamp
HB2CAP48	924 IU/mL		06/13/2017 13:24:22

Workflow

Process Steps	Name	System ID	Position	Timestamp	Clip#
Primary Pipetting	HB2CAP48	Manual	n/a	n/a	n/a
AmpliPrep Preparation	HB2CAP48	UATH-CAP1 (394923	0001 - 23	06/13/2017 10:58:48	$SC2A05E6F
TaqMan48 Amplification	HB2CAP48	UATH-CTM48 (00463	TCA 002 - 01	06/13/2017 13:24:12	$800201
TaqMan48 Detection	HB2CAP48	UATH-CTM48 (00463	TCA 002 - 01	06/13/2017 13:24:22	$800201

Measurement Details



normalized

CH1: Target Cycle#: 31.6
CH2: QS/IC Cycle#: 29.6

Reagent Kits

Reagent Kit	Lot Number	Cassette Serial Nr.	Expiration Date	Calibration
PCR Kit	X0541400000	5354	04/30/2018	A: -0.0003 B: 0.3150 C: 3.6100

Reviewing Information

Accepted by: UATHLAB
Accepted Date/Time: 06/13/2017 13:33:59

your company name

your department

AMPLILINK 3.3 Report: Sample Result Detail

Order

Order Number: UA/HBV/17/009OSJ
Order Date/Time: 07/10/2017 11:30:26
Ordered by: UATHLAB
Sample ID: UA/HBV/17/009OSJ
Diluted: No
Doctor:
Hospital:
OrderCom2:

09

Patient

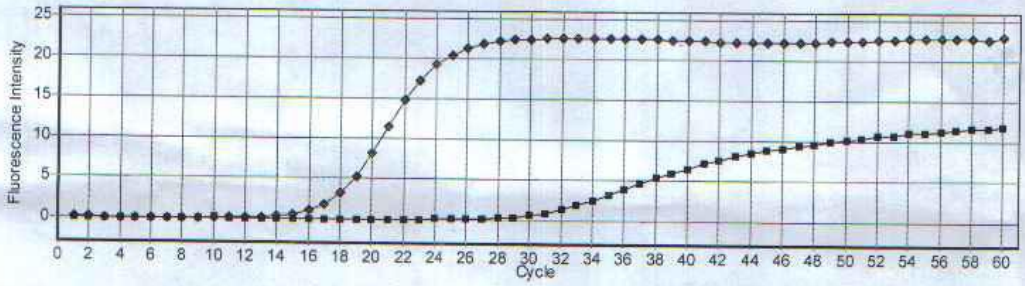
Patient ID:
Patient Name:
Date of Birth:
Sex:
PatientCom1:
PatientCom2:

Test	Result	Flag Remark	Timestamp
HB2CAP48	99520111 IU/mL		07/10/2017 16:02:24

Workflow

Process Steps	Name	System ID	Position	Timestamp	Clip#
✔ Primary Pipetting	HB2CAP48	Manual	n/a	n/a	n/a
✔ AmpliPrep Preparation	HB2CAP48	UATH-CAP1 (394923	0016 - 06	07/10/2017 12:40:41	$SB29F5B5A
✔ TaqMan48 Amplification	HB2CAP48	UATH-CTM48 (00463	TCA 002 - 18	07/10/2017 16:02:13	$800218
✔ TaqMan48 Detection	HB2CAP48	UATH-CTM48 (00463	TCA 002 - 18	07/10/2017 16:02:24	$800218

Measurement Details



normalized

CH1: Target Cycle#: 13.8
CH2: QS/IC Cycle#: 27.9

Reagent Kits

Reagent Kit	Lot Number	Cassette Serial Nr.	Expiration Date	Calibration
PCR Kit	X0541400000	5354	04/30/2018	A: -0.0003 B: 0.3150 C: 3.6100

Detail Flags

TM42: SPK_CORR-1

Reviewing Information

Accepted by: UATHLAB

Accepted Date/Time: 07/10/2017 16:11:25

your company name

your department

AMPLILINK 3.3 Report: Sample Result Detail

Order

Order Number: UA/HBV/17/6067OSJ
Order Date/Time: 07/10/2017 11:30:26
Ordered by: UATHLAB
Sample ID: UA/HBV/17/6067OSJ
Diluted: No 067
Doctor:
Hospital:
OrderCom2:

Patient

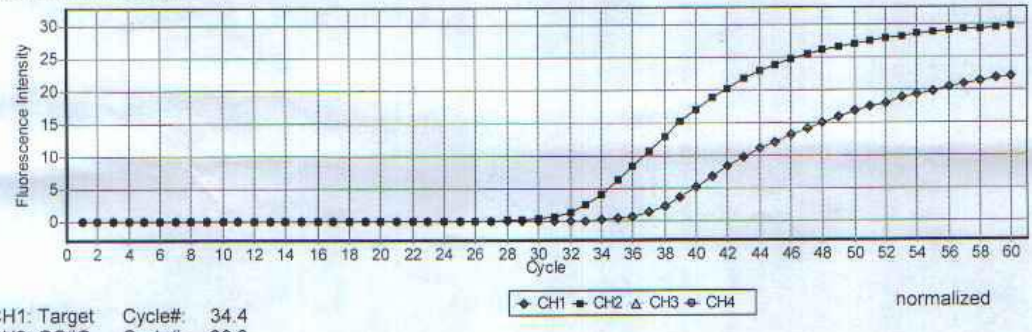
Patient ID:
Patient Name:
Date of Birth:
Sex:
PatientCom1:
PatientCom2:

Test	Result	Flag	Remark	Timestamp
HB2CAP48	92 IU/mL			07/10/2017 16:02:24

Workflow

Process Steps	Name	System ID	Position	Timestamp	Clip#
Primary Pipetting	HB2CAP48	Manual	n/a	n/a	n/a
AmpliPrep Preparation	HB2CAP48	UATH-CAP1 (394923	0016 - 08	07/10/2017 12:47:53	$SA266F59B
TaqMan48 Amplification	HB2CAP48	UATH-CTM48 (00463	TCA 002 - 09	07/10/2017 16:02:13	$800209
TaqMan48 Detection	HB2CAP48	UATH-CTM48 (00463	TCA 002 - 09	07/10/2017 16:02:24	$800209

Measurement Details



CH1: Target Cycle#: 34.4
CH2: QS/IC Cycle#: 29.2

Reagent Kits

Reagent Kit	Lot Number	Cassette Serial Nr.	Expiration Date	Calibration
PCR Kit	X0541400000	5354	04/30/2018	A: -0.0003 B: 0.3150 C: 3.6100

Reviewing Information

Accepted by: UATHLAB

Accepted Date/Time: 07/10/2017 16:11:25

Appendix 20: Some Equipment/Instruments Used During the Project

Figure 4.22: Stax fax 2100 Plate Reader and Printer used for all ELISA Procedures

Figure 4.23: Stat Fax 1904 Spectrophotometer Used for Determination of Alanine Aminotransferase (ALT)

Figure 4.24: Vortex Machines for Mixing of Samples and Reagents before Centrifugation

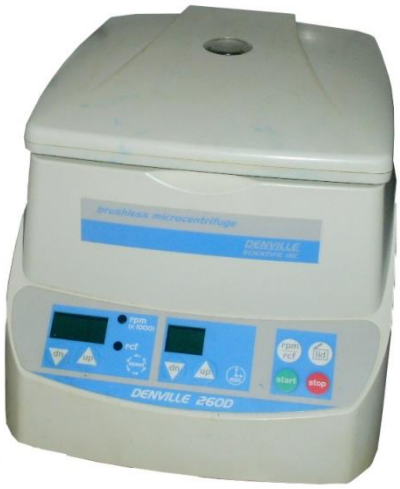


Figure 4.25: Denville 260D Microcentrifuge used for DNA Extraction for Conventional PCR

Figure 4.26: Thermo Scientific NanoDrop 1000 Spectrophotometer Used for Detection and Assessing the Quality of DNA after Extraction

Figure 4.27: Samples Preparation in Biosafety Cabinet CLASS II for Real Time PCR

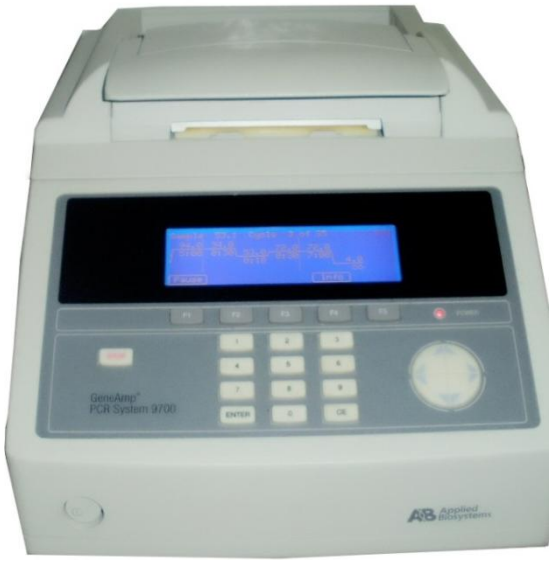


Figure 4.28: GeneAmp PCR System 9700 Thermal cycler used for Conventional PCR

Figure 4.29: Loading of Samples in COBAS AmpliPrep for Nucleic Acid extraction for Real Time PCR

Figure 4.30: UVP Trans illuminator for Gel Visualization and tank for Agarose Gel Electrophoresis

Figure 4.31: Retrieving the Results of Real Time PCR from COBAS Amplilink

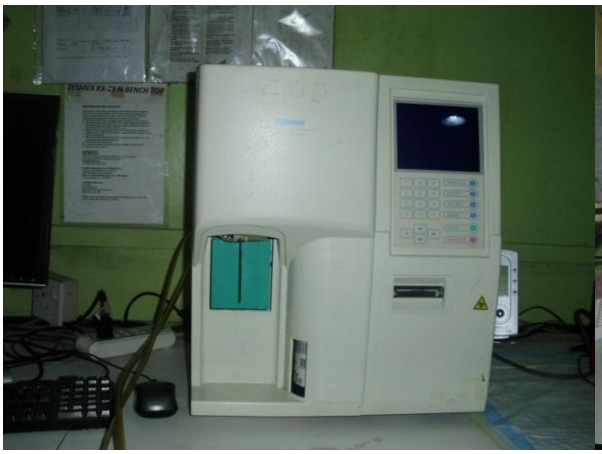


Figure 4.32: Sysmex Auto-Hematology Analyzer used for Determination of some Hematological Parameters

Figure 4.33: Cyflow Counter used for Determination of CD4 Cell Count