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**Threat of Occult Hepatitis B Virus Infection: A Major Issue in Blood Transfusion Services**

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**Summary**

*Occult hepatitis B virus infection is the existence of hepatitis B virus DNA in blood and liver tissues of hepatitis B surface antigen-negative individual as detected using molecular analysis (PCR). It is so far one of risk factor involved in transmission of HBV from blood and organ donations. Healthy individual that harbors occult HBV DNA may reactivate and yield hepatitis in potential organ and/or blood recipients depending upon host immune response and viral load. Therefore, it is pertinent to ascertain the quality of blood before blood transfusion. An 18% prevalence was reported in HBV seropositive individuals, and only 8% among seronegative individuals. In 2015, a research conducted in Nigeria revealed a prevalence of 17%. Several molecular mechanisms including deletions, gene splicing, point mutations, co-infection with HCV and Lamivudine associated mutations are implicated in pathogenesis of occult hepatitis B virus infection. Pathogenesis of HBV infection is influenced by multiple cytokines. Tumor necrotic factor (TNF) induces innate immune response and causes destruction of virally infected hepatocytes. Interleukin 12 (IL-12) functions in viral clearance and seroconversion. Interferon gamma (IFN- $\gamma$ ) has antiviral activity, while Interleukin 10 (IL-10) has anti-inflammatory and immunosuppressive function. Diagnosis of occult HBV infection is carried out by detecting circulating HBsAg, HBV DNA, anti-surface antibody, and anti-core antibody. This infection may be treated using several approaches including INF- $\alpha$ , Lamivudine, Entecavir, Adefovir and pegylated INF- $\alpha$  therapies. However, advanced approach to treatment involves employing 'Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas9)'. This review is aimed at highlighting the risk associated with transfusing blood screened only with HBsAg assays, because health conditions demanding blood transfusion is high in Nigeria, and so the need to initiate HBV DNA screening in blood transfusion*

*services.*

**Keywords:** *Occult, hepatitis B virus, Cytokines, seroconversion, CRISPR/Cas9*

**Introduction**

Hepatitis B Virus (HBV) infection is one of the major causative agent implicated in hepatocellular carcinoma. More than 400 million people have HBsAg- positive viral infection mostly acquired by vertical transmission (Raimondo *et al.*, 2008). This infection in most cases is chronic i.e. persist for more than six months in the blood or serum of an individual.

Hepatitis B Virus is a 42nm enveloped double stranded (dsDNA) virus having an RNA intermediate, belonging to group VII of Baltimore classification, and a member of *Hepadnaviridae* family (Lee, 1997). HBV envelop is made of a lipid bilayer in which are integrated Hepatitis B surface antigen, which is a 25 KDa polypeptide. HBV nucleocapsid is composed of hepatitis B envelope antigen (HBeAg) and hepatitis B core antigen (HBcAg) (Lee, 1997).

HBV infection may present signs and symptoms or exist as asymptomatic. Asymptomatic HBV infection is common in children and may present with mild symptoms that mostly go unnoticed (McMahon *et al.*, 1985). Symptomatic HBV infection is usually associated with fever, malaise, nausea, pale-colored stool, jaundice and sometimes enlargement of the liver leading to upper right quadrant discomfort (Mahoney, 1999). Increased serum transaminases and bilirubin levels are also suggestive of HBV infection.

HBV infection is hyperendemic in Sub-Saharan Africa with greater than 8% affected (Ola and odaibo, 2007). In Nigeria, prevalence of HBV

infection accounts for 13.6%, with 14.1% among pregnant women attending antenatal clinics; 11.5% among children and 14.0% among adults (Musa *et al.*, 2015). Other studies show a prevalence of 8.8% among Tanzanian Doctors (Matee *et al.*, 1999), 10.0% among Sudanese Blood donors (Elfaki *et al.*, 2008). A prevalence of 18% of occult HBV infection was reported in HBV seropositive individuals, and only 8% among seronegative individuals (Minuk *et al.*, 2005). In Nigeria, a research conducted by Opaleye *et al.* (2015) revealed a prevalence of 17%. In relation to overall prevalence of HBV infection, occult HBV accounts for 3.7% in the Americas, 4.9% in India (Jain and Nijhawan, 2008), 12.4% in Turkey, and 26.6% in Italy (Di Stefano *et al.*, 2009). Risk factors include chronic liver disease, Hepatitis C virus (HCV) infection, Human immunodeficiency virus (HIV) and haemodialysis.

Occult hepatitis B virus infection is the absence of HBsAg and presence of HBV DNA in blood and liver tissues of individual as detected by PCR. It is so far a risk factor of concern associated with transmission of HBV in blood and organ donations. Blood or organ harboring the HBV DNA may reactivate and yield hepatitis, depending upon host immune response and viral load (Mehdi, 2013).

Prevalence of Occult HBV infection in endemic areas of the world and in developed countries due to advanced medical procedures including organ transplant and hemodialysis is high. This review is aimed at highlighting the risk associated with transfusing blood screened only with HBsAg assays, because health conditions demanding blood transfusion worldwide is high, especially in Nigeria due to increased rate of postpartum haemorrhage, road accidents, insurgency, malaria endemicity and sickle cell disease. In the light of the above, advocacy exist on the need to initiate HBV DNA screening in blood transfusion services.

## ETIOLOGY

HBV infection is caused by Hepatitis B virus, belonging to *Hepadnaviridae* family, the genome being a double stranded DNA consisting of an outer envelope and inner nucleocapsid. The outer envelope contains HBsAg and the inner nucleocapsid consists of HBcAg and HBeAg (Lee, 1997). Host cellular immune response against HBV is induced by HBcAg-derived peptides, and existing viral replication is indicated by HBeAg, while DNA polymerase serves in reverse transcriptase function for synthesis of both negative and positive HBV DNA strands (Sara *et al.*, 2011).

## PATHOPHYSIOLOGY

HBV is transmitted via blood transfusion, vaginal secretions, semen, vertical transmission (mother to baby), needle prick injury, body piercings, tattoos, injections. Upon entry into the blood stream, mature virions are carried to the liver where they attach to cell membrane of hepatocytes mediated by Pre-S proteins, followed by entry. The viral genome uncoats, releasing a circular DNA, which is then transported into nucleus where genomic transcripts are transcribed by RNA polymerase of the host cell. Pre-genomic RNA is subsequently used as a template for synthesis of HBV DNA (Jasmine *et al.*, 2012).

Hepatocytes are not directly killed by HBV. Rather, liver damage is caused due to immune response to viral antigens. Antigen presenting cells presents the viral antigens in association with T lymphocytes in the lymphoid tissues. The T cells mature, expand and moves to liver triggering an inflammatory response which clears the viral DNA induced by tumor necrosis factor- $\alpha$  and interferon- $\gamma$ . This leads to downregulation of viral replication giving rise to CD8<sup>+</sup> cytotoxic T cells disintegration of infected hepatocytes (Guidotti *et al.*, 1996).

A vital replication step of HBV is transcription of pregenomic mRNA, where covalently closed circular DNA is used as template for integrating HBV DNA into the nucleus of hepatocytes, thereby causing hepatocellular carcinoma (Seeger and Mason, 2000). HBsAg is seen when a patient is considered to be infected (may be acute, chronic or carriers). Persistence of HBsAg differentiates acute from chronic infection. Existence of Anti-HBs indicates either active or passive persistent lifelong immunization (Zhang *et al.*, 2016). Antibodies to HBV are detected at different phases of infection, with Anti-HBc being the first to be detected, IgM anti-HBc detected in acute infection and during window period. IgG anti-HBc appears due to previous or ongoing infection. Presence of HBeAg indicates active disease and increased rate of viral replication and transmission. Anti-HBe indicates seroconversion and clinical improvement. HBV DNA indicates viral burden and replication and an indication for subjecting a patient to antiviral therapy (Hao *et al.*, 2016).

## OCCULT HEPATITIS B VIRUS INFECTION

This is the presence of HBV DNA while circulating HBsAg is absent in the serum of infected individual. Despite presence of other HBV antigens, absence of serum HBsAg is regarded as HBV negative. However, molecular detection of HBV DNA may

prove this wrong- for example, Raimondo *et al.* (2008) found that some individuals may have HBV DNA in their blood or Liver, but lacking circulating HbsAg, also Brechot *et al.* (2001) revealed that occult HBV infection may be linked with low HBV levels, but not HBV mutants devoid of surface proteins. Several risk factors are associated with occult HBV infection (Table 1), with organ transplant having the highest prevalence.

Occult HBV infection is due to undetectable anti-HBc and/or anti-HBs antibodies in seronegative Occult hepatitis B virus infection (Raimondo *et al.*, 2008).

Occult HBV infection may persist for years without any clinical sign and symptoms unless if triggered by

immunosuppression, drug misuse and abuse or coinfection (Jeantet *et al.*, 2004)

HBV infection can easily be diagnosed using serological assays that are targeted at detecting the presence of HBsAg. However, this is only possible when there is high HBV viral load. Therefore, people with low viral load may not be positive for HBsAg, but may harbor the viral DNA making the infection hidden and hence termed 'occult HBV infection' (Raimondo *et al.*, 2008).

Recovery from an acute HBV infection following seroconversion (absence of HBsAg in serum) may result in signs of hepatocellular carcinoma and other related liver diseases.

**Table 1. High risk groups for occult HBV Infection**

Group	Prevalence% _____
Transplant patients	64
HCV infection	15-33
Hemodialysis	27
HIV infection	10-45
Hepatocellular carcinoma	62

High risk groups (Jasmine *et al.*, 2012).

**MOLECULAR BASIS OF OCCULT HBV INFECTION**

Genetic aberrations including gene splicing, deletions, and point mutations are linked to this infection, but it is not obvious if such mutations are the cause of occult HBV infection. Most of these mutations occur at the S gene or regions associated with regulating its expression (Jasmine *et al.*, 2012).

A missense mutation at preS1 domain of HBsAg was found to be located at receptor binding site of the sequence, with eleven amino acid changes. A single mutation in TP and S domains of P protein of HBV confers resistance to antiviral therapy (Julio *et al.*, 2017). These missense mutations mostly occur between nt 350 to nt 750 of the viral genome sequence. These are important regions for antiviral resistance and escape mutants (Zhang *et al.*, 2016).

Mutations in RT domain of P protein result in defective viral replication leading to abnormal function of the HBx protein. This gives rise to downregulating mRNA expression (Zhu *et al.*, 2016). Splicing of S gene mRNA has been found to be altered due to a Guanine-to-Adenine mutation of the surface gene at position 458 thereby interfering with the HBsAg expression (Hass *et al.*, 2005). HBV

genome has been found to contain a region called the 'Posttranscriptional regulatory Element (PRE) which facilitates the transfer of the viral transcript from the nucleus to the cytoplasm and finally to the cellular translational machinery leading to the deletion of 30 nucleotides downstream (Huang and Yen, 1994). Mutation in 'a' determinant of HBsAg leads to change in conformation of the protein, making it undetectable by most HBsAg assays due to decreased immunogenicity and expression of HBsAg (Kim *et al.*, 2015).

Lamivudine associated mutations causes some changes in sequence of Amino acid in HBV polymerase and surface gene resulting in decreased binding to anti-HBs antibodies (Gaillard *et al.*, 2002). Some mutation causes Lamivudine resistance thereby limiting the replicating ability of HBV (Gaillard *et al.*, 2002). However, this can be restored by hepatitis B immune globulin-induced vaccine escape mutants causing public health concern. This is because they are detected by commercial HBsAg assays, resist treatment with lamivudine and infect vaccinated individuals (Torresi *et al.*, 2002).



Decreased number of HBV with detectable HBV DNA was found to be linked with HCV co-infection, mostly leading to hepatocellular carcinoma (Matsuoka *et al.*, 2008). Some studies found out that occult HBV patients harboring HCV genotype 1b have increased infection than those with genotype 2a (Fukuda *et al.*, 1999).

Others found out that there is decreased HBV replication and decreased HBsAg expression in hepatocytes of HBV and HCV coinfecting patients (Chu *et al.*, 1998). Replication of HBV is altered when it coexists with HCV in the same hepatocyte. Also, HBV replication is altered when HCV core protein interact with HBx protein preventing polymerase and core from binding to package signal in the pregenomic RNA (Jasmine *et al.*, 2012).

HCV NS2 protein confers an inhibitory action on HBsAg and HBeAg secretion. This inhibitory activity is related to its amino acid terminus (Dumoulin *et al.*, 2003).

#### **BLOOD TRANSFUSION AND OCCULT HEPATITIS B VIRUS INFECTION**

HBV infection is one of the transfusion transmissible infections (TTIs) causing major worldwide concern especially in Sub-Saharan Africa and South East Asia (Kafi-Abad *et al.*, 2007). Despite the fact that routine immunochromatographic test to detect HBsAg are carried out prior to collecting blood for transfusion, some patients known to be negative for HBV infection eventually become positive for it (post transfusion HBV infection). This prompted scientist to finding the mechanism behind this vague incidence as first reported by Hoofnagle *et al.*, (1978). Molecular techniques including nucleic acid testing and PCR showed occult HBV infection exist among blood donors (Dong *et al.*, 2015). Risk of transfusing blood products in which occult HBV infection is suspected depends upon the viral dose and presence of anti-HBs. The minimum DNA dosage of HBV capable of causing infection was estimated at 1049 copies (Allain, *et al.*, 2013).

#### **Phases of HBV Infection**

Chronic hepatitis B progresses in four distinct phases. Phase one being the immune tolerance phase where no clinical symptoms are seen. However, this phase shows high HBV DNA levels with normal liver enzyme-Alanine Aminotransferase (ALT).

The second phase is known as immune clearance phase where HBeAg is converted to anti-HBe. HBV clearance occurs together with destruction of

hepatocytes as seen by increased ALT level. HBeAg seroconversion results in decreased HBV DNA with normal ALT levels (Chun and Anna, 2002)

This is then followed by inactive carrier phase, indicated by presence of anti-HBe, decreased HBV DNA level and normal ALT level. The fourth phase is the chronic HBeAg-negative hepatitis B that occurs only when there is reactivation of HBV replication, and is indicated by increased ALT and HBV DNA levels. Treatment is indicted for this phase (Chun and Anna, 2002)

#### **Mechanism of HBV infection**

Infection with HBV is dependent upon immune state of the host and dosage. Presence of HBV DNA in a pint of blood is sufficient enough to cause infection. It has been found that there is likelihood of infection from blood containing anti-HBc. However, blood containing anti-HBc and anti-HBs was not infectious (Mosley, 1995).

#### **Immune response against HBV**

Immune response to HBV constitute both innate and adaptive responses (Barbara and Michelina, 2005). Innate immune response is associated with mild responses during the first week of infection and beyond, after which HBV DNA may be absent in serum. Formation of new HBV capsid is inhibited by IFN-  $\alpha$  and IFN-  $\beta$ , which are secreted in response to HBsAg and HBcAg. NK cells recognize viral infected cells, and inhibit replication as well as kill these cells by initiating INF- $\gamma$ .

Adaptive immune response is associated with vigorous CD4+ and CD8+ responses. The degree of T cell response mounted by acute HBV and chronic HBV infected patients differs. In chronic infections, patients show weak and undetectable T-cell responses, while in acute infections, patients are able to mount a strong MHC I and II responses against different epitopes of HBV antigens (Ferrari *et al.*, 1990). Response to chronic HBV infection is a Th2 type response to HBsAg and the virus can evade the T-cell immune defense mechanism leading to necrosis, fibrosis and chronic liver disease.

Humoral immune response is indicative of the stage of infection. HBcAg-specific IgM is detected early in infection, while antibodies against HBsAg and HBeAg are detected late in the infection. However, HBsAg-specific antibodies and HBcAg-specific IgG persist after recovery from infection (Barbara and Michelina, 2005).



Recovery from HBV infection is associated with immunological memory which confers a lasting protective immunity, mediated by CD4<sup>+</sup> and CD8<sup>+</sup> T cells and antibodies specific to HBsAg (Barbara and Michelina, 2005).

### **CYTOKINE PROFILE IN HBV INFECTION**

Cell mediated immunity is important in pathogenesis of hepatocellular damage and is known not to induce cytopathic effect on infected hepatocytes. However, defects in T cell response is a major factor responsible for pathogenesis of chronic hepatitis (Chisari and Ferrari, 1995).

Cytokines associated with HBV infection include; Interleukin 2, Interleukin 12, Interleukin 10, Interleukin 4, Tumour necrosis factor (TNF- $\alpha$ ) and interferon gamma (IFN- $\gamma$ ). These are released in immune response in association with T Helper 1(Th1) and T Helper 2(Th2) cells. Immune responses by Th1 are mainly directed towards inhibiting viral replication and eradicating HBV.

Th1 cytokines are responsible for delayed hypersensitivity leading to inflammation, local tissue damage, antiviral activity of macrophages, resistance to virus in neighboring cells and nitric oxide production, leading to HBV eradication.

### **Interferon gamma (IFN- $\gamma$ )**

Eradication of HBV may be achieved by the action of IFN- $\gamma$  through direct anti-viral activity, activation of macrophages, cytotoxic T lymphocyte activation and increased expression of major histocompatibility complex (MHC) (Toyonaga *et al.*, 1994). Th2 responses are directed against extracellular pathogens by regulating humoral immune responses.

### **Role of Interleukin 12 (IL-12) in HBV infection**

Interleukin 12 is a product of antigen presenting cells (APC), and consists of 2 subunits, p40 and p35 which mediate both adaptive and innate immune responses (Sun and Wei, 2004). Transcription of IFN- $\gamma$  receptor induced by transcriptional factor T-bet is expressed in Th1 cells, so genes encoding IL-5 and IL-4 are suppressed, thus initiating synthesis of IFN- $\gamma$ . This mechanism is governed by IL-12 (Sun and Wei, 2004).

Increase in IL-12 is associated with clearance of HBV and seroconversion. Th1 synergizes with IL-2 and IL-12 by stimulating TCR-CD3 and CD28 receptor to produce IFN- $\gamma$ .

### **Role of Interleukin 10 (IL-10) in HBV infection**

Immunosuppressive activity of IL-10 causes ineffective immune response that inhibits T cell activity by interrupting the maturation, differentiation and proliferation of T cell. However, it stimulates proliferation of NK cells, B lymphocytes and CD8<sup>+</sup> T cells.

IL-10 serves as an anti-inflammatory agent by inhibiting the action of macrophage-secreted cytokines (Zhang, *et al.*, 2007)

### **Tumor Necrotic Factor (TNF)**

TNF play a vital role in recruiting monocytes and neutrophils to infection site, and causes an increase in production of prostaglandins. It is produced by NK cells, T cells, mast cell as well as B cells. TNF has several antiviral activities including inhibition of viral entry into hepatocytes, disrupting viral replication and nucleocapsid formation (Ebert *et al.*, 2015)

TNF serves as a therapeutic target by inhibiting cellular inhibitor of apoptosis (cIAPs), resulting in apoptosis of virally infected cells (Ebert *et al.*, 2015).

### **DIAGNOSIS OF OCCULT HBV INFECTION**

Presently, the following markers are used for screening occult HBV infected patients; HBsAg, HBV DNA, Anti-surface antibody, and Anti-core antibody.

### **HBsAg**

World health organization (WHO) standard test kits must be employed in testing for HBsAg to overcome the quantification problems due to virus, host or test kits used. Range of detection boundary of 0.04-0.62 ng/mL are internationally recognized, with an estimated 2 million genomes/mL of blood at 0.04ng/mL (Gerlich *et al.*, 2007)

Most HBsAg assays may detect all genotypes of HBV but may miss those with mutation at the S region (Weber, 2006).

### **HBV DNA**

This is the most standard method for diagnosis of occult HBV infection. It involves studying extracted DNA from blood or liver of patients using real time PCR, nested-PCR and/or transcription-mediated amplification. Detection limit for the viral DNA may range from <10 to 425 copies/mL (Sara *et al.*, 2011), and primers used must be complementary to conserved nucleotide sequences with specificity for different HBV genomes (Raimondo *et al.*, 2008). S and X genes are amplified by PCR with S been the most sensitive in serum and X in liver.

### Anti-surface Antibody (anti-HBs)

Anti-surface Antibody is used in studying serological status of patients suspected to have occult HBV infection. It is the last antibody that appears following acute phase of the infection and is responsible for neutralization of the infection.

### Anti-core Antibody (anti-HBc)

This Antibody destroys the nucleocapsid of HBV and is the first antibody to appear. It is present in blood of patients with previous exposure to HBV and exists in all phases of infection, including recovery (Urbani *et al.*, 2010). However, it may not be detected in coinfection with HIV, or other variants of HBV (Urbani *et al.*, 2010).

### TREATMENT OF HBV INFECTION

HBV infection can be treated using different approaches ranging from CRISPR/Cas9, INF- $\alpha$ , Lamivudine, Adefovir dipivoxil, Entecavir, Pegylated INF- $\alpha$  and combination therapy.

CRISPR/Cas9 is a genome editing tool that targets a particular region, segment, receptor site of a viral genome, thereby suppressing viral replication and/or leading to reduced level of circulating antigens and viral DNA (Hao *et al.*, 2016).

The cleavage of HBV covalently closed circular DNA results in disrupting viral replication owing to the linearization of the circular genome by employing CRISPR/Cas9. This method is in contrast to the traditional methods that inhibit viral replication post-transcriptionally, failing to eliminate the cccDNA, and hence the mechanism behind occult HBV infection (Vyas *et al.*, 2015)

INF- $\alpha$  has a dual action of immunomodulatory action and antiviral activity. It activates NK-cells, cytotoxic T cells, macrophages, antibody production and induces 2,5-oligoadenyl synthetase and protein kinase (Lavanchy, 2004). Treatment with INF- $\alpha$  has been found to produce a positive response with loss of HBsAg in 87% of patients, long term effect and a complication free survival (Brunetto *et al.*, 2002).

Lamivudine, an enantiomer of 2,3-dideoxy-3-thiacytidine competes with dideoxythiacytidine triphosphate (dCTP) during DNA synthesis, leading to termination of the DNA chain. Lamivudine treatment is found to produce a moderate response rate after 12 months therapy when given in a dosage of 100mg daily. Although cheap and affordable, lamivudine has been found to produce viral resistance (Gaillard *et al.*, 2002). However, it has

been found not to be associated with any adverse effect, durability of its response is lower than that of INF- $\alpha$  and development of lamivudine-resistance mutant increases with duration of treatment.

Adefovir dipivoxil, an oral antiviral drug of the Acyclic nucleoside phosphonates class taken at a dose of 10mg daily acts on Lamivudine-resistant and wild type HBV (Tassopoulos, 2001). However, reemergence occur after therapy is stopped and there is possibility of renal impairment in patient under prolonged Adefovir therapy. There is no literature that suggests clearance of HBsAg can be achieved with only adefovir therapy.

Entecavir, also an oral antiviral drug of the class deoxyguanosine analogue is active against Lamivudine-resistant and wild type HBV with no significant side effect. (Tassopoulos, 2001). Recommended dosage is 0.5mg daily. However, higher dose is required in curbing HBV resistance.

Pegylated INF- $\alpha$  is developed by attaching polyethylene glycol molecule to INF- $\alpha$  (a process known as pegylation). The drug thus produced was found to produce twice as much HBeAg clearance and seroconversion, decreased HBV DNA and normalization of ALT than those that receive INF- $\alpha$  (Cooksley *et al.*, 2003)

Sequential or concurrent treatment with multiple drugs (combination therapy) produces more effect and efficiency in inhibiting the replication HBV.

### Conclusion

Occult HBV infection is serious issue of public health concern because of its ease of transmission and the possibility of it leading to hepatocellular carcinoma and other liver diseases. Proper health education and awareness is paramount to limiting this menace and ensuring a hepatitis B- free blood transfusion in Nigeria and the world at large. Also, government and other stakeholders are therefore encouraged to introduce testing for occult hepatitis to ensure adequate and effective ways to eliminate the risk occult HBV infection.

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