OCCULT HEPATITIS B VIRUS INFECTION AMONG BLOOD DONORS AT NATIONAL BLOOD BANK, ADDIS ABABA, ETHIOPIA.

BY

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LIST OF ACRONYMS/ ABBREVATIONS

A	Adenine
aa	Amino acids
AHRI	Armauer Hansen Research Institute
ALERT	All Africa Leprosy, Tuberculosis and Rehabilitation Training
Anti -HBS	Antibodies to the HBV surface antigen
Anti-HBC	Antibodies to the HBV core antigen
BCP	Basic core promotor
bp	Base pair
C	Cytosine
cccDNA	Covalently closed circular Deoxyribonucleic acid
cDNA	Complimentary DNA
CHB	Chronic Hepatitis B
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
G	Guanine
HBcAg	Hepatitis B core Antigen
HBeAg	Hepatitis B envelope Antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B Virus
HBx	Hepatitis B virus x protein
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C virus
HIV	Human Immunodeficiency virus
IRB	Institution Review Board
HLA	Human Leucocyte Antigen
MEGA	Molecular evolutionary Genetics Analysis
MHBS	Middle Hepatitis B surface protein
MHR	Major Hydrophilic Region
ml	Milliliter
mM	Millimolar

NAT	Nucleic acid testing
ng	Nanogram
nm	Nanometer
NTCP	sodium taurocholate co-transporting polypeptide
OBI	Occult hepatitis B virus infection
ORFs	Open Reading Frames
PCR	Polymerase Chain Reaction
rcDNA	Relaxed circular DNA
RNA	Ribonucleic Acid
RT	Room Temperature
S-gene	Surface Gene
SHBS	Small Hepatitis B surface protein
T	Thymine
TTIs	Transfusion Transmittable Infections
WHO	World Health Organization

ABSTRACT

Background: Occult hepatitis B infection (OBI) is a persistent Hepatitis B virus infection in which serological test fails to detect surface antigen, but viral DNA is detectable at a low level. The HBV screening for blood transfusion is still based only on HBsAg. As a result, OBI remains a potential threat for blood safety. Thus far, there is no data on the magnitude of occult hepatitis among blood donors. Therefore, this study sought to determine the magnitude of occult hepatitis B virus among apparently healthy blood donors in Ethiopia.

Objectives: To study the magnitude of OBI and associated risk factors among blood donors at National Blood Bank, Addis Ababa, Ethiopia.

Methods: Cross-sectional study was conducted from December 2020 to April 2021 to determine the prevalence of OBI among blood donors. A total of 973 HBsAg negative plasma samples were collected from National Blood Bank. The samples were tested for anti-HBc using an ELISA and viral DNA using quantitative real time PCR [Abbot *m2000rt*]. Along with plasma samples, demographic data were also retrieved from donor's database. Both descriptive and inferential statistics was employed for the analysis of data by SPSS 15. P-value less than (0.05) were considered as statistically significant.

Results: Of the total 973 apparently healthy blood donor study participants, 445 (45.7%) were females and mean age was 26.5 years. A total of 144 of the 973 blood samples were anti-HBc reactive (14.8%). Four (0.41% of all and 2.7% of anti HBc+ donations) samples were confirmed as OBI by DNA detection. The mean viral load was 31IU/ml with +/- 12 SD suggesting true occult hepatitis BV infections. Age was found to be risk factors for anti-core positivity and statically significant at (p=0.0001).

Conclusion: About 4 in 1000 apparently healthy blood donors screened negative for HBsAg had occult HBV infection. This shows that there is a risk of HBV transmission through blood transfusion in Ethiopia. Therefore, there is a need for further investigation and action to revise blood screening strategy including anti-HBc and HBV nucleic acid testing.

Keywords: *Occult Hepatitis B infection, Hepatitis B virus, Blood donors*

1. INTRODUCTION

1.1. Background

The International Society of Blood Transfusion is dedicated to advancing blood safety in the world with specific focus on infectious risks. The safety of blood components depends on proper selection of blood donors and complemented by sensitive screening tests to exclude transmission of infectious agents. As a result, WHO recommend mandatory Screening for four infectious diseases both in developing and developed countries for the provision of a safe blood supply: Human immune deficiency virus (HIV), Hepatitis B virus (HBV), Hepatitis C virus (HCV) and Syphilis (WHO, 2010). Among those infectious diseases, Hepatitis B virus is one of the viruses responsible for hepatocellular carcinoma (HCC) (Bréchot *et al.*, 2000).

Hepatitis B virus (HBV) is a chronic infectious pathogen belonging to the family Hepadnaviridae. HBV has a circular genome of approximately 3200bp, comprising partially double stranded DNA that contains four overlapping open reading frames. Currently, ten genotypes were reported and many sub genotypes are recognized (Lin and Kao, 2015). Hepatitis B viral infection is transmitted by both horizontal and vertical transmission. Vertically the virus can transmit from mother to fetus during pregnancy (Chen *et al.*, 2013). The horizontal transmission is quite common in endemic areas. Transmission through blood transfusion is one way of horizontal transmission of hepatitis B virus infection (Samal *et al.*, 2012).

Infection with HBV leads to develop acute hepatitis B infection, which often follows with complete immune clearance of the virus that yields lifelong immunity. However, in a few other individuals, HBV infections persist and at least three distinct clinical states of viral persistence have been defined based on serological findings: chronic hepatitis B, the silent or "healthy" carrier state, and occult hepatitis B (de la Fuente *et al.*, 2011). Both HBsAg and HBV DNA are detected in the majority of chronically infected patients. But in some cases, referred to as occult HBV infections, surface antigen was undetectable due to having low level of HBV DNA in blood, which is undiagnosed frequently (WHO, 2017b).

Occult hepatitis B, has been identified before three decades (since 1985) by sensitive PCR assays that may detect low levels of HBV DNA in the serum samples or in the liver of people who are HBsAg negative (Bréchot *et al.*, 1985). Occult hepatitis B infection may be found in blood donors as a result of various clinical conditions, including: (1) window period of acute infections; (2) end stage of chronic hepatitis B; (3) low-level viral replication after recovery from hepatitis; and (4) escape mutants not detected by current HBsAg tests (Allain, 2017, Awerkiew *et al.*, 2007).

All blood donated from OBI carriers are responsible for transmission of HBV via blood transfusion; and the clinical outcome of OBI transmission mainly depends on the immune status and copies of HBV DNA in blood products of the recipient (Seo *et al.*, 2015). The look back project in Japan and Canada showed varied possibility of post transfusion infection of transfusion transmitted occult hepatitis (Levicnik-Stezinar *et al.*, 2008, Satake *et al.*, 2007).

1.2. Statement of the problem

Despite the availability of an effective vaccine and potent antiviral treatments, chronic hepatitis B virus (HBV) infection continues to be a major public health issue worldwide (Schweitzer *et al.*, 2015) because of its involvement in liver cirrhosis and hepatocellular carcinoma (HCC) that result in considerable morbidity and mortality (Samal *et al.*, 2012). According to WHO, half of the world population has been infected with HBV (Schweitzer *et al.*, 2015). The worldwide estimated prevalence of chronic HBV infection in 2016 was 3.5% with 257 million people living with chronic infection. Prevalence was the highest in the African (6.1%) and Western Pacific regions (6.2%). In Ethiopia, meta-analysis result shows the overall prevalence of hepatitis B virus (HBV) to be 7.4%; and among blood donors, it is higher than overall population with 8.4% prevalence (Belyhun *et al.*, 2016). HBV is a leading transfusion-transmissible viral infection in Ethiopia (Dessie *et al.*, 2007, Teklemariam *et al.*, 2018). Data generated by Ethiopian National Blood Bank from blood screening in 2014 shows 3.2% rates on average of HBV infection among blood donors from different regions of the country (Unpublished data).

Worldwide survey reveals that there is high occult hepatitis B in endemic area such as Africa and Asia, while lowest in North America where hepatitis B is less prevalent (Arababadi *et al.*, 2011, Roth *et al.*, 2012). Global report on occult hepatitis among blood donors from different part of world varies; it was found to be ranging from 0.006% to 17.2% (Olotu *et al.*, 2016, Said *et al.*, 2013). Ethiopia is also one of hepatitis B virus endemic countries in Africa, and so occult hepatitis B infection is expected. There is an indicator in different group of community where recent studies show high rate of occult hepatitis B infection. As reported most recently, occult hepatitis B infection was observed 5.8% among HIV negative and positive individuals in eastern Ethiopia (Ayana *et al.*, 2020) and 19.1% among HIV patients from Northern (Gondar) (Patel *et al.*, 2020). High rate (20.3%) was also seen among pregnant women from Gondar (Meier-Stephenson *et al.*, 2020). But occult hepatitis B infection has not been reported so far in blood donors in Ethiopia. Both of the above examples might be showing a glimpse of the actual occult HBV infections in the country.

Knowing the status of occult hepatitis in blood donors is very informative to understand the escape rate of hepatitis B virus infection and possibility of its transfusion-mediated transmission rate to recipients due to the existing blood screening algorism. So that policy makers would make evidence-based algorism changes on screening of blood. Therefore, the present study was conducted for first time in Ethiopia to determine magnitude of occult hepatitis B infection among blood donors in Addis Ababa at National blood bank Ethiopia.

1.3. Significance of the study

WHO's Global Strategy for Viral Hepatitis endorsed the goal of elimination of viral hepatitis including hepatitis B, and plans to reach 100% safe blood transfusion through quality assurance and zero case of viral hepatitis by 2030 (WHO, 2017a). In line with this, MoH (Ministry of Health) of Ethiopia has also developed a 5-year strategic plan for prevention and control of HBV by considering the high burden of this chronic virus (MOH, 2005). According to report of 2020, the National Blood Bank of Ethiopia has collected over 300,000 blood unit in the year and blood collection was showing increment (unpublished data). These huge numbers of blood units go for Immune compromised patients. So, it is important see

optional way to reduce burden of transfusion transmitted hepatitis B virus infection, but we still depend on serological screening. As the result, occult hepatitis was a potential problem as post transfusion hepatitis B virus infection, which could be avoided by using nucleic acid testing which is not implemented for screening of donated blood in Ethiopia. The data of Hepatitis B virus circulation in community is helpful in taking control and prevention actions, So Ethiopia blood screening strategy should be revised based on research findings. This study was therefore designed to determine the magnitude and variant of occult hepatitis B virus among blood donors in Ethiopia in order to generate data that would serve as input for policy makers to make evidence-based decisions. Data generated from this study will encourage more studies and assist in policy decisions in the implementation of advanced diagnostic facilities in blood screening and effective interventions to assure safe blood transfusion in Ethiopia**.**

2. LITERATURE REVIEW

2.1. Hepatitis B Virus

Hepatitis B virus belongs to *Hepadnaviridae* family, genus *orthohepadnaviruses*. The hepadnaviruses is characterized by the synthesis of approximately 3.2 kb partially double-stranded, relaxed-circular DNA (rcDNA) genome by reverse transcription of RNA intermediate. The pre-genome HBV DNA virus has four overlapping open reading frames (Figure 1a). The largest ORF encodes the viral polymerase, which also has reverse transcriptase (RT) activity that generates the first strand of the DNA genome from an RNA intermediate. The second largest ORF encodes the three viral envelope proteins (HBsAg): large (L-), middle (M-), and small (S-) surface antigen (HBsAg). Another ORF encodes precore, also referred to as HBV E antigen (HBeAg), and the core protein, which makes up the viral capsid. Finally, the smallest ORF encodes the HBV X protein (HBx), a small regulatory protein that has been shown to be required for HBV replication both *in vitro* and *in vivo* (Shen and Yan, 2014).

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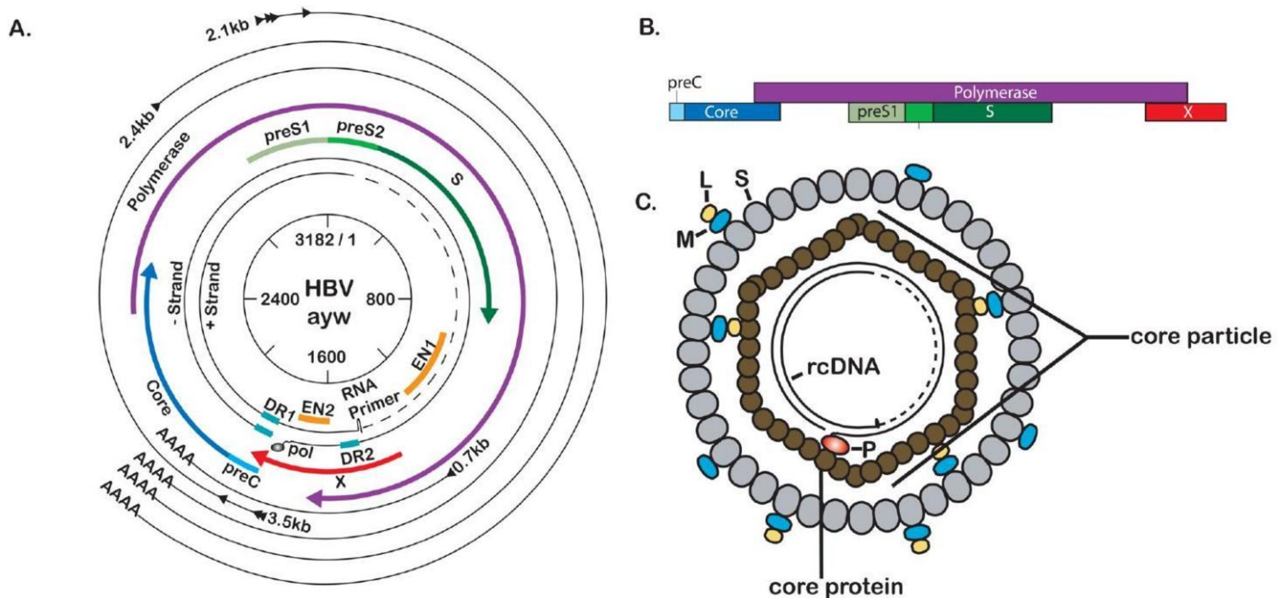

Figure 1 Molecular biology of hepatitis B virus (HBV).

(a) Scaled depiction of the HBV (genotype ayw) genome. Internal circle shows genomic position relative to EcoRI site at position 1. Partially double-stranded genome is depicted with attached RNA primer and polymerase protein. Open reading frames (ORFs) are indicated by the thicker, colored lines. The outermost black circles represent the viral transcripts with the shared polyadenylation site; (B) schematic representation of the overlapping nature of the HBV ORFs; (C) the mature HBV virion (Dane particle) consists of two main parts: a nucleocapsid (or core particle) consisting of a partially double-stranded DNA genome bound to polymerase (P) and encapsidated by dimers of core protein, and a viral envelope consisting primarily of S-HBsAg (S), with an intermediate amount of M-HBsAg (M) and lower levels of L-HBsAg (L) (Lamontagne *et al.*, 2016).

2.2. Hepatitis B Virus replication cycle

HBV infection has 6 stages of life cycle: 1) viral attachment and entry; 2) transportation into the nucleus; 3) conversion of rcDNA to cccDNA; 4) transcription and translation; 5) synthesis of progeny viral DNA genomes; and 6) assembly of viral particles and secretion. The viral DNA is associated with DNA polymerase, an enzyme, which is important for the replication of the virus. The initial stage of HBV infection involves the attachment of virus to the membrane of hepatocyte using a specific receptor recognition domain identified in the preS domain of surface protein. In addition, sodium taurocholate co-transporting polypeptide (NTCP), a functional cellular receptor, mediates HBV entry through binding of the preS domain Peptide (Tsukuda and Watashi, 2020). Viral replication takes place via an RNA intermediate utilizing reverse transcriptase, which lacks proof reading activity. Transcription starts from the core promoter to yield the 3.5 kb pre-genomic RNA, which is packaged with polymerase into immature core particles, and then serves as a template for reverse transcription and negative strand DNA synthesis. The incomplete positive strand DNA is then synthesized. The mature core particles are packed into HBsAg and pre-S proteins in the endoplasmic reticulum (ER) then exported from the cell (Figure 2) (Urban *et al.*, 2010).

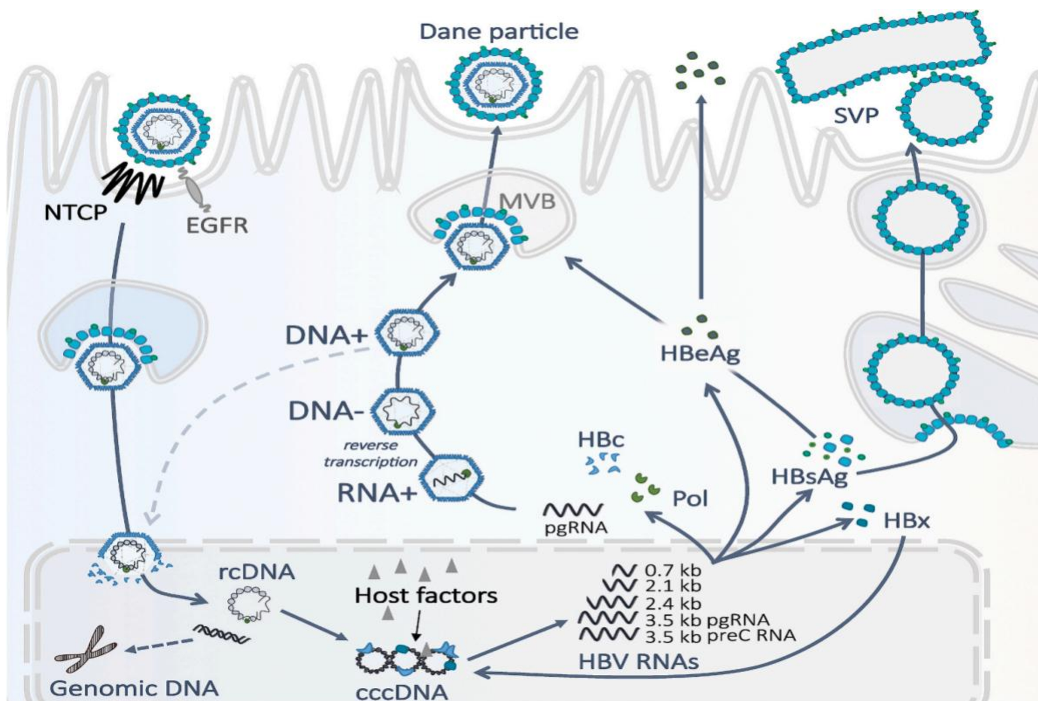

Figure 2 Schematic overview of the HBV life cycle (Tsukuda and Watashi, 2020).

2.3. Transmission and risk groups

Transmission is through parenteral (blood exposure), sexual and vertical (from mother to baby). The groups most at risk of HBV infection are: Intravenous drug users, healthcare workers, blood and blood product recipients, tattoos/ body piercing/ acupuncture, patients undergoing dental or surgical treatment, multiple sexual partners, men who have sex with men, sex workers and babies born to mothers who are chronic carriers of HBV or who have acute HBV infection during pregnancy (Kudesia and Wreghitt, 2009).

2.4. Immune response and Pathogenesis of HBV

The incubation period of HBV following successful infection is typically 6 to 25 weeks (Gitlin, 1997). After infection, the clinical presentation ranges from subclinical and asymptomatic carriage to

acute self-limiting hepatitis or fulminant hepatitis to chronic hepatitis. Infection during childhood may cause acute or fulminant hepatitis, chronic hepatitis, liver cirrhosis and liver cancer during childhood or later in adulthood (Hsu *et al*., 1997). The key determinant of chronic infection is the age of infection. Chronic infection occurs in 90% of infected neonates and infants but in less than 5% of patients who acquire infection in adulthood (Indolfi *et al*., 2019). Approximately 90% of the infants of hepatitis B e antigen (HBeAg) seropositive mothers become HBsAg positive (Stevens *et al.*, 1975). In adult acute HBV infection 65% cases subclinical infection while 35% leads to acute hepatitis from this only 1% ended up with fulminant hepatitis which leads to death. From HBV infection 10% progress to chronic hepatitis and some of chronic infection leads to cirrhosis finally cause hepatocellular carcinoma (Feitelson, 1992).

In HBV immune response both Innate and Adaptive immunity involvement was identified. But at early infection HBV is poorly sensed by the innate immune system and can escape innate immune recognition (Ferrari, 2015). This is because the HBV infection is relatively invisible to innate sensing machinery unlike other viruses due to the fact that it retains the transcriptional templets in the nucleus, which instead produces capped and polyadenylated viral mRNAs that resemble the structure of normal cellular transcription (Bertoletti A, 2010). Because of the poor induction of innate intracellular immunity, adaptive responses are efficiently and timely induced immediately after active virus replication begins (Tan *et al.*, 2015). As adaptive immune response, HBV-specific T cells, especially CD8 cells, at the site of infection are believed to be the main cause of the early non-cytolytic clearance of HBV. HBV-specific CD8+ T cells enter the liver, recognize antigen, kill infected cells and secrete IFN_{γ}, which triggers a broad-based cascade that amplifies the inflammatory process (Ferrari, 2015). However, establishment of an effective adaptive antiviral immune response is dependent on CD4+ T cells (Loggi *et al.*, 2014). CD4 T cells are robust producers of cytokines and required for the efficient development of effector CD8 CTLs and B-cell antibody production. CD8 T cells clear HBV-infected hepatocytes through cytolytic and noncytolytic mechanisms, reducing the levels of circulating virus (Chisari, 1978).

The humoral response is evoked against the structural antigens of the virus, namely the core (HBcAg) and envelope antigens (HBsAg). Antibodies response to the hepatitis B surface antigen (HBsAg) neutralizes HBV infection presumably by preventing virus attachment to hepatocytes. They also

facilitate viral clearance by complexing with free viral particles and removing them from the circulation (Milich and McLachlan, 1986). Anti-core (antiHBc)-specific IgM is the earliest marker of infection, whereas antibodies against HBsAg and HBeAg, a circulating none particulate form of HBcAg, appear much later and indicate a favorable outcome of the infection (Loggi *et al.*, 2014).

HBV is considered as a non-cytopathic virus, but it induces hepatic cell apoptosis by different mechanisms (Baumert and Blum, 2000). Virus-host interactions, especially the virus-specific T cell response, are the key factors accounting for the pathogenesis of HBV infection (Baumert *et al.*, 2007).

HBV also has the immune modulation mechanism to persist in the host by producing a soluble form of HBsAg and HBeAg in excessive amounts over whole virions. It is believed that soluble viral antigens can inhibit antigen-presenting function; by altering their ability to produce cytokines, and inhibit the induction of HBV-specific T cells (Tan *et al.*, 2015). The inefficient immune response to HBV during chronic HBV infection is results in low-level liver cell destruction and regeneration over long periods of time that lead to fibrosis, cirrhosis, steatosis and eventually HCC (Lamontagne *et al.*, 2016).

2.5. Serological profile and diagnostic markers in different stages of |HBV infection

Hepatitis B surface antigen detection is a primary target for diagnosis and immunoprophylaxis of HBV infection both in the routine virology laboratory and for blood donation screening (El-Sherif *et al.*, 2007). The most common and important markers are HBsAb, HBcAb total, HBeAb, HBeAg and HBV DNA; and different stages of infection characterized based on those markers as indicators (Trepo *et al.*, 1993) **Table 1**.

Table 1.Typical HBV serology and different clinical conditions. (Lelie *et al.*, 2017)

HBV infection category/stage	Pattern HBV markers on index donation				
	HBV-DNA	HBsAg	Anti-HBc	IgM-anti-HBc	Anti-HBs
1. Pre-HBsAg window period (WP) infection	+	-	-	-	-
2. Acute occult HBV infection (OBI) (or primary OBI)	+	-	-	-	-
3. Anti-HBs (or vaccine) breakthrough or abortive infection	+	-	-	-	+
4. Concordant HBsAg and HBV-DNA positive infection	+	+			
5. Post-HBsAg WP infection	+	-	+	+	
6. Low viral load HBsAg positive/HBV-DNA negative carrier	-	+	+		
7. Anti-HBs negative OBI	+	-	+	-	-
8. Anti-HBs positive OBI	+	-	+	-	+
9. OBI with anti-HBs only	+	-	-	-	+
10. OBI without serologic marker	+	-	±	-	-

Occult hepatitis B virus (HBV) infection (OBI) is classified in to three by International workshop on occult hepatitis infection based on their serologic profiles, in which two groups are seropositive OBI and in one case seronegative (Raimondo *et al.*, 2008) In seropositive occult hepatitis infection, hepatitis B core antibody (anti-HBc) and/or hepatitis B surface antibody (anti-HBs) are positive, whereas in seronegative OBI there is total absence of detectable serological markers (Raimondo *et al.*, 2019). Nevertheless, most blood donors with OBI were anti-HBc antibody positive but negative for anti-HBs antibody (Ramezani *et al.*, 2010). The observation of anti-HBc percentage can reach up to 66% of occult hepatitis B infection cases (Tsega *et al.*, 1987). There are also findings in which all OBI donors were anti-HBc positive (Jutavijittum *et al.*, 2014); and anti-HBs positive but not anti-HBc (Awerkiew *et al.*, 2007). In addition, anti- HBc/anti- HBe and anti- HBc/anti- HBs/anti- HBe positivity have been reported in small proportion of occult hepatitis B infections (Katsoulidou *et al.*, 2009). Rare sero-negative cases of OBI occur in individuals with no markers of HBV infection other than HBV DNA (Jutavijittum *et al.*, 2014). Anti-HBc could reflect occult HBV infection in high risk cases but not in

low-risk individuals (Ramezani *et al.*, 2010). So, detection of anti-HBc in the blood is often used as a surrogate for diagnosis of occult hepatitis from serology tests.

2.6. Molecular mechanisms of occult HBV infection

In occult HBV infection, low viral replication and gene expression are usually observed. The molecular mechanism by which this occult HB infection occurs is not fully known (Pollicino *et al.*, 2007). However, some host and viral factors that contribute for the development of OBI (occult hepatitis B infection) have been identified. Viral factors include: (1) mutation of (surface protein, core protein, pre-core protein, X protein and polymerase) (2) low viral load (3) co-infection with HCV and (4) epigenetic. On the other hand, host factors are linked to immunology and genetics of the patients (Yip and Wong, 2019).

2.6.1. Viral Mechanisms

Viral covalently closed circular DNA (cccDNA) persists as an episome in the nucleus of infected hepatocytes. HBV DNA may also persist after integration into the DNA of hepatocytes (Yip and Wong, 2019). In the nucleus, HBV genomic DNA is modified by cellular factors. The Pol-linked terminal redundant sequence in the 5′-end of the minus strand DNA and the RNA oligonucleotide attached at the 5′ end of the plus strand DNA are removed from the rcDNA, and the gaps in both strands are filled and ligated to generate cccDNA (Guo, 2007). As the inherently stable cccDNA resides episomally in the nucleus, it functions as a template for viral replication over the long term. Nonetheless, immune responses and cytokine stimulation are major factors that affect cccDNA maintenance (Hu *et al.*, 2019). One of evolutionary benefits of HBV persistence is the integration of viral DNA into host cells, which has possible role in hepatocellular carcinoma. Integrated HBV DNA is shorter than episomal cccDNA and unable to produce pgRNA, which as a result represents a replicative dead-end for the virus. Integration disrupt the stability of chromosome of the infected host cells and leads to HCC (Tu *et al.*, 2017).

Mutation in viral genome is one of the mechanisms for persistence of the virus, and amino acid substitutions in the major hydrophilic region (MHR) can cause reduced binding to anti-HBs antibodies, resulting in immune escape. Mutations in the HBsAg gene bring about the structural arrangement of the protein, which may lead to the development of undetectable HBsAg by commercial HBsAg test kits. The most common MHR mutation G145R, was initially described in 1990 (Carman *et al.*, 1990). Second, mutations in the S region have been associated with reduced expression of HBV surface proteins; mutations in preS1/preS2 promoters are frequently observed in OBI patients (Chaudhuri *et al.*, 2004).

Additionally, HBV variants with mutations in the S-gene promoter and splice variants have also been reported to affect HBsAg production/secretion and to be responsible for some cases of OBI (Raimondo *et al.*, 2019). Stop codon mutations within the pre-S/S proteins, S region and pre-core mutation is also higher (13.6 %) in the OBI group (Thedja *et al.*, 2010). Low viral load also lead to low production of HBsAg, which has the same problem of detectability with current immunoassay (Araujo *et al.*, 2008).

2.6.2. Co-infection with other viruses

The other factor contributing to persistence of Hepatitis B virus DNA is Co-infection with other infectious agents. The most common co-infection correlating with occult hepatitis B infection was HDV (hepatitis D virus) and HCV (Hepatitis C virus. In case of co-infection of HBV and with HCV of the same hepatocytes, HCV core protein (a part of the viral capsid) inhibits HBV replication by binding to HBx protein, which has HBV replication enhancer activity. As a result, HCV molecules inhibit the replication of HBV, resulting in OBI with low replication of HBV DNA and low surface antigen, which can't be detected with conventional immunoassay (Samal *et al.*, 2012). There is also a case of more HBV S gene mutation during HBV-HDV co-infected than mono-infected patients. This might indicate the evolution of HBV S gene under selection pressures generated from HDV coinfection (Baig *et al.*, 2018).

2.6.3. Host mechanisms

Host immune response is also one factor for persistence of HBV DNA. Host immune response to hepatitis B virus infection is affected by age and genetics; and most of neonates who got hepatitis infection through vertical transmission can't clear infection, due to weak immunity that leads to viral DNA persistence (Cote *et al.*, 2000). The specific T-cell responses is also higher in occult and suppress viral replication to low levels leading HBsAg expression to undetectable levels (Bes *et al.*, 2012). Normally any HBV-infected hepatocytes undergo apoptosis by expressing *Fas* surface protein, but soluble forms of *Fas* (*sFas*) antagonize *Fas*-mediated cytotoxicity by binding to and inactivating the ligand of killer cells. But in the case of occult hepatitis, soluble *Fas* has been found to be lower when compared to chronic HBV (Hayashi and Mita, 1999).

From host genetic side, Human Leucocyte Antigen (HLA) DPA1/DPB1 genes, which are located on the short arm of chromosome 6, influence different type of HBV infection responses (Kamatani *et al.*, 2009). A recent study in Indonesia confirmed that HLA-DPA1 and HLA-DPB1 variants are associated with undesirable outcomes of HBV infection such as susceptibility, persistent infection, and disease progression (Wasityastuti *et al.*, 2016). The minor allele of rs3077 (T) in the HLA-DPA1 gene was related to an increased risk of OBI in seropositive Indonesian blood donors (Mardian *et al.*, 2017).

2.7. Clinical significance of occult hepatitis B in immunocompromised groups

Occult HBV is associated with increased risk of hepatocellular carcinoma, and reactivation to chronic HBV during immune suppression. HBV reactivation occasionally occurs in patients with occult HBV infection who receive intensive immunosuppressive chemotherapy; infected with immunosuppressive infection like HIV, Rheumatological disease, cancer patient, diabetes patient and those who use hemodialysis. The prevalence and reactivation of occult hepatitis B virus infection in those groups is summarized in the following table (**Table 2**).

Table 2 Prevalence and frequency of reactivation OBI infection in immunocompromised groups

No	Type of immuno-compromised group	Prevalence of reactivation of occult HBV	Study Popu-lation	Country	Reference
1	Immunosuppressive chemotherapy	56.5% of reactivation	23	Italy	(Coppola *et al.*, 2011)
2	HIV	19.8%	86	Italy	(Filippini *et al.*, 2006)
3	B cell non-Hodgkin's lymphoma	6%	471	Taiwan	(Chen *et al.*, 2008)
4	Diabetes	11%	100	Turkey	(Demir *et al.*, 2008)
5	Hemodialysis Patients	6.2%	289	Iran	(Aghakhani *et al.*, 2010)
6	Chronic hepatitis C patient	14.8%	210	Taiwan	(Kao *et al.*, 2002)

2.8. Global prevalence of occult HBV infection

Occult HBV infection is elusive but a world-wide problem whose prevalence in blood donors is related to the prevalence of overt HBV infection in that geographical area and population. Globally, Hepatitis B virus infection, as detected by surface antigen positivity, is estimated to be 1.99% among blood donors (Berger, 2018). HBV is more prevalent in Africa than other continents and it varies from low intermediate to highly endemic countries: North western Africa with high; northern Africa and the Middle Eastern region with low intermediate; and southern and eastern part of Africa including Ethiopia with high intermediate prevalence (Ott *et al.*, 2012).

Prevalence of occult hepatitis infection is greatly varied over the world. It is less prevalent in western countries but is frequently detected in the developing countries. The prevalence of OBI is in the range of 1:100-1,000 in high prevalence areas (East Asia and West Africa) but below 1:5,000 in Western Europe, North America and Australia (Candotti *et al.*, 2012). From the percentage of overall infection, OBI remains small ranging between 0.1% and 0.6% globally (Allain, 2017). However, its overall global prevalence among blood donors was estimated to be 8.55 per 1 million donations, according to a 2008 international survey (Roth *et al.*, 2012).

2.9. Genotypes involved in occult Hepatitis infection

A genotype is defined as the genetic constitution of an organism. Hepatitis B virus has distinctive reverse transcriptase enzyme and due to spontaneous error of reverse transcriptase, there is nucleotide substitution at an estimated rate of $1.4\text{-}3.2*10^{-5}$/sites/yr in the genome of HBV (Lau and Wright, 1993). This error-prone replication leads to the occurrence of various genotypes, subtypes, mutants, recombinants, and even quasispecies in the context of the long-term evolution of HBV that resulted in the existence of currently ten HBV genotypes (A to J), four serotypes and more than 31 sub genotype (Lin and Kao, 2015). Hepatitis B virus classification into genotypes is based on >8% intergroup sequence divergence (Yu *et al.*, 2010).

The genotypes of hepatitis B virus show a distinct geographical distribution between regions and even within same region (Kramvis, 2014). Blood donors from South East Asia harbor genotypes B and C (60% and 34%, respectively) in agreement with the genotype distribution in chronically infected donors in the region (Candotti *et al.*, 2012). Genotype A has been found mainly in North America, USA, Europe, India and Africa; and genotype F is common in Central and South America, including among indigenous populations (Kramvis, 2014, Norder *et al.*, 2004). The genotypes D and E are reported to be high for OBI globally (Allain, 2017). In China, 86% of genotype reported in occult infection is genotype C, which is the genotype endemic to the country (Zheng *et al.*, 2011). However, mixture of genotypes can be detected in some countries and regions. For example, a study on blood donors with occult hepatitis B infection from Italy reveals different genotype with different proportion: A, 33.1%; C, 9.4%; and D, 57.5% (Velati *et al.*, 2008).

HBV is also endemic to Africa with varied genotypes distributed distinctly by sub-region: genotype A is found mainly in southern, eastern and central Africa; genotype D prevails in northern Africa countries; and genotype E in western and central Africa (Kramvis and Kew, 2007). Genotype E is emerging in east African according to a study from Rwanda (Hübschen *et al.*, 2009). In Kenya which is bordering to Ethiopia, HBV/A1 and HBV/D4 are dominant among blood donors (Kwange *et al.*, 2013). Even though limited research was done on genotyping in Ethiopia, there is an indication that genetic variability of hepatitis B virus exists in the country (Hundie *et al.*, 2016). Where genotypes A and D are common among the general community and blood donors; and a novel hepatitis sub genotype D10 was reported to be circulating (Ambachew *et al.*, 2018, Hundie *et al.*, 2016). In northern part of Ethiopia,

HBV genotype D was the predominant genotype (81%) among OHB-positive individuals from HIV patients (Meier-Stephenson *et al.*, 2020).

Testing and diagnosis of hepatitis B virus infection is critical for prevention and treatment, which provides an opportunity to reduce HBV transmission. In general, literatures indicate that knowing the magnitudes and distribution of occult hepatitis B virus infection is helpful in deciding blood screening approach.

3. OBJECTIVES

3.1. General objective

To quantify the magnitude of occult HBV infection among apparently healthy blood donors at National Blood Bank of Ethiopia from December 2020 to April2021.

3.2. Specific objectives

1. To determine the magnitude of occult HBV infection among blood donors
2. To determine the HBV viral load on viremic HBsAg-negative but HBc positive blood donors
3. To associated risk factors to anti-Hbc marker positive blood donors

4. MATERIAL AND METHODS

4.1. Study Area

The study was done at Addis Ababa at National Blood Bank of Ethiopia, Addis Ababa. Study area was selected purposively for accessibility. Addis Ababa is capital of Ethiopia and from central part of country. Administratively, the city is divided in to 10 sub cities and 116 Woredas. The total area of the city is 54,000 hectares. According to the 2013 population estimation, the total population of Addis Ababa is more than 4 million. The Ethiopian National Blood Bank collected nearly 300,000 units of blood from donors in 2020, from this 112,153 blood units were collected from Addis Ababa city blood collecting centers and mobile team. Normally, the Blood Bank screens for Human immune deficiency virus (HIV), Hepatitis B virus (HBV), Hepatitis C virus (HCV) and Syphilis. The donated blood is processed to whole blood, red cell (CRC), platelet, plasma (FFP), and cryoprecipitate. Ethiopian National Blood Bank have been using ELISA targeting surface antigen for screening HBV, which is sandwich type of ELISA (Wantai Biological Farm (China)).

4.2. Study period

The study was conducted at National blood bank from December, 2020 to April 2021.

4.3. Study Design

A cross-sectional study was conducted.

4.4. Population

4.4.1. Source population

All blood donors who donate blood at the Blood Bank in Addis Ababa.

4.4.2. Sampling frame

The study sampling frame was the list of samples, which are all surface antigen screening test negative at national Blood Bank.

4.4.3. Study participants

All blood donors donating blood in different sites for National Blood Banks whose serum samples meet the inclusion criteria.

4.5. Inclusion criteria

Inclusion criteria were samples surface antigen screening negatives with sufficient serum volume properly labeled and stored.

4.5. Exclusion criteria

Exclusion criteria were samples surface antigen screening positive, not properly labeled, insufficient volume and those which are not stored properly.

4.6. Sample size determination and sampling technique

Sample size (n) was calculated manually. The minimum sample size required for this study was determined by using the following sample determination according to probable sampling formula.

$$n=\frac{1.96^2(Pexp\ (1-Pexp)}{d^2}$$

Where: n= required sample size; 1.96= the value of z at 95% of confidences level. Pexp= prevalence of occult hepatitis B was calculated by considering 50%, since there is no any previous data in the study area and by considering expected occult hepatitis prevalence to be lower; d = desired absolute precision level at 95% confidence. According to the formula, a minimum of 384 blood unit samples was calculated. But because of the nature of occult hepatitis B virus infection in which viral DNA is found in serum intermittently, the sample size was increased, and 973 serum samples were collected for the study.

4.7. Study variables

4.7.1. Dependent Variable

The presence of antibody to core antigen and hepatitis B virus DNA in serum samples were considered as dependent variables.

4.7.2. Independent Variable

Sociodemographic information such as age, sex, donation history, number of donation and data regarding site of collection were considered as independent variable.

4.8. Data collection procedures

4.8.1. Socio demographic characteristics

Data collected by Blood Bank was used and information related to socio demographic characteristics (**Appendix II**) were extracted with respect to selected serum samples from National Blood Bank's data documentation system with their identification number.

4.9. Sample collection transportation and storage

Nine hundred and seventy-three serum samples were randomly selected from a pool of hepatitis B surface antigen-negative sera from anonymized leftovers from initial blood testing of blood donors at the National Blood Bank in Addis Ababa. The leftover plasma/serum was aliquotted into tubes (2 ml capacity). Collection tubes were labeled with unique codes for identification, date of collection and other necessary information. Collected specimen was transported under cold chain in ice box to Armauer Hansen Research Institute (AHRI) for further tests. Transported samples were stored at -80°C till use.

4.10. Laboratory diagnosis

4.10.1. Serological tests

ELISA was used in the routine blood bank screening procedures with sandwich ELISA kit (Wantai biological pharmacy, China). In this study, all specimens tested negative for HBsAg during the screening procedures at National Blood Bank and collected for the study were retested for further confirmation at AHRI for surface antigen with commercially available ELISA kits whose lower limit of detection is estimated to be 0.025 IU/ml at 450/620-700 nm spectrophotometric reading. Detailed procedure for this assay (Monolisa™ HBs Ag ULTRA (France)) is shown in **appendix III.**

Surface antigen test negative samples were subjected to ELISA anti-HBc test to assess previous exposure to hepatitis B virus infection. Commercial kit of Enzyme Immunoassay (EIA) (Monolisa™anti- HBC ULTRA (France)) with (sensitivity lower than 2 IU/ml at 450/620-700 nm spectrophotometric) was used for the detection and quantitation of anti-nucleocapsid antigen (core) antibody. Detailed procedure for assay is shown in (**Appendix III**).

4.10.2. Molecular tests

a. DNA extraction and quantification using ABBOTT *m2000rt* system

HBV viral load quantification was carried out on anti-HBC positive plasma samples using the ABBOTT real time automated DNA extraction and amplification equipment. Hepatitis B virus DNA was extracted from 200μl plasma according to manufacturer's recommendation. The extracted DNA was immediately used for amplification and quantification in ABBOTT m2000rt system (ABBOTT Real Time PCR, Abbott Molecular Inc.). The upper limit of quantitation (ULQ) for the ABBOTT Real Time HBV assay is 1 billion IU/mL and the lower limit of quantitation is equivalent to LOD (10 IU/mL for the 0.5 mL sample preparation procedure, 15 IU/mL for the 0.2 mL sample preparation procedure).

The target sequence for the ABBOTT Real Time HBV assay is in the Surface gene of the HBV genome. This region is specific for HBV and is highly conserved (Abbott, 2021). The primers are

designed to hybridize to this region with the fewest possible mismatches among HBV genotypes A through H (Abbott, 2021). The *m2000rt* System ABBOTT gives the result as printed out with their corresponding identification numbers. Sample with viral load <200 IU/mL was confirmed as true occult hepatitis infection. Detail procedure is described in appendix part (**appendix III**).

b. DNA extraction and Nested Polymerase chain reaction

Those serum samples with detectable viral load from *m2000rt* ABBOTT System were subjected to manual DNA extraction and conventional type amplification. DNA was extracted from 400µl of plasma by QAIGEN extraction kits as recommended by manufacturer and eluted by 200 µl elution buffer. The eluted DNA was ultra-centrifuged by vacuum centrifuge, then resuspended by 22ml TE buffer to 10% concentration.

A nested PCR was performed from this suspension using paired primers for both portions of HBV S and Pol genes, which are designed to amplify around 952bp. The outer prime HBVm11_F (5'-ACT CGT GGT GGA CTT CTC TCA-3) and HBVm11_R (5'-GGG TTG CGT CAG CAA ACA C-3') and inner primer forward HBVm01 '5-AAATTGCAGTCCCCAACC-3' and reverse primer 5'-CAAAAGAAAATTGGTAATAGAGGTA-3' amplify 509bp were used with Q5 high-fidelity 2X master mix shown in For detailed PCR reaction preparation see (**Appendix III**).
The thermocycling conditions for first round PCR consisted of initial denaturation 98 °C for 30 sec; followed by denaturation at 98 °C for 10 sec; primer annealing 58 °C for 30 sec, elongation at 72 °C for 45 sec which was run for 35 times; and final extension elongation at 72 °C for 2 minutes and cooling at 4°C.

c. Gel electrophoresis
Detection of the amplified 509bp PCR products was performed using 1.20% agarose gel. The gel was prepared by dissolving 0.9 g of agarose powder into 75 mL of 1X TAE buffer and heating 2 minutes to a boiling temperature. Then 2.5 µl ethidium bromides were added. The agarose was poured into a mold and cooled to about 50^{0}C. Then, 5µl of the PCR product mixed with 2µl of blue 6x loading dye containing bromophenol blue, xylene cynol, and orange G (Promega, Madison, USA) were loaded into

the wells on the gel. The loaded agarose gel was then subjected to 120 Volts of a unidirectional electrical current for duration of 50 minutes, while submerged in a 1X Tris-acetate-EDTA (TAE) buffer. The migrated sample DNA bands and 1kb+ DNA ladder in the agarose gel were visualized using ultraviolet transillumination and corresponded to positive controls. A digital image of the gel was taken using a gel documentation system (GEL DOC. BIORAD).

4.11. Quality assurance

All specimens were properly labeled as parallel to the demographic data; the serological and molecular diagnostic reagent kits were stored per recommendation and used as per the manufacturer’s instructions with good laboratory practices. Samples were stored at - 80 °C until processed. Standard operating procedures (SOP) and pre-analytical, analytical and post analytical quality control measures were applied. Enzyme Linked Immuno Sorbent Assay (ELISA) test results were determined based on the cut-of values following the manufacturer’s instruction. In ABBOTT *2000rt* system, Internal control kit with HBV negative, HBV low positive and HBV high positive were used with each run according to the manufacturer’s instruction.

4.12. Data Management and analysis

Raw data were coded and entered into excel spreadsheet. A back-up of all data was kept regularly using flash disks to avoid any loss. All the statistical analyses were performed using SPSS version 15. Both descriptive and inferential statistics was employed for the analysis of data. Frequencies were used to determine the prevalence of occult hepatitis infection in blood donors. Bivariate logistic regression and chi-square were employed to assess the significantly associated risk factors with occult hepatitis B infection. Prevalence figures were calculated for the total study population and the association between variables was calculated. P-value less than (**0.05**) were considered statistically significant.

4.13. Ethical considerations

The project was ethically approved by the department of Microbiology, Immunology & Parasitology research ethics committee (DRERC), College of Health Sciences, Addis Ababa University (Reference no DRERC/01/2021) and exempted from review by AHRI/ALERT Ethics committee (protocol number PO/37/20). Official permission was obtained from National Blood Bank. Blood banks have obtained donors written consents and therefore we were granted with waiver for seeking separate participants' consent for this research. Confidentiality was maintained at all levels of the study by using codes rather than names of the study participants. Data collected from Ethiopia National Blood Bank and results of laboratory tests were kept confidential and used only for the research purpose.

4.15. Dissemination of results

The result will be submitted to Addis Ababa University and ALERT/AHRI. The finding of this study will be useful to improve blood transfusion safety. Thus, the finding will be presented at public thesis defense, communicated to the Blood Bank, and presented at various scientific conferences. The finding will also be published in peer reviewed journals.

4.16. Operational definitions

Occult hepatitis B virus infection: Presence of hepatitis B virus DNA in a surface antigen negative sample without viral load range.

True occult hepatitis B virus: Presence of hepatitis B virus DNA in a surface antigen negative sample with viral load less than 200 IU/mL

5. RESULTS

5.1. Sociodemographic data

A total of nine hundred and seventy-three serum samples were collected from national blood bank along with the respective demographic data. The age of participants was between 18 years to 61 years, with median age of 24 years and mean of 26.5±8.7 years. From the total study participants, 45.7% were females. The majorities (almost 90% of samples) of blood units were collected from schools, blood bank blood collection centers and temporary blood collection centers. The remaining were from spiritual organization, different institutions and health facilities (**Figure 3**).

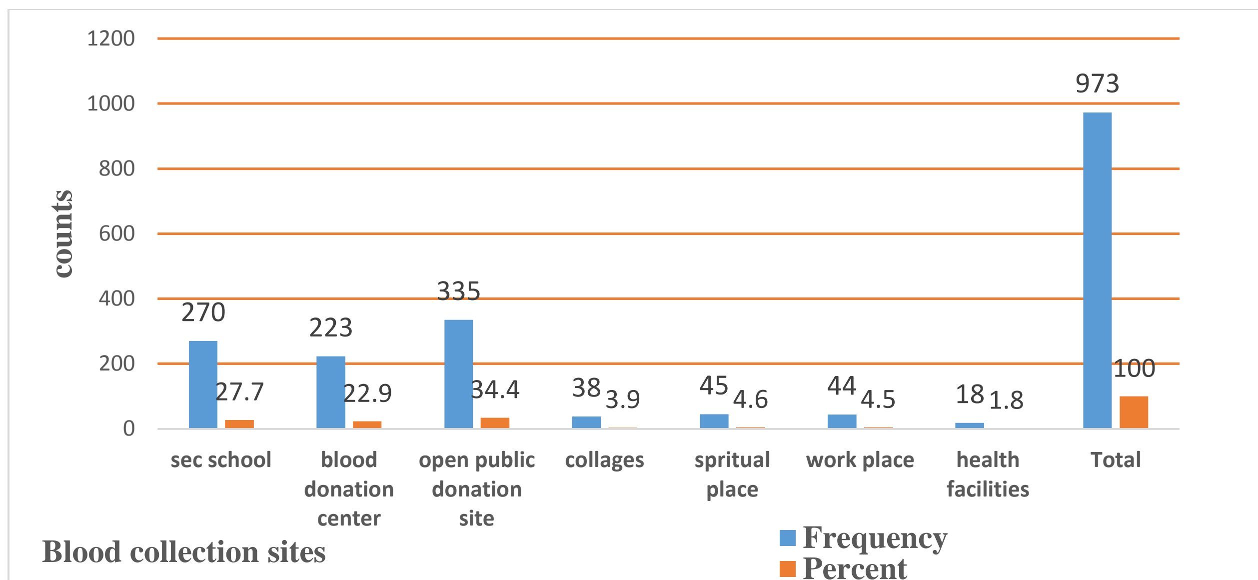

Figure 3 Frequencies and percentage of blood samples collected from different sites

5.2. Prevalence of occult hepatitis

The sero-positivity rate for anti-core of HBV among the 973 HBsAg sero-negative blood donors was 144 (14.8%). The HBV DNA was detected in 4/144 among these anti-HBc positive blood donors, indicating the prevalence of occult hepatitis B virus infection as 2.8% among anti-HBc positive blood donors and 0.41% among the total HBsAg sero-negative blood donors (**Figure 4**).

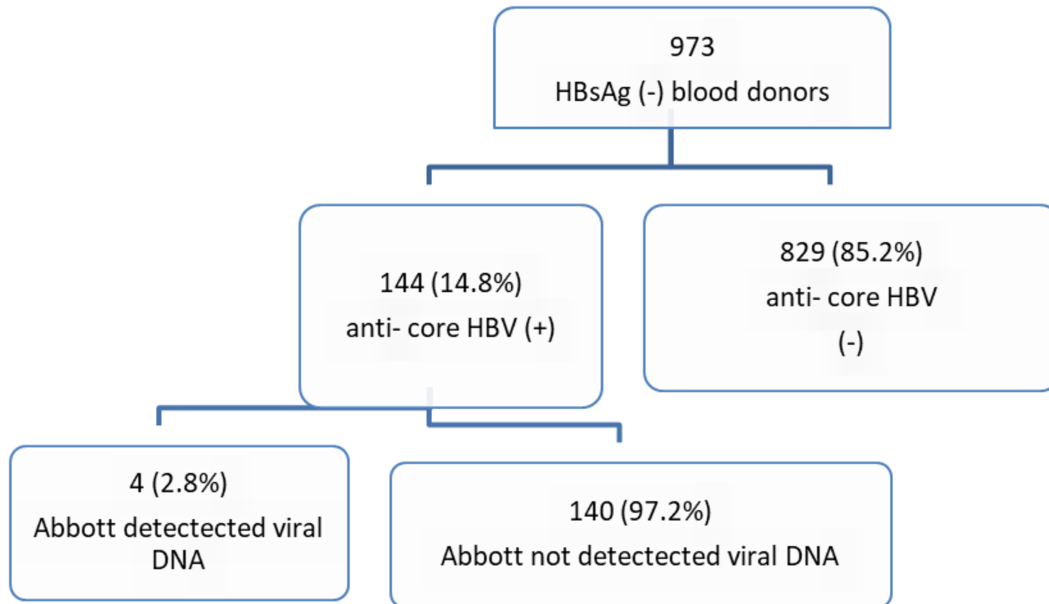

Figure 4 Flow chart of laboratory processing and results

5.2.1. HBV DNA among reactive hepatitis B virus (HBV) core antigen

From the total of 973 HBsAg negative blood donors, amplification and quantification was conducted for 144 anti HBc positive individuals. Of these 144 anti HBc positive individuals, 140 (97.2%) had no quantifiable plasma HBV DNA and only four had quantifiable HBV DNA in their plasma that make the overall OBI prevalence to be 2.8%. The mean HBV DNA load in viremic HBc^{+} individuals was 31 IU/ml with a range of 20 - 48 IU/ml.

5.2.2. Nested PCR

All samples with detectable viral load by Abbott Realtime PCR were also detected with nested PCR. The length of amplified region gave a typical band size of 509 bp when comparing to standard 1kb+ ladder as shown below (Figure 5).

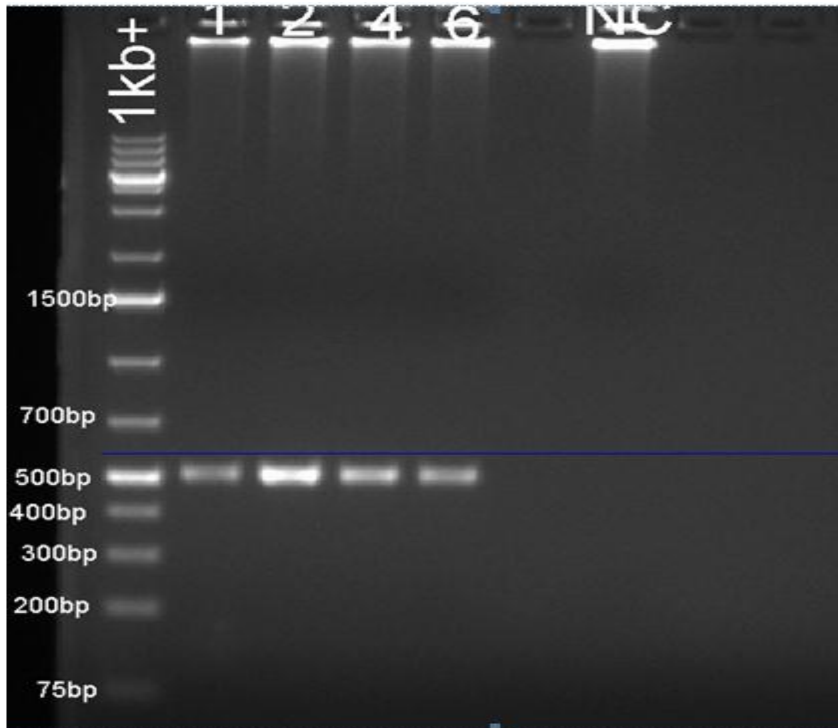

Figure 5 An example of an agarose gel documentation of pol and S gene amplicons

5.3. Demographic associated risk factors

There is difference in sero-posetivity for anti-core between different age groups: it was 42.5% among age group >45 years old donors, 27.5% among ages between 36-45 years old, and 8% among those aged between 18-25 years old donors. Age was one of the risk factors to have had a statistically significant association with anti-HBc seropositivity at significance level of (p=0.0001). As frequency of blood donation increased the prevalence of anti-core seemed to have

increased significantly (p=0.001), where 31.8% of blood donors who donated more than eight times had higher prevalence of anti-HBc compared to first time donors whose anti-HBc prevalence was at the rate of only 13.3%. Similarly, age and frequency of previous blood donation were correlated with anti-HBc seropositivity (p=0.0001), where the highest prevalence (42.55%) was observed among the highest age group (46-65 yrs) compared to the lowest prevalence (7.4%) among the younger donor age group (18-25 yrs). The prevalence of anti-core was higher in male (16.1%) than in female (13.2%), but the difference was not statically significant (**Table 3**).

Table 3. Associated risk factors of Anti-HBc

Characteristics	**Categories**	**Anti-HBc**		**Total**	**P value**	**Odds ratio**
		Anti-HBc negative	**Anti-HBc positive N (%)**			
Age	18-25	510	41 (7.4%)	551		
	26-35	218	55 (20.14%)	273		
	36-45	74	28(27.45%)	102		
	46-65	27	20 (42.55%)	47		
	total	829	144	973	0.0001	0.225
Blood donation frequency	0-2	653	102 (13.5%)	755		
	3-5	123	23 (15.7%)	146		
	6-7	23	5 (17.85%)	28		
	>8	30	14 (31.8%)	44		
	Total	829	144	973	0.0001	
sex	F	386	59 (13.25%)	445		
	M	443	85 (16.1%)	528		
	Total	829	144	973	0.239	

6. DISCUSSION

6.1. Anti-HBc marker among HBsAg negative voluntary blood donors

Blood transfusion is a life-saving intervention and expected to be safe for receipts. To reduce risk of transfusion transmittable diseases, World Health Organization recommend mandatory blood screening for infectious diseases like HBV for both developing and developed countries. WHO also plan to reach 100% hepatitis B free blood transfusion to make transfusion safe (WHO, 2010). But largely blood screening depends on ELISA targeting only HBsAg, which can't detect occult hepatitis due to different reasons. As a result, still there is risk of transmission of HBV especially through blood transfusion.

The current study was carried out to identify occurrence of occult hepatitis among blood donors who are thought to play a major role in the transmission of HBV infection to recipients. From the total of 973 HBsAg-negative donated blood samples and tested in Addis Ababa, 144 (14.8%) had serological markers for anti-HBc, which show a previous exposure to HBV. To our knowledge there is no recent data of anti-HBc among blood donors and there is only one study done far back showing high rate (66%) (Tsega *et al.*, 1987), which is could not represent the current situation. Compared to the latter report, the anti-HBc positivity rate from our study is significantly lower. Several potential reasons could be attributed for this discrepancy between the two studies. One possibility could be that HBV infection among blood donors might have been in a reducing trend during the time between then (over three decades ago) and now due to recent implementation of preventive intervention such as child immunization, pregnant mother screening for treatment, and blood screening with more sensitive immunoassay starting from 2007 (Shiferaw *et al.*, 2016). . In fact, recently Anti-HBc detection studies from non-blood donor groups also show a lower rate than the one reported by Tsega et al. (1987), as exemplified by the following four reports: 19.5% among children aged 5-8 years from Hawassa city (Argaw *et al*., 2020); overall anti-HBc positivity of 5.8% (5.6% in HIV negative and 6% in HIV positive) from three hospitals in the eastern part of Ethiopia (Ayana *et al*., 2020); 21% among HIV-positive adults in three hospitals in

eastern Ethiopia (Ayana *et al.,* 2019); and 26.8% among pregnant women from Gondar (Meier-Stephenson *et al*., 2020).

An alternative explanation could be that blood donors nowadays are selected strictly on voluntary basis, unlike over three decades ago where replacement donors were the predominant ones. Voluntary blood donors are non-remunerated and usually healthy because they usually have better health seeking behavior than the replacement blood donors (Motayo *et al*., 2015), whereas replacement donors, who are socioeconomically marginalized people and so are forced to "sell" their blood, are prone to be exposed for repeated needle piercing that would in turn expose them to blood-mediated infections. There are a number of recent observations that anti-HBc marker is indeed lower among voluntary healthy blood donors than other groups from whom samples from reasonably similar time periods were analyzed. For example, it was found that the following non-blood donor members of high-risk groups including the general community had higher Anti-HBc positivity 36% and 30% reports respectively from Gondar (Geta *et al.*, 2019) and Jimma (Taye *et al.*, 2019) among pregnant women; 39.6%, 39.4%, 17.1% among HIV patients, medical waste handler and none medical waste handler, respectively (Amsalu *et al.*, 2016).

On the other hand, both higher and lower anti-HBc positivity from blood donors were documented domestically and from elsewhere globally (e.g. higher rates from Brazil (22%), Burkina Faso (20.1%), Pakistani (17.28%), Cameroon (48.7%) blood donors (Bhatti *et al.*, 2007, Diarra *et al.*, 2018, Fopa *et al.*, 2019, Moresco *et al.*, 2014) and lower rates in children from Gondar (6.3%) (Ayalew *et al.*, 2019), Mexico (6.4%) (García-Montalvo and Ventura-Zapata, 2016), and Iran (5.18%)(García-Montalvo and Ventura-Zapata, 2016, Jafarzadeh *et al.*, 2008). One study from Egypt reported a 14.2% (Said *et al.*, 2013). Age differences, endemicity of HBV in the general population, and investigation approach might be responsible for such discrepant prevalent reports from the same study groups (healthy blood donors).

6.2. Occult HBV among voluntary blood donors

Due to lack of molecular assays in blood screening, there is limited knowledge on the rate of occult hepatitis B infection, especially in blood donor in Ethiopia. The present study showed the

prevalence of occult hepatitis among anti-HBc positive blood donors to be 4/144 (2.8%). The result was lower than other finding (5.6%, 6% and 19.1%) (Ayana *et al.*, 2020, Patel *et al.*, 2020) among HIV negative, HIV patient and pregnant Mothers, respectively, which is expected due to the latter being members of risk groups. For HIV negative individuals, level of endemicity of HBV in the respective local areas and age group difference could be possible reasons for having occult HBV greater than that seen in our study where median age of the participants was 24 years. Similarly, although comparable prevalence rate was documented in some countries (e.g. Pakistan (2.9%) (Bhatti *et al.*, 2007) and Brazil (2.7%) (Moresco *et al.*, 2014), the 2.8% prevalence rate among blood donors in this study is lower than rates reported from many countries including those from the following: Sudan (16 %) (Abakar, 2018); Egypt (14.2%) (Said *et al.*, 2013); Nigerian (17%) (Oluyinka *et al.*, 2015); Egypt (14.2%) (Mabunda *et al.*, 2020); and Burkina Faso (20.1%) (Diarra *et al.*, 2018); and Saudi Arabia (8.6%) (Alshayea *et al.*, 2016). However, lower prevalence rates are also reported among blood donors from few African countries: 0.56% from Cameroon (Allain, 2017); 0.1% from Libya (Fopa *et al.*, 2019); and 0.025% from South Africa (Shambesh *et al.*, 2016).

In regards to HBV viral load, the maximum viral load detected in occult hepatitis B infection in this study was 48IU/ml, which is higher than those reported from Nigeria (1.6IU/ml) (Oluyinka *et al.*, 2015), Cameroon (<6 IU/mL median 5IU/mL) (Fopa *et al.*, 2019), and China (14 IU/ml)(Zheng *et al.*, 2011); but lower than those reported from Saudi Arabia (186 IU/mL) (Alshayea *et al.*, 2016), Italy (108 IU/mL) (Manzini *et al.*, 2007), and Loa PDR (3510 IU/ml) (Jutavijittum *et al.*, 2014).

This study shown that as age increased had high anti-HBc in there blood (OR = .225; 95% CI:0.121–0.417) and found to be associated risk factors from demographic data analysis, others author result support this finding (Demir *et al.*, 2008, Fopa *et al.*, 2019).

7. LIMITATIONS OF THE STUDY

- ❖ This study also assessed only the anti-HBc positive OBI individuals due to resource limitations, which may have affected detection of the true prevalence of OBI; it should have also included tests like anti-HBsAg testing. It would also be more informative if anti-HBc negative samples were included and tested for anti-HBsAg seropositivity along with NAT.

8. CONCLUSION AND RECOMMENDATION

8.1. Conclusion

From this cross-sectional study we can conclude that from 1000 blood units considered to be safe, four blood units had occult HBV infection. And from total blood donors, 14.8% had the history of exposure to HBV infection in the past. The frequencies of blood donation history and donors' age group were found to be associated risk factors. The mean viral load of occult hepatitis B virus in blood donors was found to be 31 IU/mL, which can be undetectable in convectional PCR. HBsAg alone is not sufficient to eliminate the risk of HBV transfusion-transmission.

8.2. Recommendation

- Further investigation should be undertaken to gate full picture of the magnitude occult hepatitis in blood donors
- Based on research action should be taken to revise blood screening strategy including anti-HBc and HBV nucleic acid testing when possible is warranted.

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10. APPENDICES

Appendix I: Letter for asking waiver for informed consent

In this study the participant did not give consent, rather in routine blood bank blood collection they have used questionnaires with consent form and I was asked Blood Banks for waiver by letter.

Date_______________

Asking for Waiver of informed consent to Ethiopian National Blood Bank

Title of the project: Occult Hepatitis B Virus infection and genotype distribution among blood donors in selected blood bank of different region of Ethiopia

Principal Investigator's: Gizachew Gemechu (DVM, MSc candidate in Medical Microbiology)

Addis Ababa University, School of medicine, department of Medical microbiology, Immunology and Parasitology

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The research was conducted using previously archived plasma have no adverse effect on the rights and welfare of the subjects. There is no intervention to be done and no consent was needed for this study. As a result I need a waiver letter for informed consent for the above study to use data of questioner already collected along with all of the study sample (973) plasma left over from blood screening of blood donors from Addis Ababa Blood Bank. Thank you in advance

With best regards,

Investigator signature: ______________________ Date:_______________

Appendix II. Demographic data collection format sheet

Donor ID	Sex	Age	Donation history	If yes, how many times?	Donation site

Appendix III. **L**aboratory procedures, Material and reagent

General information on laboratory procedures

This section outlines the list of tasks required to complete this procedure. These tasks should be assigned to individual(s) capable of their execution and their name entered beside the task listed in the table below.

Tasks and responsible person

Tasks	**Study personnel**
Preparation of reagents and laboratory equipments	PI and AHRI lab staff
Serological immunoassay tests	PI and AHRI lab staff
DNA extraction and quantification	PI and AHRI lab staff
Amplification	PI and AHRI lab staff
Sequencing	PI and AHRI lab staff

SOP for preparation, testing and interpretation of result for serology detection of surface antigen

1. SEROLOGY

1.1. Surface Antigen test

1.1.1.1. General information on laboratory procedures

This section outlines the list of tasks required to complete this procedure. These tasks should be assigned to individual(s) capable of their execution and their name entered beside the task listed in the above table.

1.1.1.2. Principles of test

The assay is a qualitative one-step enzyme immunoassay based on the principle of 'sandwich' type using monoclonal antibodies and polyclonal antibodies selected for their ability to bind themselves to the various subtypes of HBsAg in serum or plasma samples. If a sample is positive in HBs antigen, the anti-HBs antibodies in the neutralization reagent saturate the HBs antigen determinants of the sample which can no longer bind to the antibody fixed on the solid phase. A decrease in optical density was observed when comparing the same sample in which the neutralization reagent is replaced by a negative control diluent not containing anti-HBs antibodies. Several dilutions of the sample was analyzed, as required, to take into account variations in HBs Ag concentration.

1.1.1.3. Materials and Equipment

- ✓ Pencil
- ✓ Pipettes
- ✓ ELISA kit with plate
- ✓ Materials required but not provided
- ✓ Distilled water.
- ✓ Sodium hypochlorite (household bleach).
- ✓ Absorbent paper.
- ✓ Disposable gloves.
- ✓ Safety glasses.
- ✓ Disposable tubes.

- ✓ Automatic or semiautomatic, adjustable or preset pipettes or multipipettes to measure and dispense 50 µl, 100 µl, 1 000 µl and 10 ml.
- ✓ Graduated cylinders of 100 ml, 1000 ml capacity.
- ✓ Automatic, semi-automatic or manual microplate washer system.
- ✓ Water-bath or equivalent microplate incubator thermostatically set at 37°C ± 1°C.
- ✓ Container for biohazardous waste.
- ✓ Microplate reader equipped with 450, 490 nm and 620-700 nm filters

The ELISA HBs Ag test is used to confirm the presence of hepatitis B surface Ag (HBs Ag) in samples of human serum or plasma found reactive by the screening test.

1.1.1.4. Assay Procedure

We will follow the following Good Laboratory Practice:

1. Carefully establish the sample distribution and identification plan.
2. Prepare the diluted washing solution R2 (refer to reagent preparation).
3. Prepare the conjugate R6+R7 working solution (refer to reagent preparation).
4. Take out from the protective packing the support frame and the necessary number of strips (R1). Put the unused strips back in their packing. Close the packaging and replace it at +2-8°C.
5. Distribute in the wells in the following order (advisable plate distribution) :
   - 100 µl of negative control (R3) in wells A1, B1, C1 and D1
   - 100 µl of positive control (R4) in well E1
   - 100 µl of the first unknown sample in well F1 if this well is not used as control well for the validation of the sample and conjugate deposition (optional)
   - 100 µl of unknown sample in wells G1, H1, etc
6. Quickly dispense 50 µl of conjugate solution (R6 + R7) into all wells; the conjugate solution must be shaken before use. Homogenize the reaction mixture.
7. When possible, cover the plate with new adhesive film.
8. Incubate the microplate for 1 hour and 30 minutes (± 5 min.) at 37°C ± 1°C.

9. If necessary, remove the adhesive film. Aspirate the contents of all wells into a liquid waste container and add a minimum of 370 µl of washing solution into each well. Aspirate again and repeat the washing a minimum of 4 times. The residual volume must be lower than 10µl
10. Prepare development solution (reagent R8+R9)
11. Quickly dispense into each well 100 µl of prepared development solution (R8+R9), freshly prepared before use. Allow the reaction develop in the dark for 30 minutes (± 5 min.) at room temperature (18-30°C).
12. Add 100 µl stopping solution (R10) by using the same sequence and rate of distribution as for the substrate solution. Homogenize the reaction mixture.
13. Carefully wipe each plate bottom. Wait at least 4 minutes after stopping solution addition before reading and within 30 minutes of stopping the reaction, read the optical density at 450/620-700 nm using a plate reader.
14. Check for agreement between the spectrophotometric and visual readings and against the plate and sample distribution and identification plan.

4.1.5. Calculation and Interpretation and Interpretation of the test result

The cut-off is determined with negative control R3: Calculate the mean measured absorbance value for negative control R3. Calculate the cut-off value: mean OD negative control + 0.050. The presence or absence of HBs Ag is determined by comparing the registered absorbency to the calculated cut-off value for each sample. The following ratio is calculated for each sample:

Ratio = OD of the sample / CO Value

Samples with an optical density value lower than cut-off value are considered to be negative (ratio < 1). Results just below the cut-off value (CO-10% < O.D < CO, ratio between 0.9 and 1) wasretested. Samples with optical density values greater or equal to the cut-off (ratio ≥ 1) are considered to be initially positive wasretested in duplicate before final interpretation.

4.1.6. Quality Control

Use the negative control (R3) and positive control (R4) in each run of test to validate the assay as described by manufacturer.

4.2. Anti-HBc test

2.2.1. Principle of test

Anti-HBc is an enzyme immunoassay (indirect ELISA type) for the simultaneous detection of total antibodies to hepatitis B virus core in human serum or plasma. The absorbance measured for a sample allows the presence or absence of antibodies to HBc to be determined. The colour intensity is proportional to the quantity of anti-HBc antibodies bound on the solid phase

2.2.2. Materials and Equipment

Materials and Equipment listed in above procedure was used

2.2.3. Assay procedure

1. Carefully define the sample distribution and identification plan.
2. Prepare the wash solution to working strength.
3. Remove the microplate frame and ready to use strips (R1) from their protective bag.
4. Add quickly, directly and in succession :
   - ✓ 200 µl of diluent (R6) into each well
   - ✓ 20 µl of negative control serum (R3) in A1, B1
   - ✓ 20 µl of positive control serum (R4) in C1, D1, E1
   - ✓ 20 µl of the first sample in F1 and continue for other samples
5. Cover the wells with adhesive film by pressing over the whole surface to ensure tightness.
6. Incubate the microplate in a thermostat-controlled water-bath or in a dry microplate incubator for : 30 min ± 5 min at 37°C ± 1°C
7. Remove the adhesive film and wash three times with washing solution
8. Distribute quickly 200 µl of the conjugate solution into all wells
9. Cover with new adhesive film and incubate for: 60 min ± 5 min at 37°C ± 1°C
10. Remove the adhesive film, empty all wells by aspiration and wash 4 times as previously described
11. Prepare the substrate solution (see section 8, reagent R8 + R9) and quickly dispense into each well 100µl of prepared development solution (R8+R9), freshly prepared before use. Allow the reaction to develop in the dark for 30 ± 5 minutes at room temperature
12. Add 100 µl stopping solution (R10) by using the same sequence and rate of distribution as for the substrate solution. Homogenize the reaction mixture

13. read the optical density at 450/620-700 nm using a plate reader

2.2.4. Calculation and interpretation of the results

The presence or absence of anti-HBc antibodies is determined by comparing for each sample the recorded absorbance with that of the calculated cut-off value.

Mean of OD R4= Total optical density divided by 3

Calculation of the cut-off value (Vs), $Vs = \frac{\text{mean of OD R4}}{5}$

4.2.5. Validation

Validation criteria were as follows:

a) For the negative control: each individual measured absorbance value must be less than 0.100

b) For the positive control: Each absorbance value must be greater than, or equal to, 1.000 and less than, or equal to 2.900. If one of the positive control value is out of these norms or differs by more than 30% from the mean value, carry out the calculation again with the two remaining positive control values. The test should be repeated if more than one positive control value is outside the limits set above.

3. MOLECULER TECHNIQUES

3.1. DNA extraction and quantification using ABBOTT system

a. Material and equipment

1. Abbott RealTime HBV Control Kit
2. Abbott RealTime HBV Calibrator Kit
3. Abbott Real time m2000sp machine
4. Laboratory marking pen
5. Disposable gloves
6. Laboratory tissue wipes
7. Ice bucket and ice
8. Abbott mSample Preparation System DNA (4 x 24 preps)
9. Abbott Proteinase K (List No. 3L78-60)
10. Abbott RealTime HBV m2000 System ROW Combined Application CD-ROM
11. Sample Racks
12. 200 mL Reagent Vessels
13. 5 mL Reaction Vessels
14. Master Mix Vial
15. Abbott 96-Well Optical Reaction Plate
16. Abbott 96-Deep Well Plate
17. Abbott Splash-Free Support Base
18. Abbott Optical Adhesive Cover
19. Abbott Adhesive Cover Applicator
20. Round-bottom 12.5 x 75 mm Sample Tubes
21. Vortex Mixer
22. 50 mL Polypropylene Centrifuge Tubes
23. Centrifuge capable of 2000 g

24. Calibrated Precision Pipettes capable of delivering 10 µL-1000 µL
25. 20 µL-1000 µL Aerosol Barrier Pipette Tips for precision pipettes
26. USP Grade 190-200 Proof Ethanols (95-100% Ethanol). Do not use ethanol that contains denaturants.
27. Serological Pipettes
28. Graduated Cylinder, 100 mL
29. Molecular Biology Grade Water (Eppendorf Scientific, Inc. or equivalent)
30. 1.7 mL Molecular Biology Grade Microcentrifuge Tubes (Dot Scientific, Inc. or equivalent)*
31. Cotton Tip Applicators (Puritan or Equivalent)
32. Heating block or water bath set at 56°C
33. Biological safety cabinet approved for working with
34. Infectious materials.
35. Sealable plastic bags

b. Extraction and sample preparation procedure

1. A total of 48 samples can be processed in each run. A negative control, a low positive control, and a high positive control must be included in each run, therefore allowing a maximum of 45 specimens to be processed per run.
2. Thaw assay controls and Internal Control (IC) at 15 to 30°C or at 2 to 8°C.
3. Thaw amplification reagents at 15 to 30°C or at 2 to 8°C until required for the amplification master mix procedure. This step can be initiated before completion of the sample preparation procedure.
4. Open the Abbott Proteinase K reagent pack. Add 17.15 mL of Molecular Biology Grade water to a 50 mL polypropylene centrifuge tube. Pipet 2.45 mL of Proteinase K into the container of water. Mix by gentle inversion 10 to 15 times.
5. Open the Abbott mSample Preparation System DNA pack.
6. If crystals are observed in any of the reagent bottles upon opening, allow the reagents to equilibrate at room temperature until the crystals disappear. Do not use the reagents until the crystals have dissolved.

7. Prepare the mWash2DNA by adding 70 mL of USP Grade 190-200 Proof Ethanol (95%-100% Ethanol) to the mWash2DNA bottle as described in the Abbott mSample Preparation System DNA product information. Do not use ethanol that contains denaturants
8. Vortex the IC vial(s) three times for 2 to 3 seconds before use. Using a calibrated precision PIPETTE DEDICATED FOR INTERNAL CONTROL USE ONLY, add 100 µL of IC to a bottle of mLysis Buffer. Mix by gently inverting the container 5 to 10 times to minimize foaming.
9. Gently invert the Abbott mSample Preparation System DNA bottles to ensure a homogeneous solution and pour the contents into the appropriate reagent vessels per the Abbott m2000sp Operations Manual, Operating Instructions.
10. Place the low and high positive controls, the negative control, the calibrators (if applicable), and the patient specimens into the m2000sp sample rack.
11. Place the 5 mL Reaction Vessels into the m2000sp 1 mL subsystem carrier.
12. Load the carrier racks containing the Abbott mSample Preparation System DNA reagents and Proteinase K, and the Abbott 96-Deep Well Plate, on the Abbott m2000sp worktable as described in the Abbott m2000sp Operations Manual, Operating Instructions.
13. From the Run Sample Extraction screen, select the appropriate application file corresponding to the sample volume being tested. Initiate the sample extraction protocol as described in the m2000sp Operations Manual, Operating Instruction.
14. Load the amplification reagents and the master mix vial on the m2000sp worktable after sample preparation is completed.
15. Select the appropriate deep well plate from the Run Master Mix Addition screen that matches the corresponding sample preparation extraction. Initiate the Abbott m2000sp Master Mix Addition protocol. Follow the instructions as described in the Abbott m2000sp Operations Manual, Operating Instructions section.
16. The DNA was either directly used amplify in m2000rt automatic system or stored at -80°C.

3.2. Amplification with Abbott RealTime HBV

Principles

The Abbott RealTime HBV assay uses PCR to generate amplified product from the DNA genome of HBV in clinical specimens. A DNA sequence that is unrelated to the HBV target sequence is introduced into each specimen at the beginning of sample preparation. This unrelated DNA sequence is simultaneously amplified by PCR and serves as an internal control (IC) to demonstrate that the process has proceeded correctly for each sample. The amount of HBV target sequence that is present at each amplification cycle is measured through the use of fluorescent-labeled oligonucleotide specifically bound to the amplified product. The amplification cycle at which fluorescent signal is detected by the Abbott m2000rt is inversely proportional to the log of the HBV DNA concentration present in the original sample.

a. Materials and equipment

1. Abbott m2000rt
2. Abbott RealTime HBV m2000 System ROW Combined Application CD-ROM
3. Abbott m2000rt Optical Calibration Kit
4. Biological safety cabinet approved for working with infectious materials.
5. Sealable plastic bags

b. Procedures

1. Switch on and initialize the Abbott m2000rt in the amplification area
2. Seal the Abbott 96-Well Optical Reaction Plate after the Abbott m2000sp instrument has completed addition of samples and master mix according to the Abbott m2000sp Operations Manual, Operating Instructions section.
3. Place the 96-Well Optical Reaction Plate into the Splash-Free. Support Base for transfer to the Abbott m2000rt instrument.
4. Export the completed 96-Well Optical Reaction Plate results to a CD or Network Drive

5. Place the Abbott 96-Well Optical Reaction Plate in the Abbott m2000rt instrument. Import the m2000sp test order via CD or network Drive per the Import Order instructions in the Abbott m2000rt Operations Manual, Operating Instructions section
6. Remove the Abbott 96-Deep Well Plate from the worktable and dispose according to the Abbott m2000sp Operations Manual
7. Place the Abbott 96-Well Optical Reaction Plate in a sealable plastic bag and dispose according to the Abbott m2000rt Operations manual along with the gloves used to handle the plate
8. Clean the Splash Free Support Base before next use, according to the Abbott m2000rt Operations Manual.

3.3. DNA extraction and Amplification with Nested PCR

3.3. 1. DNA extraction

Materials and equipment needed

- ✓ Ethanol (96–100%)*
- ✓ 1.5 ml microcentrifuge tubes
- ✓ Pipet tips with aerosol barrier
- ✓ Microcentrifuge (with rotor for 2 ml tubes)
- ✓ Vortexer
- ✓ Water bath or heating block at 56°C
- ✓ Phosphate-buffered saline (PBS) may be required for some samples

Procedure

1. Pipet 40 µl QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube.

2. Add 400 µl sample to the microcentrifuge tube. Use up to 400 µl, plasma, serum

3. Add 400 µl Buffer AL to the sample. Mix by pulse-vortexing for 15 s. Note: Do not add QIAGEN Protease or proteinase K directly to Buffer AL.

4. Incubate at 56°C for 10 min. DNA yield reaches a maximum after lysis for 10 min at 56°C.

5. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

6. Add 400 µl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
7. Carefully apply the mixture from step 6 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate. Close each spin column to avoid aerosol formation during centrifugation.
8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.
9. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min
10. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer AW2 carryover.
11. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not Provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.

3.3.2. Nested PCR

a. Materials and equipment

- ✓ Sterile 2.0 mL microcentrifuge tubes, nuclease-free
- ✓ P20 aerosol barrier (plugged) pipette tips
- ✓ P200 aerosol barrier (plugged) pipette tips
- ✓ P1000 aerosol barrier (plugged) pipette tips
- ✓ Pipets
- ✓ PCR machine

- ✓ Water (Brau Water (Braun aqua n aqua Mgcl2
- ✓ Q5 high-fidelity master mix
- ✓ Outer Forward Primer
- ✓ Outer Reverse Primer
- ✓ Inner Forward Primer
- ✓ Inner Reverse Primer PCR water

b. Procedures

PCR master mix were prepared and loaded to PCR machine. The preparation of reagents for first PCR and nested PCR was as the following Table.

	Ser.NO	Components	25 REACTION	6X
First PCR	1	Q5 High-Fidelity 2X Master Mix	125 µl	75 µl
	2	10 µM Forward Primer	1 µl	6 µl
	3	10 µM Reverse Primer	1 µl	6µl
	4	Nuclease-Free Water	5.5 µl	33 µl
	5	Templets	5 µl	
NESTED PCR	1	Q5 High-Fidelity 2X Master Mix	125 µl	75 µl
	2	10 µM Forward Primer	1 µl	6 µl
	3	10 µM Reverse Primer	1 µl	6µl
	4	Nuclease-Free Water	9.5 µl	57 µl
	5	Templets	1 µl	

Running PCR

1. On the PCR machine
2. Set temperature and time as follow:

Thermal condition	temperature	time
Initial denaturation	98°C	10 sec
Denaturation	98 °C	30 sec
Primer annealing	58 °C	30sec
Elongation	72 °C	45sec
Final elongation	72 °C	2 min
Cooling	4 °C	for ever

3.4. Detection of Amplicon on gel

a. Material and equipment

1. Agarose gel powder
2. Box to hold the gel
3. Comb
4. Positive and negative electrodes to create the electrical current
5. Power supply
6. Gel photo imaging system
7. Buffer solution (TEA)

b. Preparing agarose gel

- ✓ Dissolving 0.9 g of agarose gel into 75 mL of 1X TAE buffer by heating.
- ✓ Cool to about 50°C
- ✓ Place the comb over casting tray
- ✓ Pour gel on electrophoresis casting tray
- ✓ Remove the comb
- ✓ Add buffer solution
- ✓ Briefly add 5µl of the PCR product mixed with 2µl of blue 6x loading dye and ladder
- ✓ Load mixed into the wells on the gel
- ✓ loaded agarose gel will then subjected to 120 Volts of a unidirectional (from the anode to the cathode) electrical current for a duration of 50 minutes,

- ✓ Follow the migration direction
- ✓ The migrated sample DNA bands from first PCR 952bp and from nested 509bp in comparison of DNA ladder in the agarose gel was visualized using ultraviolet transillumination and corresponded to positive controls.
- ✓ A digital image of the gel was taken using a gel documentation system.
- ✓ Image was labelled with ID number and stored on soft copy.

11. DECLARATION SHEET

I undersigned, Clinical Laboratory Sciences in (Diagnostic and Public Health Microbiology) Student declare that this thesis is my original work in partial fulfillment of the requirement for Master of Science degree in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology).

Name of Principal Investigator: - Gizachew Gemechu (DVM)

Signature: --------------------

Place of Submission:-Addis Ababa University, College of Health Sciences, Department of Medical Laboratory Sciences

Date of Submission: ---------/--------------/------------------

Approved by my advisors:

Advisors: This thesis work has been submitted for examination with my approval as

University and research institute advisors.

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1.	Dr. Woldaregay Erku Abegaz	____________________	_____________
2.	Dr. Andergachew Mulu	____________________	_____________
3.	Dr. Adane Mihret	____________________	_____________