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Antibody level against HBV after Hepatitis B vaccination and Sero-prevalence of HBV in children in Addis Ababa, Ethiopia

By

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List of Acronyms

CHB	Chronic Hepatitis B
DNA	Deoxyribonucleic Acid
EPI	Expanded Programme on Immunization
FDA	US Food and Drug Administration
GAVI	Global Alliance for Vaccine and Immunization
HBIg	Hepatitis B Immunoglobulin
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
NA	Nucleotide Analogue
OBI	Occult Hepatitis B infection
UNICEF	United Nations Children Fund
USA	United States of America
UK	United Kingdom
WHO	World Health Organization

Abstract

Background: Around two billion people have been infected with HBV worldwide, and more than 240 million are chronic carriers. Vaccine introduction for HBV in children was officially launched by WHO in 1980. Since then the vaccine response level was determined in different countries. However, there is no any study conducted in Ethiopia to assess the response level and effectiveness of HBV vaccine against the virus since the initiation of the vaccine in 2007.

Objectives: The aim of the study was to determine antibody level against HBV after hepatitis B vaccination and sero-prevalence of HBV in children in Addis Ababa, Ethiopia.

Methods: A cross sectional study was conducted and a multi stage probability sampling techniques was applied. Four hundred and fifty children between the age of 5 and 8 years who are living in three sub cities of Addis Ababa were included. Three to four ml of blood was collected and questionnaire was obtained. Finally ELISA was done to determine antibody level against HBV after hepatitis B vaccination and sero-prevalence of HBV infection in children.

Result: The mean age of the children was 7$\pm$1(SD) years. Protective antibody levels were detected in 208(54.3%) of children with a slightly high response level in females 98(54.7%) than males 110(53.9%). The overall vaccine coverage was 85.1 %. The protective level is declined as the age of the child increased and it was 52.6%, 60%, 43.5% and 37.1% at the age of 5, 6, 7 and 8 years, respectively. The sero prevalence of HBsAg was 0.4% whereas Anti HBc was 5.6%. Age has negatively correlated and significantly associated with the response level (p=0.001) whereas sex and previous disease status of the child has no significant association. Age of the child was also having significant association with sero- prevalence of anti HBc (p=0.003). Other factors like vaccination status and dose of vaccination were also significantly associated with a vaccine response level (p=<0.001). All partially vaccinated children didn't respond to the vaccine.

Conclusion: The vaccine coverage in the country is worthy but antibody response against HBV vaccine is low. We also showed a low Sero- prevalence of the virus in children. However, the low response level to the vaccine should have to be the concern and revaccination or booster doses should be given for non responded children in order to enhance the vaccine response.

Key words: *HBV, antibody response level, vaccination status, HBsAg, Anti HBsAg, Anti HBc*

1. Introduction

1.1 Background

Chronic hepatitis B virus (HBV) infection is a serious global public health problem. According to the world health organization (WHO), two billion people have been infected with HBV worldwide, and more than 240 million (4%-6% of the world population) are chronic carriers. Chronically infected individuals have a 25% risk of dying from the result of chronic HBV infection, such as cirrhosis and hepatocellular carcinoma (HCC) (WHO, 2013).

Although HBV is predominantly a hepatotropic virus, there is increasing evidence documenting that the immune (lymphoid) system is also an important site for maintaining viral persistence (Pontisso P et al., 2008). HBV genomes and viral proteins have been detected within a variety of immune cell subpopulations and, in some reports the virus appears to specifically target B cells and monocytes (Chemin I et al., 1994). Infection with HBV results in acute hepatitis followed by recovery in 85%–95% of human adults. Recovery occurs when the organism mounts adequate immune responses against the virus. Such responses include production of protective, neutralizing antibodies against HBV surface antigen (HBsAg), activation of strong and diversified CD4 and CD8 T-cells, expression of antiviral cytokines in the liver, such as gamma interferon and tumor necrosis factor alpha, and generation of cells that are protected from re-infection (Ciupe SM et al., 2014).

HBV infection is one of the vaccine-preventable infectious diseases and its vaccine became commercially available in the 1980s. In 1991, WHO recommended the integration of the HB vaccine into the national immunization programs in countries with an HBV carrier prevalence of 8% or higher by 1995. In 2004, hepatitis B vaccine had been introduced into the routine infant immunization Programme in 153 (79.7%) of the 192 WHO Member States. By the end of 2005, this proportion had increased to 158/192 (82.3%) (WHO, 2002, 2006).

Hepatitis B vaccine for infants was introduced nationwide in 183 countries by the end of 2013. Global coverage with three doses of hepatitis B containing vaccine is estimated at 81% and is as high as 92% in the Western Pacific. A birth dose for hepatitis B vaccine was introduced in 93 countries by 2013, and global coverage was estimated at 38%, reaching 79% in the Western Pacific, but only 11% in the African Region (WHO, 2013).

Most of the countries in the WHO African Region have implemented hepatitis B vaccine in their national Expanded Programme on Immunization (EPI). This strategy is important for effective control of HBV infection and its effect is mass vaccination of neonates and children. In 2007, the sub-Saharan region was very close to complying with the original recommendation: almost all countries had Hepatitis B in their national programme (Françoisa G et al., 2008).

Antibody to hepatitis B surface antigen (anti-HBs) in vaccinated children declines with time, especially during the first few years of vaccination **(**Hassan S et al., 2007). Currently, the efficacy of vaccination is only determined by assessing the level of antibodies to HBsAg in blood. The vaccination response can be determined by measuring the concentration of antibodies against the HBsAg. The responsiveness to hepatitis B vaccine should usually be determined by antibody measurement within 2-6 months after the third vaccine dose. Most patients produce a high concentration of antibodies. However, a small group of vaccines (5-10%) produce no (<10 mIU/mL) or suboptimal concentrations (10-100 mIU/mL) of antibodies, which are known as non-responders and low-responders respectively. The responders have high concentration (>100mIU/mL).These non- or low-responders have no protection against hepatitis B or are protected for only a few years (Faas H et al., 2011; Leonardi S et al., 2012).

The site of injection and mode of administration are critical factors in achieving an optimal response. The vaccine should be given intramuscularly into the deltoid region in children (≥1year of age) or into the anterolateral thigh in newborns and infants (< 1year of age). Hepatitis B vaccines are well tolerated. Side effects are generally mild, transient and confined to the site of injection (erythema, swelling, and indurations). Systemic reactions (fatigue, slight fever, headache, nausea, abdominal pain) are uncommon (Franco E et al., 2012).

Booster doses of hepatitis B vaccine after primary vaccination in immunocompetent individuals are currently not recommended by WHO, decrease of antibody concentrations below sero protection level or even below detection levels is not considered as an indicator of loss of protection (Van Damme P and Van Herck K., 2007). Booster doses of Hepatitis B vaccine are recommended only in certain circumstances, for example, for hemodialysis patients, and for those with an ongoing risk of exposure. Annual anti-HBs testing and a booster dose should be administered when anti-HBsAg levels decline to < 10 mIU/mL (Gabbuti A et al., 2007). With the passage of time and longer experience, protection has been shown to last for at least 25 years in

those who showed an adequate initial response to the primary course of vaccinations (Van Damme P and Van Herck K., 2007).

1.2 Statement of the problem

There are an estimated 600,000 deaths annually from complications of HBV-related liver disease (Gao S et al., 2015). In highly endemic areas, the incidence of HBV infection is greater than 8%, and is often acquired at birth or in early childhood from exposure to HBV infected mothers or family members. About 90% of unvaccinated infants born to mothers with chronic hepatitis B (CHB) will became chronic carriers, and the risk of CHB is up to 30% in children infected at 1-4 years of Age (Hyams KC, 1995). Despite implementation of widespread childhood vaccination programs, the incidence and mortality of HBV-related cirrhosis and HCC continues to increase due to the enormous burden of chronically infected carriers worldwide (Gao S et a.,2015; Mphahlele MJ et al., 2002).

In South Africa overall HBsAg positivity was, 10.4% of the children tested. There was a high rate of positivity in the 0–6 and 7–12 month age groups accounting for 8.1% and 8.9%, respectively, suggesting a higher rate of early acquisition. The proportion of HBsAg-positive children increased significantly with increasing age reaching 15.7% in the 61–72-month age group (Vardas E et al., 1999).

There are debates over the long-term persistence of post vaccination immunity to hepatitis B and the need for booster doses of the vaccine. Long-term follow-up studies of newborn vaccination demonstrated that antibodies become negative in 15-50% among the vaccine responders within 5 to 10 years. Approximately 5-10% of vaccinated person do not achieve detectable antibody response (non responders) (Kwon SY and Lee CH, 2011). A study conducted in Iran revealed that protective antibody levels were detected in 65% of children one year after vaccination, which declined significantly over time to 24% in 15 years after vaccination (Aghakhani A et al., 2011). In a study in China, 50% of vaccinated children had protective levels of anti-HBsAg at 15 years of age (Liao SS et al., 1999).

The effectiveness of routine infant hepatitis B immunization in significantly reducing the prevalence of chronic HBV infection has been demonstrated in a variety of countries (Wright TL, 2006). A review paper revealed that, with the introduction of infant universal vaccination,

the prevalence of HBsAg declined from 1.6% to 0.04% in the United States, 7% to 2% in Colombia, 9.8% to 1.3% in Taiwan, 9%-12% to < 1% in China, 13.4% to 0.91% in Italy, 8%-9% to 0.9% in South Africa, 13.3% to 0.6% in Gambia (Keneba), and from 35% to 1% in Gambia (Mandar). The highly endemic countries in particular showed a remarkable reduction in the prevalence of HBsAg (Komatsu H, 2014).

HBV vaccine was introduced in Ethiopia with other vaccines called pentavalent vaccine in 2007 and from that time, vaccine coverage in EPI program is increasing. In Ethiopia, the vaccine is given in 3 doses at 6, 10 and 14 weeks of birth following the recommendations of WHO (WHO, 2002, 2006). The routine immunization programme is funded primarily by partners like Global Alliance for Vaccine and Immunization (GAVI), National and sub national Government, United Nations Children Fund (UNICEF) and WHO (FMOH, 2010).

In Ethiopia, the national coverage survey conducted in 2012 indicated a pentavalent 3 coverage was 65%. However, EPI coverage stagnated between 83 – 86% for three successive years after 2010 (WHO, 2014). WHO and UNICEF also estimated the Ethiopian Immunization Coverage Survey of Hepatitis B in 2012 based on card or history results. The overall immunization coverage was 60 % and 65 % respectively in which 1st dose card or history coverage was 80 %, 1st dose card only coverage was 59 % and 3rd dose card only coverage of 48 % in Hepatitis B (WHO and UNICEF, 2016).

1.3 Significance of the study

Many studies conducted in other countries revealed that the level of antibodies produced after vaccination is decreasing as age increases and some of them also recommend the administration of booster doses after some years (Aghakhani A et al., 2011). What about the Ethiopian situation? After all these years from the introduction of hepatitis B vaccine in the country what is the level of their immune status against HBV? What about the response level to vaccine? What about the sero prevalence of the HBV? And how many of them really completed full dose of the vaccine? From a demographic point of view, the analysis of a universal vaccination program is important to take collective or individual measures to raise the vaccination response level. It will give also for Ministry of Health and other stake holders about the response to the vaccine that help them to direct the future immunization strategy. It will also give a base line data for sero-

prevalence of HBV in children which helps the ministry to take actions and also as an initiation for future studies. Therefore, this study will answer the above questions and provide the overall prevalence and response level of antibody against HBV in Ethiopian children.

2. Literature review

2.1 Virology of Hepatitis B virus

Hepatitis B virus is the prototype member of the *Hepadnaviridae* family which includes various avian and mammalian viruses sharing similar genome structure and organism tropisms (Seeger C et al and Mason WS 2006). It is a small Deoxyribonucleic Acid (DNA) virus with approximately 3.2 Kb partially double stranded relaxed circular DNA (rcDNA) genome within a nucleocapsid surrounded by a lipid envelope. The full-length virus negative-strand has an approximately 7-9 nucleotide redundancy and the complementary positive-strand is approximately 50%-70% full genome length (Yan et al., 2012). There are ten major HBV genotypes (A-J) worldwide, which are identified by greater than 7.5% divergence across the HBV full genome between each genotype (Kramvis et al., 2008; Tatematsu et al., 2009).

Genotypes A and D are widely distributed in all the continents, but A is predominant in northern Europe, D prevails in the Mediterranean area. Genotypes B and C are found mainly in eastern Asia and the Far East, the Near and Middle East and south Asia, while genotype E is indigenous to sub-Saharan West Africa, genotype F is likely to exist in populations with origins on the American continent, and genotype G is found in the USA and Mexico and Europe, genotype H is closely related to F and prevails in Central and North America (Liu et al., 2002).

2.2 Epidemiology of Hepatitis B virus

Hepatitis B virus infection is endemic worldwide, but its prevalence differs greatly among regions. Several factors can impact the prevalence of HBV: male sex, being an adult aged 20 years, a family history of hepatitis B, drinking, smoking, being a migrant worker, and/or having some specific occupations are the major ones (Yang et al., 2012). The highest numbers of HBsAg carrier rates are found in developing countries with primitive or limited medical facilities. The world can be divided into three areas where the prevalence of chronic HBV infection is high (>8%), intermediate (2-8%) and low (<2%). High endemic areas include South-east Asia and the Pacific Basin (excluding Japan, Australia and New Zealand), sub-Sahara Africa, the Amazon Basin, parts of Middle East, the central Asian Republics and some countries in Eastern Europe is about 70-90% of the population becomes HBV infected before age of 40 and 8-20% of people are HBV carrier (Chen and Chang, 2010; Negero A et al., 2011).

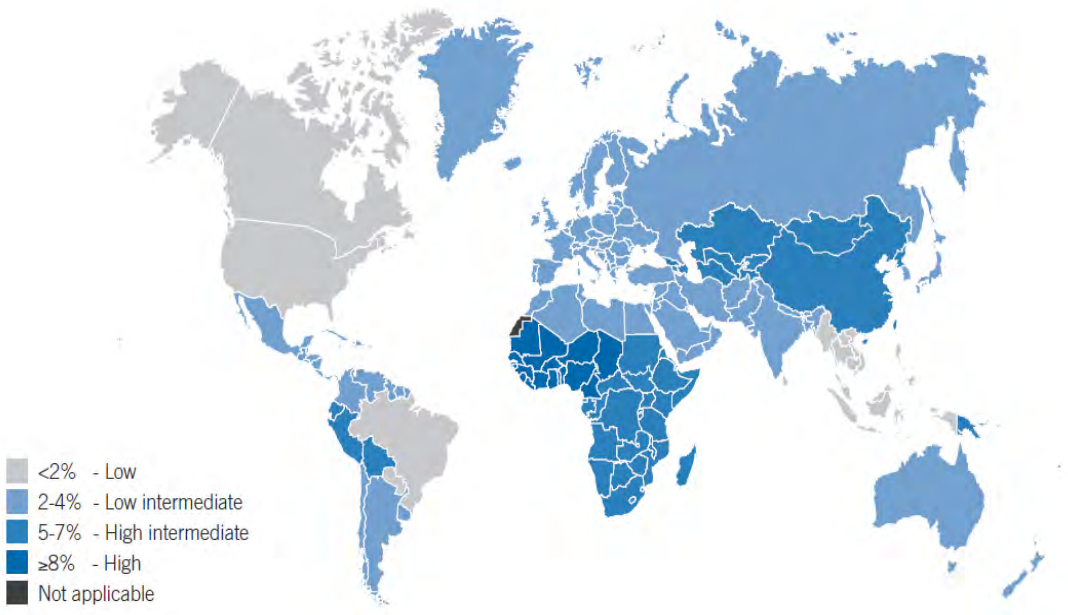

Figure 1: Prevalence of Hepatitis B infection, children 5-9 years, 2005 (WHO guidelines 2015)

2.3 Clinical Outcome of Hepatitis B virus

HBV infection can be either acute or chronic, and may range from asymptomatic infection or mild disease to severe hepatitis. Acute hepatitis B is usually a self-limiting disease marked by acute inflammation and hepatocellular necrosis, with a case fatality rate of 0.5–1%. Chronic hepatitis B (CHB) infection encompasses a spectrum of disease, and is defined as persistent HBV infection with or without associated active viral replication and evidence of hepatocellular injury and inflammation (Lavanchy et al., 2004*)*, chronicity is common following acute infection in neonates (90% of neonates born to hepatitis B e antigen [HBeAg]-positive mothers) and in young children under the age of 5 years (20–60%), but occurs rarely (<5%) when infection is acquired in adulthood(Hoofnagle et al., 2007). Worldwide, the majority of persons with CHB were infected at birth or in early childhood (WHO, 2015).

The spectrum of disease and natural history of chronic HBV infection are diverse. In some people, CHB is inactive and does not lead to significant liver disease. In others, it may cause progressive liver fibrosis, leading to cirrhosis with end-stage liver disease, and a markedly

increased risk of hepatocellular carcinoma, independent of the presence of cirrhosis – usually many years after initial infection (Lok and McMahon, 2007). In those with cirrhosis, there is an approximately 20% annual risk of hepatic decomposition and the annual incidence of hepatitis B-related HCC is high, ranging from <1% to 5% (Fattovich et al., 2004).

Untreated patients with decompensate cirrhosis have a poor prognosis, with 15–40% survival at five years. Several host and viral factors, especially coinfections with Human immune deficiency virus (HIV), Hepatitis C virus (HCV) and Hepatitis D virus (HDV), together with other cofactors such as alcohol use, may increase the rate of disease progression and risk of developing HCC (Fattovich et al., 2004; McMahon, 2004*).*

2.4 Occult HBV Infection

It is defined by detection of HBV DNA in serum and/or liver of patients and the absence of HBsAg, with presence or absence of anti-HBc or anti-HBs and it is characterized by a low level of virus replication and absence of HBsAg in screening tests(Alborzi et al.,2013).

Seropositive occult hepatitis B infection (OBI) is characterized by the detection of anti-HBc antibody with or without anti-HBs antibody, while seronegative OBI is described by undetectable both anti-HBc and anti-HBs antibodies. Majority of OBI cases which can be attributed to the larger proportion of resolved HBV infections. It has been reported that more than 20% of OBI cases are seronegative for all the HBV markers (Torbenson and Thomas, 2002).

Occult HBV infection has been found in patients with HCC, past HBV infection, or chronic hepatitis C, and individuals without HBV serological markers. The frequency of the diagnosis depends on the relative sensitivity of HBV DNA assays and the prevalence of HBV infection in the population. Collectively, around 30% to 35% of HBsAg-negative subjects with chronic hepatitis with or without HCC have positive serum HBV DNA (range from 5% to 55%). The prevalence of HBV DNA is higher in anti-HBc-positive, but anti-HBs-negative patients, ranging from 7% to 60% in populations highly exposed to HBV (Brechot et al., 2001).

Viral DNA persistence is not restricted to patients with liver disease and may be observed in subjects with normal liver parameters, however, it can be found in blood and/or organ donors. Overall, occult HBV infection is seen in 7%-13% of anti-HBc-positive and/or anti-HBs-positive subjects, and in 0% to 17% of blood donors. Occult HBV infection represents a potential transmission source of HBV via blood transfusion or organ transplantation. In addition, occult HBV infection has been associated with chronic hepatitis and hepatocellular carcinoma (Hou et al., 2005).

Real-time fluorescence-based quantitative polymerase chain reaction (PCR) and Nested PCR were used to measure HBV DNA levels in serum samples. The prevalence of OBI in HB-vaccinated infants from HBsAg-positive mothers was 4.92% in China. Occult infection was associated with absent anti-HBs or with low anti HBs level, high maternal viral loads and escape mutations in the S gene (Su et al., 2013).

2.5 Immunology of HBV infection

Antibodies to the hepatitis B surface antigen are mainly targeted to bind the amino acid hydrophilic region, referred to as a determinant of HBsAg. This provides protection against infection with all HBV genotypes and is responsible for the broad immunity afforded by hepatitis B vaccination (Zanetti et al., 2008).

There are four major terms that are usually expressed for progressive immune response and virus replication. The term “immune tolerant” where HBeAg positivity occurs most commonly in HBsAg-positive children and young adults infected in the perinatal or early childhood period. “immune-active” is when HBeAg-positive and this phase may last from several weeks to years, and may result in successful seroconversion from an HBeAg-positive state to an anti-HBe positive state and its called chronic hepatitis. “Immune-control” is during HBeAg negative, anti-HBe positive. This stage usually called inactive chronic hepatitis and “immune-escape” or HBeAg-negative chronic hepatitis. In this stage HBeAg is undetectable and anti-HBe detectable which represents a later phase of disease, generally in older persons (WHO, 2015, Bertoletti and Kennedy, 2014).

2.6 Diagnosis of Hepatitis B virus

Detection of HBsAg allowed for the first time for screening of apparently infected blood donors for a dangerous pathogen. The need to diagnose clinically silent HBV infections was a strong driving force in the development of modern virus diagnostics. HBsAg was the first infection marker to be assayed with a highly sensitive radio immune assay (Gerlich, 2013).

HBV serological markers

Previous HBV infection is characterized by the presence of antibodies (anti-HBs and anti-HBc). Immunity to HBV infection after vaccination is characterized by the presence of only anti-HBs. CHB is defined as the persistence of HBsAg for more than 6 months. Recently, quantitative HBsAg level determination has been proposed to differentiate inactive HBsAg carriers from persons with active disease (Brunetto et al., 2010*).* Serum HBV DNA concentrations quantified by real-time PCR correlate with disease progression and are used to differentiate active HBeAg-negative disease from inactive chronic infection, and for decisions to treat and subsequent monitoring (Lok and McMahon, 2009*).*

2.7 Transmission and risk factors of Hepatitis B virus

HBV is transmitted parenterally by contact with blood or body fluids of an infected person. There are factors for the transmission of HBV like unprotected sex, multiple sexual partners, needle pricks, clinical operation, blood transfusion, dental scaling/ surgery and having history of abortion also increased the risk of having HBV infection more than twice (Uddin et al., 2014; Qureshi et al.,2009 and Awole and Gebre-Selassie ,2005). The virus is also an association with HIV (Mbaawuaga, et al., 2014).

Perinatal transmission is the major route of HBV transmission in many parts of the world, and an important factor in maintaining the reservoir of the infection in some regions, particularly in China and South East Asia. In the absence of prophylaxis, a large proportion of HBV positive mothers, especially those who are seropositive for HBeAg, transmit the infection to their infants at the time of or shortly after birth (Beasley et al., 1983*).*

2.8 Treatment and prevention of Hepatitis B virus

Although HBV infection can be prevented by vaccination, it is important to treat persons with CHB at high risk of progression to reduce the considerable morbidity associated with CHB. Currently, seven antiviral agents (lamivudine, adefovir, entecavir, telbivudine, tenofovir, emtricitabine, and pegylated interferon) are approved for the treatment of CHB in high-income countries, and have been shown to delay the progression of cirrhosis, reduce the incidence of HCC and improve long-term survival. Although nucleotide analogues (NAs) are effective inhibitors of HBV replication, they rarely result in cure, and clearance of HBsAg is rare. Therefore, at present, long-term (potentially lifelong) NA therapy is required (WHO, 2015; EASL, 2012*)*.

The majority of children will not require antiviral therapy, early identification and monitoring of children at risk for progression of liver disease guided by liver histology and a family history of HCC remains important. The use of non invasive tests and identification of appropriate cut-offs have not yet been defined in children. Only conventional interfetron, lamivudine and adefovir have been evaluated for safety and efficacy, but children generally have a similar response as adults. Interferon cannot be used in infants aged less than 1 year. US Food and Drug Administration (FDA) have approved tenofovir for use in adolescents and children above the age of 12 years for HBV treatment (Jonas et al., 2010).

Infection rate can be reduced through a modification of behavior and improving individual education. Testing of all blood donations and assuring asepsis in clinical practice reduce the risk of contracting HBV. Moreover, screening of all pregnant women helps to avoid mother to child transmission at birth. Administration of human hepatitis B immunoglobulin (HBIg) contributes for prevention of neonatal infection and can be used after exposure to HBV as prophylaxis. Vaccination is the most effective means of preventing hepatitis B, cirrhosis and hepatocellular carcinoma worldwide (Franco et al., 2012).

Passive and active immunizations are the most effective measures to prevent HBV infection and its consequences. Two vaccine programs are being conducted worldwide to control and eradicate HBV infection. The first program is universal vaccination, which integrates a three or four-dose series Hepatitis B vaccine into routine vaccination programs. The other is selective vaccination, which targets high-risk individuals identified by assessments of chronic diseases, lifestyle, and

occupation. The WHO strongly recommends universal vaccination in all countries, and nearly all of the countries throughout the world are adopting such a program (WHO, 2002).

Most vaccines against hepatitis B use a single hepatitis B antigen. This antigen is the viral envelope protein: the hepatitis surface antigen. The configuration of the HBsAg used in current vaccine formulations contains a determinant which is located between amino acids 121-149 of the HBsAg immunogenic epitope, that trigger the production of polyclonal antibodies against the HBV major surface protein (HBsAb) (Abushady et al., 2011). Currently, several therapeutic vaccines for CHB are in experimental or clinical studies, including adenovirus-based therapeutic vaccine, hepatitis B DNA vaccine, HBsAg-HBIG immunogenic complex and so on (Qin et al., 2016)

2.9 Vaccine response and its effectiveness against HBV

A study conducted in Alaska, United States of America on Levels and Protection after Hepatitis B Vaccine showed that the protection afforded by primary immunization with plasma-derived hepatitis B vaccine during childhood and adulthood lasts at least 22 years, in which 60% of vaccinated individuals had an anti-HBs level 10 mIU/mL (McMahon et al., 2009).Another study conducted on American Samoa, only 39% of vaccinated children had protective levels of anti-HBs when tested at a mean of 8.75 years of age (Williams et al., 2003).

Long-Term protection against carriage of Hepatitis B virus after Infant Vaccination is also seen in a study conducted in Gambia showed that the Overall vaccine efficacy against infection and carriage was 83.4% and 96.5% respectively and Both vaccine efficacy and levels of hepatitis B surface antibody decreased with age, resulting in a vaccine efficacy against infection and carriage among 20–24-year-old participants of 70.9% and 9.11%, respectively (Van der Sande et al., 2006).

A meta-analysis was conducted in Germany on determinants of long-term protection after hepatitis B vaccination in infancy. The study analyzed 46 studies reporting information on the anti-HBs $\geq$ 10 mIU/mL 5 to 20 years after primary immunization and 29 studies providing information on booster response were identified. From 46 studies which had a total of 38524 children, many of them were excluded due to different reasons leaving only 28329 individuals of whom 15944 (56.3%) were protected by anti-HBs $\geq$ 10 mIU/mL at the time of follow up. And

from the booster dose group they analyzed 3235 individuals of whom 2663 (82.3 %) responded to the booster vaccination (Schönberger et al., 2013).

Another study in Italy The long-term immunogenicity of recombinant hepatitis B virus (HBV) vaccine, the efficacy of anti-HBV vaccination was 100% due to that All vaccinated students were HBsAg/ anti-HBc negative. This assessment also showed the level of antibody response against HBsAg, of the 588 students vaccinated in infancy during that period, 23% had an anti-HBs titer lower than 10 IU/L, 67.3% had a titer between 10 and 400 IU/L, and 9.7% had a titer over 400 IU/L; whereas of the 202 vaccinated during adolescence in the same period, the prevalence were 6.4%, 60.4%, and 33.2% in the above groups respectively (Coppola et al., 2015).

A study conducted on decreased immune response to hepatitis B eight years after routine vaccination reported that 77.1% of children in Israel at 8 years after vaccination had detectable antibody levels (≥10 mIU/ mL) and 48.4% of them had high antibody levels (>100 mIU/mL) (Gold et al., 2003).

Another study in Iran on persistence of anti-HBs antibody and immunological memory in children vaccinated with hepatitis B vaccine at birth. The results showed that at 10 years after primary vaccination with recombinant HB vaccine, 47.9% of the children had protective levels of anti-HBs antibody (Jafarzadeh et al., 2006). In the same country but different area another study that has been conducted on serum levels of anti-Hepatitis B surface Antibody Among Vaccinated Population Aged 1 to 18 Years in Ahvaz City Southwest of Iran showed that the overall rate of protection among studied subjects was 55.2% and Protective antibody levels were detected in 90% of the children, one year after vaccination. They were decreased thereafter to 63.6%, 54.2%, 35.7%, and 48.9% after five, ten, fifteen, and eighteen years of vaccination, respectively (Norouzirad et al., 2014).

A Cluster Randomized Controlled Trial Conducted in China also showed that the efficacy of the vaccine in the vaccination group was 84% against HBV related Primary liver cancer development before age 30 and 70% against severe end-stage chronic liver diseases. The HBsAg sero positive rates in the vaccination group were 2.16% at age 10–11, and 1.83% at 19–28 years, respectively, which were significantly lower than those in the control group and the protection

efficacy of neonatal vaccination against HBsAg Sero-positivity in this age group was 78% and 72% respectively (Qu et al., 2014).

A Population-Based Study conducted in China on examining Hepatitis B Virus Infection and Immunization rates in Northwest China revealed that prevalence of HBsAg in children of the age group 1-4 year is 1.3% and 5-9 year is 2.5 %. This study also assessed the vaccine induced protection and that was varied from 66.6%, 54.22%, 49.3% and 39.3% among ages of 1, 2, 3 and 4 years of age which indicate the level of antibodies decreases in increasing age (Ji et al., 2014).

A 10-Year Follow-Up conducted in Taiwan to determine the long-term efficacy of hepatitis B vaccination among high-risk infants in 805 vaccine responders showed that cumulative persistence of antibody to hepatitis B surface antigen (anti-HBs) was 85%, and cumulative incidence of HBV infection was 15% at 10 years after vaccination (Wu et al., 1999).
A study conducted in Thailand on Persistence and immune memory to hepatitis B vaccine 20 years after primary vaccination of Thai infants, born from HBsAg and HBeAg positive mothers by classifying them in two groups. Half of the subjects enrolled received an interim booster dose at the age of 5and the other half of the subjects enrolled did not. The result indicted that the percentage of subjects with anti-HBs antibody concentrations >10 mIU/mL in the boosted and unboosted groups at 20 year time point was 84.2% and 44% respectively (Poovorawan et al., 2012).

A study conducted in India on Impact of hepatitis B immunization among the Nicobarese tribe - antibody titers & Seroprotection five years after vaccination indicates among those who received three doses of vaccination, 85.9% had anti-HBs antibody levels of $\geq$10 mIU/mL, indicating seroprotection. The difference in the seroprotection rates among those who received three doses of vaccination (85.9%) and those who received less than three doses (58.3%) was significant. Although about 15% of the vaccinated persons lost seroprotection by the end of the third year, no further loss in seroprotection was observed between the third year and the fifth year (Sugunan et al., 2014).

In a community based study conducted in Brazil on Antibody levels in children after 10 years of vaccination against hepatitis B also revealed that about 54.8% had protective levels of anti-HBs after 10 years of vaccination (Fagundes et al., 2012). Antibody levels against hepatitis B virus

after hepatitis B vaccination in Egyptian diabetic children and adolescents also showed that 60% of them in healthy children had a protective antibody (≥ 10 IU/L) whereas the level of antibody in diabetic children was only 30.2% (Elrashidy et al., 2013). In another study on Long-term protection of hepatitis B vaccination among Egyptian children showed that 81% and 48% of children had ≥ 10 mIU/ml after 5 and 10 years of vaccination respectively (El-Sayed et al., 2011).

A study conducted in Gambia on vaccinated and unvaccinated young adults born during 1986–90 showed that full infant HBV vaccination does protect strongly against chronic HBV infection with 94% vaccine efficacy and there was less prevalence of HBV infection in vaccinated groups which was 0.8% but it was 12.4% in unvaccinated groups (Peto et al., 2014). In another Observational Study conducted on Vaccine Efficacy 24 Years after the Start of Hepatitis B Vaccination in Two Gambian Villages revealed that there was 95.1% vaccine efficacy against chronic infection with hepatitis B virus (Mendy et al., 2013).

3. Objective of the study

3.1General objective

To determine antibody level against HBV after hepatitis B vaccination and sero-prevalence of HBV in children in Addis Ababa, Ethiopia.

3.2 Specific objective

- To assess the concentration of antibody after HBV vaccination
- To determine the response level of the vaccine in partially and fully vaccinated children
- To determine sero-prevalence of HBV infection among vaccinated and unvaccinated children
- To assess the risk factors that affects the response level of the vaccine

4. Materials and methods

4.1 Study design

A cross sectional study design was conducted to determine the antibody level against HBV infection after HBV vaccination and sero-prevalence of HBV in children.

4.2 Study area

The study was conducted in Addis Ababa which is a capital city of Ethiopia with a population of 3,384,569 according to the 2007 population census with annual growth rate of 3.8%. The City is divided into 10 Sub-cities. Kolfe keranio is the largest and most populated sub city. 24% its population is under the age of fifteen. The town has latitude and longitude of 9.022736 N and 38.746799E respectively with an elevation of 2,326 meters above sea level. All of the population is urban inhabitants.

There are 15 public and 23 private hospitals in the city. Of which 5 are owned by Federal Ministry of Health, 5 by Addis Ababa Regional Health Bureau, 2 by Non-Governmental Organization, 3 by Defense Public Hospital, and the rest 23 are private hospitals based on Addis Ababa Health Bureau data. Almost all health facilities in the city deliver pentavalent 3 vaccine and according to the findings of the EPI cluster survey in 2014, the immunization coverage in Addis Ababa was 96.4%.

4.3 Study period

The study was conducted from April 2016 to May 2017.

4.4 Source and study populations

4.4.1 Source population

Children between the age of 5 and 8 who were living in Addis Ababa, Ethiopia

4.4.2 Study population

Children between the age of 5 and 8 who were vaccinated or unvaccinated against HBV

4.4.3 Sample size determination and Sampling technique

The required samples sizes were calculated using 50% of prevalence in vaccine response using the following formula

$$n = \frac{\left(\frac{Z\alpha}{2}\right)^2 pq}{d^2}$$

$$\text{where, } n = \text{sample size}$$

$$Z = \text{confidence interval}$$

$$p = \text{proportion}$$

$$q = 1 - p$$

Using confidence interval of 95%, Z=1.96 and d=0.5

$$N = \frac{(1.96)^2 \text{ X } 0.5 \text{ X} 0.5}{(0.5)^2}$$

=385

Total number of the sample with 10% non-respondents rate should be 424.

The overall sample size of the study participants was 459. A multi stage probability sampling techniques was applied to get the number of children within the age group. We applied lottery method to take three sub cities among ten sub cities of Addis Ababa namely Gulele, Lideta and Kirkos and from these sub cities we took eight woredas by using lottery method. From each woreda 50-60 children were selected randomly. Our recruitment strategy was door to door with the assistance of health extension workers.

4.4.4 Inclusion and exclusion criteria

4.4.4.1 Inclusion criteria

Children were included if:-

- They were apparently healthy

- Their parents were volunteered for the study
- Aged between 5-8 years
- Vaccinated or unvaccinated against HBV

4.4.4.2 Exclusion criteria

- Children whose parents were not willing to participate in the study
- Children that are not apparently healthy

4.5 Study variable

4.5.1 Dependent variable

- Level of antibody against HBV
- Sero-prevalence of HBV infection

4.5.2 Independent variable

- Age
- Sex
- Vaccination status
- Number of vaccination
- Previous disease status of the child

4.6 Data collection

4.6.1 Data collection tools

Structured questionnaire was employed to collect all relevant information about the study participants. Socio demographic data of the mother and child, risk factors and other relevant information was collected by principal investigator using the questionnaire.

4.6.2 Data and Blood collection procedure

After the participant's family agreed to take part in the study, sign an informed consent form, baseline information was collected, and the participant's family was asked to complete a brief questionnaire and was collected and organized by the principal investigator. The vaccination

card was observed for those who had the card and those who haven't had a vaccination card, we used recall method by questioning if they vaccinated their child or not. Three to four ml of blood was collected from study participants. Then serum was separated by centrifuging at 3000 rpm for 3 min and stored at -20˚C until processed. Anti-HBsAg, anti-HBc and HBsAg detection was performed by using Enzyme-linked Immunosorbent assay (ELISA).

4.6.3 Enzyme-linked Immunosorbent assay (ELISA)

The collected serum was used to detect HBsAg, anti-HBcAg and anti-HBsAg using ELISA. Samples which were not appropriate or not sufficient were not analyzed. ELSIA was done according to the manufacturer's instruction (BIO-RAD, Monolisa test kits, France). The test kits have a high sensitivity and specificity. For the detection of anti HBsAg 99.2 % and 99.4%, for anti HBcAb 99.53% and 99.5% and for HBsAg it had 100% and 99.28% sensitivity and specificity was obtained.

The assay is a one-step enzyme immunoassay based on the principle of the "sandwich" type using antigens/ monoclonal antibodies bounded to the well that detects the antibodies or antigens in the sample respectively. The results of each assays was determined based on WHO Hepatitis B panel interpretation (Annex V)

Procedure for Enzyme-Linked Immunosorbent Assay

First we need to bring all reagents and specimens to room temperature. And then we have to prepare conjugate working solution and diluted washing Solution by using the manufacturer's instruction. After that we dilute the specimens, calibrators and controls with the Specimen diluent. For each tests we have different diluent and proportions and add a conjugate (Ab or Ag coated with peroxidase). Then incubate the plates at 37 ± 2°C at least 30 minutes (the time is different for each tests) and wash the plate after the incubation. Add quickly substrate (tetramethyl benzidine (TMB)) and put it in the dark place for 30 minutes. Then we wash the plate at least 5 times and quickly add a stopping Solution (1N sulphuric acid solution) and homogenize the reaction mixture. Finally, wipe the plate bottom and read at the optical density at 450/620-700 nm at least 4 minutes after stopping solution addition and within 30 minutes of stopping the reaction.

4.7 Data Quality Assurance

During data collection all important variables were included in the questionnaire and redundancy of the questions and study participants was avoided. Every laboratory method has been performed accordance with standard operating procedure of that specific method. Positive and Negative controls were done during each tests performed.

4.8 Data analysis and interpretation

We used SPSS Software statistical package version 20.0 to analyze the data. The association was determined by chi-square. The significant of the value obtained in the study is determined based on p-values. P-values less than 0.05 were considered as statistically significant. Logistic regression analyses were used to see the association of different variables. The result figured out by using percentages, chart and graph.

4.9 Ethical clearance

Ethical clearance was obtained from the Department of Microbiology, Immunology, and Parasitology ethical review committee (DERC) and Armauer Hansen Research Institute (AHRI). Ethical clearance and letter from Addis Ababa Health Bureau and a letter from each sub city Health bureau was also obtained. Written and verbal informed consent from participant's family was obtained. Confidentiality of the participants was guaranteed and those participants who didn't respond to the vaccine and unvaccinated children will be revaccinated or vaccinated, respectively.

5. Results

5.1 Socio-demographic characteristics

A total of 459 children aged 5-8 years were included in the study. Due to sample rejection criteria and missed samples, nine of them were excluded and 450(98%) samples were analyzed. Four samples were hemolysed, insufficient and the rest five samples were not collected or lost. The mean age of the study participants were 7±1 (SD) years. Among these, 244 (54.2%) were males and 383 (85.1%) were vaccinated. Sixty six (14.7%) children had history of any kind of disease and 3(0.7%) were HIV positive.

Table1: Socio demographic characteristics of children between the age of 5 and 8 years old in Addis Ababa Ethiopia

Socio demographic characteristics	**Categories**	**Number (n)**	**Percent (%)**
Sex	Male	204	54.2
	Female	206	45.8
Age	5	95	21.1
	6	130	28.9
	7	124	27.6
	8	101	22.4
Place of birth	Urban	422	93.8
	Rural	28	6.2
Sub city	Gulele	45	10
	Kirkos	92	20.4
	Lideta	313	69.5
History of previous disease status	Yes	66	14.7
	No	384	85.3
Disease type	Infectious	49	10.9
	Non Infectious	15	3.3
	Immunocompromised	3	0.7
	None	383	85.1
Vaccination status	Vaccinated for HBV	383	85.1
	Non vaccinated for HBV	67	14.9

5.2 Mother's base line information

The mean age of the mothers was 26.26±5.52 SD years and most of them (58%) didn't have an occupation. Around sixty six (65.5%) of mothers took below 30 minutes to travel from their home to health institution.

Table 2: Socio demographic characteristics of children's mothers, in Addis Ababa Ethiopia

Socio demographic characteristics	Categories	Number (n)	Percent (%)
Age	16-20	65	14.4
	21-25	169	37.6
	26-30	114	25.3
	31-35	80	17.8
	36-40	14	3.1
	41-45	8	1.8
Educational Status	Below certificate	398	88.4
	Certificate	30	6.7
	Diploma	18	4.0
	Degree and above	4	0.9
Occupation	Government	48	10.7
	Private	141	31.3
	Housewives	261	58.0
Institution Delivered	Home	33	7.3
	Private hospital/clinic	31	6.9
	Health center	190	42.2
	Government hospital	196	43.6
Number of child	One	82	18.2
	Two	175	38.9
	Three	118	26.2
	Four and above	75	16.7

5.3 Vaccination status

The overall vaccinated children were 383(85.1%) and the rest 67(14.9%) were unvaccinated. The number of vaccinated females was more than vaccinated males and it was 83.6 % of males and 86.9 % of females were vaccinated and 99.2 % (380) finished the three dose vaccination. Only 3 of them (0.8%) took two dose of the vaccine. There was no one who took one dose of the vaccine.

Table3: The coverage of hepatitis B vaccine among male and female children between the age of 5 and 8 years old in Addis Ababa Ethiopia.

Status	**Male**		**Female**		**Total**		**P value**
	Number (n)	Percent (%)	Number (n)	Percent (%)	Number (n)	Percent (%)	0.329
Vaccinated	204	83.6	179	86.9	383	85.1	
Non vaccinated	40	16.4	27	13.1	67	14.9	
Total	244	100	206	100	450	100	

5.4 Concentration of Anti HBsAg

The overall Anti HBsAg concentration was > 10mIU/ml protective antibody in 214 (47.6%) children. From the total of 383 vaccinated children, 208 (54.3%) had an Anti HBsAg concentration of > 10mIU/ml which are called responders while the remaining 47.3% were non responders and from 67 unvaccinated children 6 (9%) had an Anti HBsAg > 10mIU/ml and 91% had Anti HBsAg < 10mIU/ml. Vaccination for HBV has significantly associated with the concentration of Anti HBsAg (p=0.000)

Table 4: The level of Anti-Hbs antibody among vaccinated and non-vaccinated children in Addis Ababa Ethiopia

Vaccination status		<10mIU/ml	10-100mIU/ml	100-400mIU/ml	400-1000mIU/ml	>1000mIU/ml	Total
Yes	No	175	157	41	8	2	383
	%	45.7	41.0	10.7	2.1	0.5	100.0
No	No	61	6	0	0	0	67
	%	91.0	9.0	0.0	0.0	0.0	100.0
Total	No	236	163	41	8	2	450
	%	52.4	36.2	9.1	1.8	0.4	100.0

From the 208 responded children against the HBV vaccine (concentration of Anti HBsAg > 10mIU/ml), 157(75.48%) had a concentration of Anti HBsAg between 10mIU/ml and 100mIU/ml, 41(19.71%) had concentration of Anti HBsAg between 100mIU/ml and 400mIU/ml, 8(3.84%) had concentration of Anti HBsAg between 400mIU/ml and 1000mIU/ml and 2(0.97%) had concentration of Anti HBsAg greater than 1000mIU/ml. But in all 6 (100%) unvaccinated children, the concentration of Anti HBsAg was between 10mIU/ml and 100mIU/ml. The response level of the vaccine have a significant association with vaccination status (p =<0.005).

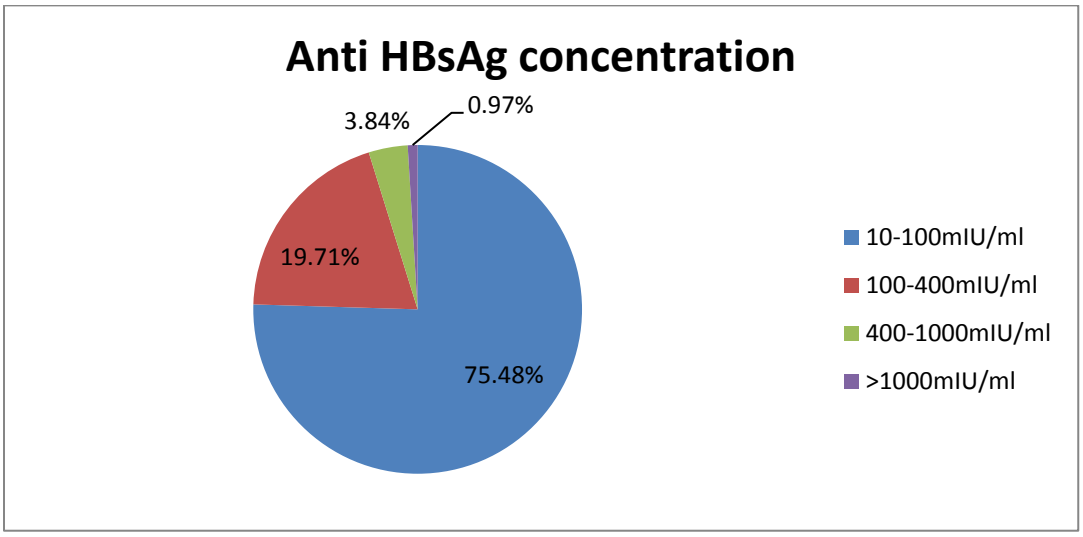

Figure 2: Antibody response against Hepatitis B vaccine in children between 5-8 years, Addis Ababa Ethiopia

Sex Vs Anti HBsAg concentration

From all vaccinated children, 208(54.3%) had Anti HBsAg concentration > 10mIU/ml. From these 110(52.9%) were male children. The response rates to the vaccine in male and female children were different in which female children have a slight higher protective antibody than male children. From 204 vaccinated male children only 110 (53.9%) and among 179 vaccinated female children, 98(54.7%) responded for the vaccination. The rest 46.1% male and 45.3% female children fail to respond for HBV vaccination. There is no statistical significance between sexes (p =0.433).

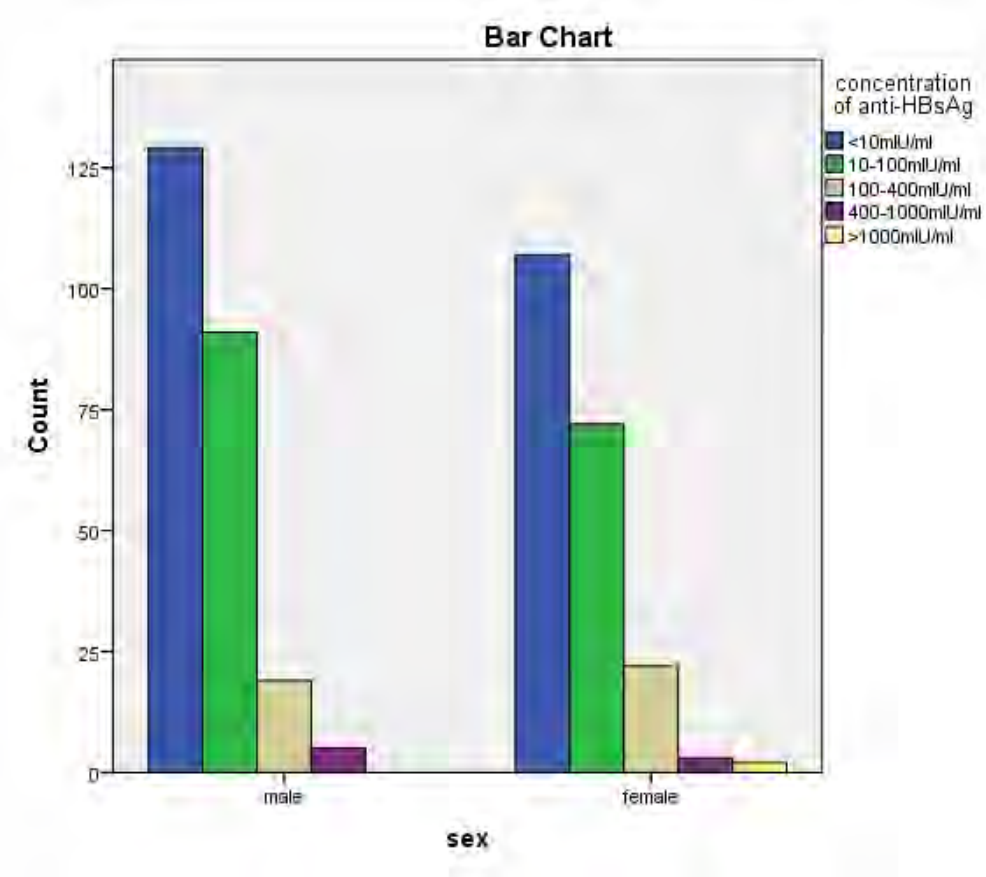

Figure 3: Antibody response to Hepatitis B virus in male and female children between the age of 5 and 8 in Addis Ababa Ethiopia

Age Vs Anti HBsAg concentration

The effective response level to the vaccine within the different age groups was 52.6%, 60%, 43.5% and 37.1% at the age of 5, 6, 7 and 8 respectively. There is a significant association between age group and the concentration of anti HBsAg (p=0.001) and the level of antibody concentration decreases as the age of the children decreases. And from three HIV infected patients, two of them were vaccinated and they didn't respond for the vaccination.

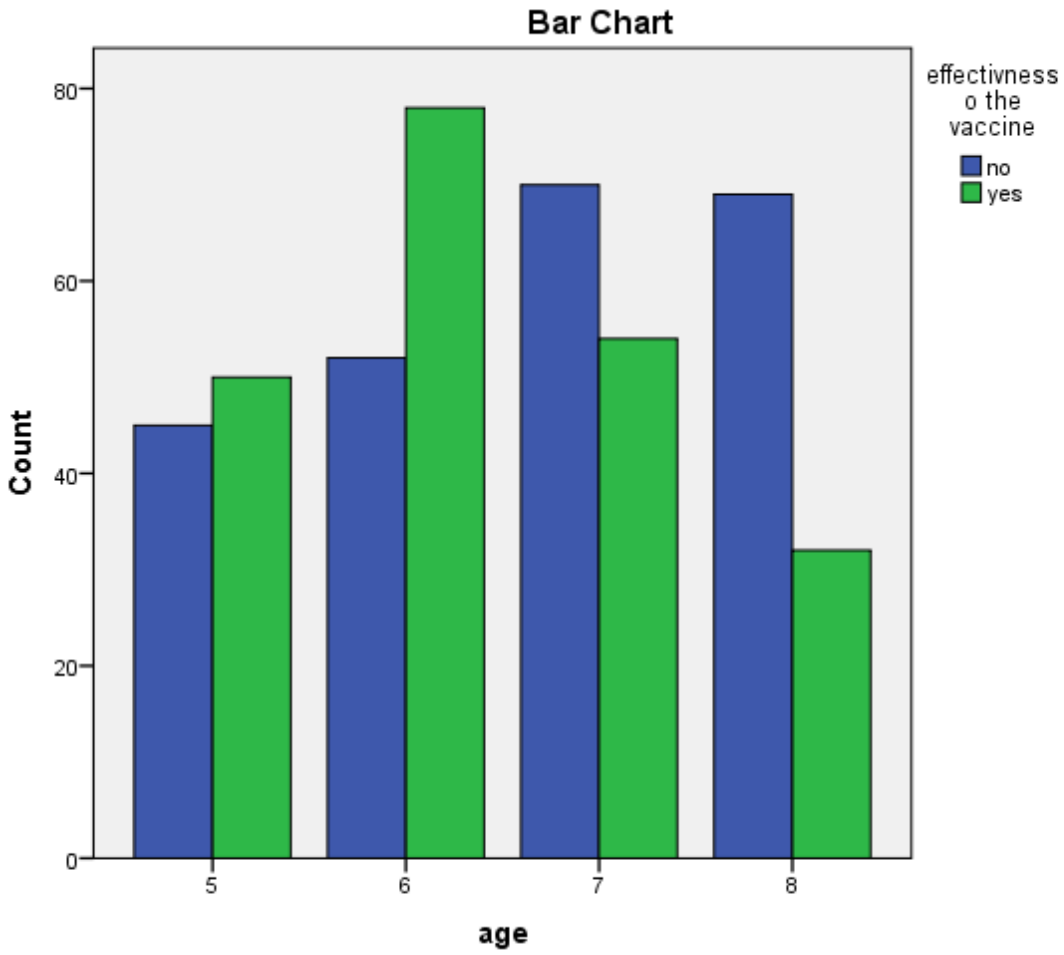

Figure 4: Protective antibody level against Hepatitis B vaccine in children between the age of 5 and 8 in Addis Ababa, Ethiopia.

Number of Dose of the vaccine Vs concentration of anti HBsAg

All (100%) of the children who took only two dose of the vaccine didn't respond whereas children who took all three doses of the vaccine, 54.7% of them were responded to the vaccine with a concentration of >10 mIU/ml and the rest 45.3% didn't respond.

5.5 Sero-prevalence of hepatitis B and risk factors

From a total of 450 children only two (0.4%) were positive for HBsAg and 25(5.6%) were positive for anti HBc. Both HBsAg and anti HBc were positive in 1(0.2%) child however, the other child was negative for anti HBc. Both children who are positive for HBsAg were female, 5 year old, non-diseased and vaccinated children. All factors are not significantly associated with sero prevalence of HBsAg

Among anti HBc positive children, 15(6.1%) were male and 10(4.9%) were female. There is no significant association between sex and anti HBcAb with a p value of 0.551. Ten (2.6%) of anti HBc positive children were also vaccinated children. From non-vaccinated children, 15 (22.4%) were anti HBc positive. There is a negative correlation and a significant association between vaccination status and anti HBc positive (p=0.000).

The sero-prevalence of Anti HBc in each age was also determined and 4.2%, 2.3%, 4% and 12.9% was observed in 5,6,7 and 8 years of the children, respectively. Age is significantly associated with the sero-prevalence (p=0.03).

Table 5 : Sero prevalence of Anti HBc in children between the age of 5 and 8 in Addis Ababa Ethiopia.

Variables	Categories	Number (n)	Percent (%)	P value
Sex	Male	15(244)	6.1	0.551
	Female	10(196)	4.9	
Vaccination status	Vaccinated	10(383)	2.6	0.000
	Non vaccinated	15(67)	22.4	
Age	5	4(95)	4.2	0.03
	6	3(130)	2.3	
	7	5(124)	4.0	
	8	13(101)	12.9	
Previous disease status	Yes	5(66)	7.6	0.438
	No	20(384)	5.2	

In multivariate logistic regression analysis, variables like distance from home to vaccination center, income, birth institution and family history of hepatitis doesn't have any significant association for the response level of the vaccine. But mother's education level, weight of the child had a significant association with a p =0.03 and p=0.008 respectively.

6. Discussion

This was the first study to evaluate the long-term antibody persistence among 5-8 years old children vaccinated against HBV in Addis Ababa, Ethiopia. In this study, we examined the persistence of anti- HBsAg levels in children between the ages of 5 to 8 years which were born after the implementation of national HBV vaccination program in 2007. The vaccination coverage in our study is 85.1% and from vaccinated children we have found that 54.3% of children had protective levels of anti-HBs antibody with decrease of protection level as their age increases.

HBV vaccine immunization started in 2007 in Ethiopia. Since then the vaccine coverage throughout the country is expanding. The vaccine coverage in our study also shows how the immunization coverage looks like before 5 years. The outcome of our study was comparable with the estimated 86% of national coverage of Hepatitis B vaccination in our country within the year of 2015 (WHO and UNICEF, 2015).

Moreover, this result is comparable with other studies conducted in different countries. In Nepal, also they found that 86% of children were vaccinated (Upreti et al. 2014). This similarity could be due to the similarity in sample population, methodology and the time in which the initiation of immunization could be the possible reasons. But in Yemen, 69.9% vaccine coverage was obtained and male children had higher coverage than female children (Al-Shamahy et al., 2011). In their study the vaccine coverage was lower than ours (69.9% vs. 85.1%). This difference in vaccine coverage may occur due to availability of health institutions (lesser health facilities in the country may result less vaccine coverage), year of immunization started (Yemen started early 2000) and sampling method difference (they took children in the age group of 1-10).

The primary goal of the HBV vaccine is to induce the immune system and have an effective antibody response against the HBV. Anti HBsAg is effective if the concentration is greater than 10mIU/ml which is important to defend the virus. The vaccine response below this value is not sufficient to neutralize the virus and the person is called non responder. In our study we found that 54.3% vaccine response was observed and when we see the response level of antibody against Hepatitis B vaccine in other studies, we may find that a similarity and also a difference in different areas.

A study conducted in Yemen revealed that 54.8 % of children responded to the vaccine with a concentration of ≥10mIU/ml. The level of antibody also decreases as the age increases (Al-Shamahy et al., 2011). In Iran also they reported that protection level against HBV vaccine was determined in 56.3% among children within the age group of 6-10 years with higher respondents of male than female children (Norouzirad et al., 2014). This similarity could be attributed to the similarity in socio economic status, similarity in the initiation of immunization and vaccine type.

In some areas however, higher antibody response was reported in comparison with our studies. A study conducted in Iran showed a higher response level within the age of 5 to 10 years. In their study 78% of children did have protective antibody with the decrement of concentration as age of the child increases. Male children had higher protection level than female children (Rezaei et al., 2014). In the same country (Yazdanpanah et al., 20), on the age group of 5 to 7 years revealed, 84 % had protective antibody and a study on children under the age of 7 years showed 87.6% of the children had a protective antibody with Anti HBsAg concentration ≥10mIU/ml (Esmaili and Seyedkolal, 2003).

A study that was conducted in Spain also revealed that 85% of children at the age of seven responded to the vaccine (Gonzalez et al., 1993). This discrepancy may be attributed largely to differences in the age because they took only seven year children but ours was five to eight years, the dosage in which higher dose of the vaccine will increase the response level, route(intradermal vs. intramuscular), intradermal route is effective for better response and nature of vaccine (i.e., plasma derived or recombinant). In a study conducted in Taiwan on recombinant and plasma derived vaccine showed the difference in response level to the vaccine which was 82% and 96% respectively (West and Calandra, 1996).

In contrast to our study, lower response level was detected in other studies. A study in Egypt resulted a low response level against the vaccine and they reported that 39. 3 % of children within the age of 6-12 years had a concentration of ≥ 10mIU/ml (Shaaban et al., 2007). The low response level in their study could be the wider age group than ours, sample size and sampling technique was also different. They took samples from children attending the health insurance clinic. They used children who came for medical advice in government clinic.

In Iran also only 47.9% children responded to the vaccine effectively with a higher response level in female children than male children (Jafarzadeh et al., 2006). The decrement in vaccine response could be the age difference in which they have done it in the age groups of 10-11 years. A lower response level was also observed in the same country found out only 48 % (Rafizade et al., 2004) and in another study 30 % (Aghakhani et al., 2011) of the children responded to the vaccine. This difference may occurred due to sampling difference, type of vaccine used during study time and dose of the vaccine.

In US also lower response level was observed (Williams et al., 2003) and it was only 41 % of the children who responded to the vaccine. In Italy also the study resulted that any of the children participated in the study could not able to respond to the vaccine after 5 years of vaccination (Resti et al., 1993). The low response level in the above countries were seen due to difference in schedule of immunization, dosage of the vaccine, route and type of vaccination could be the possible factors.

In our study the concentration of anti HBsAg between 10-100mIU/ml and 100-400mIU/ml was 75.48% and 19.71% respectively with decreased number of responders for higher concentration. When we compared our result to a study done in Italy, 10-100mIU/ml and 100-400mIU/ml anti HBsAg concentrations was found in 57.9% and 26.3% vaccinated children respectively (Coppola et al., 2015). Thirty six percent responses were seen with a concentration of 10-100mIU/ml in Brazil (Fagundes et al., 2012).The rationale for this difference could be variation in the sample population, vaccine type or vaccine dose.

Sero prevalence of HBsAg among vaccinated children is different among different countries ranging from 0-2.5%. In Yemen, the Sero prevalence of HBsAg was 1.8% (Al-Shamahy et al., 2011), 0.13% in Nepal (Upreti et al., 2014), 2.3% in Papua New Guinea (Kitau et al., 2015), 0.77% (Huang et al., 2015) and 2.5% in China (Ji et al., 2014). But in our study 0.4% of HBsAg seropositivity was observed. This difference in Sero prevalence of HBV infection could be due to the occurrence of infection before vaccination was given, perinatal transmission (Salama et al., 2015), time of the dosage (Kitau et al., 2015) and difference in vaccination coverage and vaccination failure. Vaccination failure occurs mainly due to the mutation of Hepatitis B virus in Pre S and S region (Tajiri and Shimizu, 2015, and Romanò et al., 2015).

In our study, the Sero prevalence of HBsAg was higher in females than males whereas in a community based Sero epidemiological survey conducted in Addis Ababa, it has been reported that 7% of the population was HBsAg positive and the infection was higher in males than females (Abebe et al., 2003). This discrepancy may happen due to age difference, difference in population type and study type. In our study 0.4% of the children were positive for HBsAg but in Sekota, Ethiopia the Sero prevalence in the age group of 0-14 was 2.3 % during the study period of 2012-2014 with the decrease of Sero prevalence from year to year (Gebreegziabher et al., 2016). The possible explanation for this might be the difference in demographic characteristics of the study population, vaccination status and vaccine coverage. If vaccine coverage was lower in the specific area that may lead to a higher sero-prevalence

In our study the Sero prevalence of Anti HBc is 5.6% which is lower than a study conducted in Gambia that showed 17.7% of children who were vaccinated were positive against Anti HBc antibody (Van der Sande et al., 2007) and another study conducted in two cities Keneba and Manduar in Gambia also showed that 6.1% and 5.2 % sero prevalence of Anti HBc in the age group of 5 to 9 years (Van der Sande et al., 2006). In China, 14.1% of children between the ages of 5 to 9 years were Sero positive (Ji et al., 2014) and 7.5% of children were positive for anti-HBc antibody in Iran (Jafarzadeh et al., 2006). The reason for these discrepancies in Sero prevalence could be attributed to the age difference, race, prevalence of HBV, and response level.

In our study, Socio economic factors like residence, family size and sex did not affect the effectiveness or response level of the vaccine. This is similar with the study conducted in Italy (Zanetti et al., 2005), but age and number of doses have an association with the response level of the HB vaccine (Aghakhani et al., 2011; Gerlich, 2013 and Wu et al., 1999).

Because of the progressive decline of anti-HBs and increased possibility of development of new HBV infections, some investigators recommended the use of a booster dose of vaccine for adolescents to raise the immunity rate against HBV during adulthood (Aghakhani et al., 2011; Coppola et al., 2015). Our results were also suggestive of the use of a booster vaccination although the proper timing of HBV booster administration is uncertain.

7. Limitation of the study

All children didn't come with their vaccination card.

Some serological markers like HBeAg were not examined.

There was unequal number of vaccinated and non-vaccinated children.

The study design did not account for occult infections.

8. Conclusion

This study showed 54.3% of vaccine efficacy with vaccine coverage of 85.1%. The study revealed a low protective level of antibody against HBV vaccine. But the vaccine coverage is similar with that the government estimation in 2014. The findings also discovered a decline in anti-HBsAg concentration over the time after vaccination. Other serological markers that were examined in this study for Hepatitis B virus were low. It was 0.4% for HBsAg and 5.6% of Anti HBc Sero prevalence were detected. These figures can possibly illustrate the Sero prevalence of HBV is declining through time to time in the country.

Sex, previous disease status and other variables which were determined in our study didn't affect the response level and Sero prevalence of HBV where as Age, vaccination status and number of doses was significantly associated with the response level of the vaccine. But all factors for HBsAg and all factors except age and vaccination status for Anti HBc were not significantly associated.

Persistence of anti-HBs antibodies is necessary for the long-term protection against hepatitis B virus infection. Even if different factors can contribute for the low response level of the vaccine, we need to follow up children after vaccination, especially after the infant vaccinated. Our study also revealed that age influences their response level and the concentration of antibody decreases as the child age increases. Full vaccination is also mandatory to get the maximum response. Those partially vaccinated children didn't respond to the vaccination well.

Nowadays, mutant Hepatitis B viruses are spreading globally. Vaccination regime and vaccine type should also be considered when we administer the vaccine to the child. High Sero prevalence of Anti HBc in vaccinated children may indicate the occult HBV infection which is now days a concern for everyone and we need to address this issue too.

9. Recommendations

Ethiopian program of hepatitis B vaccination has resulted in substantial progress towards the prevention and control of Hepatitis B infection. Ten years after the implementation of the program, the population under 10 years of age protected against infection with the Hepatitis B virus. However, lower response level to the vaccine is our concern that needs further analysis particularly in determining serotype and mutational changes of HBV for effective management and control of the disease.

Last but not least, we recommend that the time in which the child started vaccine should be reconsidered. The first dose of the vaccine was given in 6 weeks; therefore an infection could be introduced during this period. If it is possible, it should be within 48 hours of delivery which is done in some countries. And for those who didn't respond for the vaccine, booster dose should be given to enhance the immunological response to the vaccine.

Finally, further studies should be undertaken to determine the duration of antibody response against HBV vaccine and follow up is needed for those children who are administered with booster doses.

10. References

Abebe A, Nokes DJ, Dejene A, Enquselassie F, Messele T, Cutts FT. (2003). Sero-epidemiology of hepatitis B virus in Addis Ababa, Ethiopia: transmission patterns and vaccine control. *Epidemiol Infect*, 131, 757–770.

Abushady EA, Gameel MA, Klena JD, Ahmed SF, Abdel-Wahab KS, Fahmy SM. (2011). HBVvaccine efficacy and detection and genotyping of vaccineé asymptomatic breakthrough HBV infection in Egypt. *World J Hepatol,* 3(6), 147-156.

Aghakhani A, Banifazl M, Izadi N, McFarland W, Sofian M, Khadem-Sadegh A, Pournasiri Z, Foroughi M, Eslamifar A, Ramezani A, (2011). Persistence of antibody to hepatitis B surface antigen among vaccinated children in a low hepatitis B virus endemic area. *World J Pediatri*, *7*(4), 358-360.

Alborzi AM, Kiani Ghalesardi O, Bamdad T, Pourfathollah AA, Jalalifar MA, Shahjahani M, Kazemi Arababadi M, Dehghani Fard A, Saki N, (2013). Occult Hepatitis B Infection and Its Role in Blood Safety: a Review. *Iranian J blood and cancer*, *5*(2), 61-75.

Al-Shamahy HA, Hanash SH, Rabbad IA, Al-Madhaji NM, Naser SM. (2011). Hepatitis B Vaccine Coverage and the immune Response in Children under ten years old in Sana'a, Yemen: *SQU Med J,* 11(1), 77-82.

Awole M, Gebre-Selassie S. (2005). Sero-prevalence of HBsAg and its risk factors among pregnant women in Jimma, Southwest Ethiopia *Ethiop.J.Healh Dev,* 19(1), 45-50

Beasley RP, Lee GCY, Roan CH, Hwang LY, Lan CC, Huang FY, Chen CL, (1984). Prevention of Perinatally Transmitted Hepatitis B Virus Infections with Hepatitis B Immune Globulin and Hepatitis B Vaccine. *Obst & Gyn Survey*, *39*(6), 367-368.

Bertoletti A, Kennedy PT, (2015). The immune tolerant phase of chronic HBV infection: new perspectives on an old concept. *Cell & molecular immune, 12*(3), 258-263.

Brechot C, Thiers V, Kremsdorf D, Nalpas B, Pol S, Paterlini-Brechot P. (2001). Persistent hepatitis B virus infection in subjects without hepatitis B surface antigen: clinically significant or purely "occult"? *Hepatol*, 34, 194-203.

Brunetto MR, Oliveri F, Colombatto P, Moriconi F, Ciccorossi, P, Coco B, Romagnoli V, Cherubini B, Moscato G, Maina AM, Cavallone D. (2010). Hepatitis B surface antigen

serum levels help to distinguish active from inactive hepatitis B virus genotype D carriers. *Gastroenter, 139*(2), 483-490.

Chemin I, Vermot-Desroches C, Baginski I, Saurin JC, Laurent F, Zoulim F, Bernaud J, Lamelin J.P, Hantz O, Rigal D. (1991). Selective detection of human hepatitis B virus surface and core antigens in some peripheral blood mononuclear cell subsets by flow cytometry. *J clin & lab immune, 38*(2), 63-71.

Chen ST, Chang MH. (2010). Epidemiology and natural history of hepatitis B in children. *Viral Hepat in Child,* 13-28.

Ciupe SM, Ribeiro RM, Perelson AS. (2014). Antibody Responses during Hepatitis B Viral Infection. *PLoS Comput Biol,* 10(7), 1-16.

Coppola N, Corvino AR, De Pascalis S, Signoriello G, Di Fiore E, Nienhaus A, Sagnelli E, Lamberti M. (2015). The long-term immunogenicity of recombinant hepatitis B virus (HBV) vaccine: contribution of universal HBV vaccination in Italy. *BMC infectious diseases*, *15*(1), 149.

Elrashidy H, Elbahrawy A, El-Didamony G, Mostafa M, George NM, Elwassief A, Saeid Mohamed AG, Elmestikawy A, Morsy, MH, Hashim A, Abdelbasseer MA, (2013). Antibody levels against hepatitis B virus after hepatitis B vaccination in Egyptian diabetic children and adolescents. *Human vacc & immunotherapeut*, *9*(9), 2002-2006.

El-Sayed B, El-Guindi M, El-Shaarawy A, Salama EI, Sobhy GA. (2011). Long-term protection of hepatitis B vaccination among Egyptian children. *Egypt J Pediatr Allergy Immunol,* 9(1), 35-40.

Esmaili M, Seyedkolal F. (2003). Evaluation of anti HBS levels in vaccinated children against Hepatitis B, Amirkola Hospital, Babol. *KAUMS J FEYZ,* 6(4), 45-49.

Ethiopia. (2015). WHO and UNICEF estimate of immunization coverage: 2015 revision, Available on *URL:http//www.data.unicef.org.* Accessed on July 2016

EASL. (2012). EASL Clinical Practice Guidelines: management of chronic hepatitis B virus infection. *J Hepatol,* 57, 167–-185.

Faas H, Van Dooren FH, Litjens N. (2011). Hepatitis B vaccination: effects of diminishing HBV immunity, non response and a review of the vaccination protocol in the Netherlands. *Eras J Med,* 2(1), 34-37.

Fagundes GD, Tabalipa FO and Silva J. (2012). Antibody levels in children after 10 years of vaccination against hepatitis B: a Brazilian community-based study. *Revista da Soci Brasileira de Med Trop,* 45(2), 260-262.

Fattovich G, Stroffolini T, Zagni I, Donato F. (2004). Hepatocellular carcinoma in cirrhosis: incidence and risk factors. *Gastroenterol,* 127, S35–S50.

FMOH. (2010). Ethiopia National Expanded Programme on Immunization. Comprehensive multi-year plan.Available on URL: http*//www.nationalplanningcycles.org.*December, 2011 – 2015.

Franco E, Bagnato B, Marino MG, Meleleo C, Serino L, Zaratti L. (2012). Hepatitis B: Epidemiology and prevention in developing countries. *World J Hepatol,* 4(3), 74-80.

Françoisa G, Dochezb C, Mphahlelec MJ, Burnettd R, VanHala G, Meheus A. (2008). Hepatitis B vaccination in Africa: mission accomplished? *The South Afri J Epid and Infect,* 23 (1), 24-28.

Gabbuti A, Romanò L, Blanc P, Meacci F, Amendola A, Mele A, Mazzotta F Zanetti AR. (2007). Long-term immunogenicity of hepatitis B vaccination in a cohort of Italian healthy adolescents. *Vaccine*, *25*(16), 3129-3132.

Gao S, Duan ZP Coffin CS. (2015). Clinical relevance of hepatitis B virus variants. *World J hepa*, *7*(8), 1086.

Gebreegziabher D, Asfeha GG and Gebreyesus HA. (2016). Sero-prevalence of hepatitis B virus surface antigen (HBsAg) among clients visiting 'Tefera Hailu' memorial hospital, Sekota, Northern Ethiopia. *BMC Infect Dis,* 16, 383.

Gerlich WH, (2013). Medical virology of hepatitis B: how it began and where we are now. *Virol J, 10*(1), 239.

Gold Y, Somech R, Mandel D, Peled Y, Reif S. (2003). Decreased immune response to hepatitis B eight years after routine vaccination in Israel. *Acta Paediatr,* 92, 1158-1162.

Gonzalez ML, Gonzalez JB, Salva F, Lardinois RA. (1993). 7-year follow-up of newborns vaccinated against hepatitis B. *Vaccine,* 11, 1033-1036.

Hassan S, Ziba F. (2007). Antibody titer in Iranian children 6 years after hepatitis B vaccine administration. *Vaccine,* 25, 3511- 3514.

Hoofnagle JH, Doo E, Liang TJ, Fleischer R, Lok AS. (2007). Management of hepatitis B: summary of a clinical research workshop. *Hepatol,* 45(4), 1056–75.

Hou J, Liu Z, Gu F. (2005). Epidemiology and Prevention of Hepatitis B Virus Infection. *International J Med Science,* 2(1), 50-57.

Huang P, Zhu LG, Zhu YF, Yue M, Su J, Zhu FC, Yang HT, Zhang Y, Shen HB, Yu RB Zhai XJ. (2015). Seroepidemiology of hepatitis B virus infection and impact of vaccination. *World J Gastroentero*, *21*(25), 7842.

Hyams KC. (1995). Risks of chronicity following acute hepatitis B virus infection: a review. *Clin Infect Dis,* 20, 992-1000.

Jafarzadeh A, Montazerifar SJ. (2006). Persistence of anti-HBs antibody and immunological memory in children vaccinated with hepatitis B vaccine at birth. *J Ayub Med Coll Abbottabad,* 18, 4-9.

Ji Z, Wang T, Shao Z, Huang D, Wang A, Guo Z, Long Y, Zhang L, Su H, Zhang Q Yan Y, (2014). A population-based study examining hepatitis B virus infection and immunization rates in Northwest China. *PloS one*, *9*(5), e97474.

Jonas MM, Block JM, Haber BA, Karpen SJ, London WT, Murray KF, Narkewicz MR, Rosenthal P, Schwarz KB McMahon BJ. (2010). Treatment of children with chronic hepatitis B virus infection in the United States: patient selection and therapeutic options. *Hepatology*, *52*(6), 2192-2205.

Kitau R, Datta SS, Patel MK, Hennessey K, Wannemuehler K, Sui G Lagani W, (2015). Hepatitis B surface antigen sero-prevalence among children in Papua New Guinea, 2012–2013. *American J TMH*, *92*(3), 501-506.

Komatsu H. (2014). Hepatitis B virus: Where do we stand and what is the next step for eradication? *World J Gastroenterol,* 20(27), 8998-9016.

Kramvis A, Arakawa K, Yu MC, Nogueira R, Stram DO Kew MC. (2008). Relationship of serological subtype, basic core promoter and precore mutations to genotypes/sub genotypes of hepatitis B virus. *J med virol*, *80*(1), 27-46.

Kwon SY, Lee CH. (2011). Epidemiology and prevention of hepatitis B virus infection. *The Korean J Hepatol,* 17, 87-95.

Lavanchy D. (2004). Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J Viral Hepatol,* 11(2), 97–107.

Leonardi S, Praticò AD, Lionetti E, Spina M, Vitaliti G, La Rosa M. (2012). Intramuscular vs intradermal route for hepatitis B booster vaccine in celiac children. *World J Gastroenterol,* 18(40), 5729-5733.

Liao SS, Li RC, Li H, Yang JY, Zeng XJ, Gong J, Wang SS, Li YP, Zhang KL. (1999). Long-term efficacy of plasma-derived hepatitis B vaccine: a 15-year follow-up study among Chinese children. *Vaccine*, *17*(20), 2661-2666.

Liu CJ, Kao JH, Chen PJ, Lai MY Chen DS. (2002). Molecular epidemiology of hepatitis B viral serotypes and genotypes in Taiwan. *JBC*, *9*(2), 166-170.

Lok AS, McMahon BJ. (2007). Chronic hepatitis B. *Hepatol,* 45 (2), 507–39.

Lok AS, McMahon BJ. (2009).Chronic hepatitis B: update 2009. *Hepatol,* 50(3), 661–662.

Mbaawuaga EM, Iroegbu CU, Anthony Chibuogwu Ike AC, Jombo GTA. (2014). Studies on prevalence, co-infection and associated risk factors of hepatitis B virus (HBV) and Human immunodeficiency virus (HIV) in Benue State, Nigeria. *SJPH,* 2(6), 569-576.

McMahon BJ. (2004). The natural history of chronic hepatitis B virus infection. *Seminar in Liver Disease,* 24 (1), 17–21.

McMahon BJ, Dentinger CM, Bruden D, Zanis C, Peters H, Hurlburt D, Bulkow L, Fiore AE, Bell BP Hennessy TW. (2009). Antibody levels and protection after hepatitis B vaccine: results of a 22-year follow-up study and response to a booster dose. *J infect dis*, *200*(9), 1390-1396.

Mendy M, Peterson I, Hossin S, Peto T, Jobarteh ML, Jeng-Barry A, Sidibeh M, Jatta A., Moore SE, Hall AJ Whittle H. (2013). Observational study of vaccine efficacy 24 years after the start of hepatitis B vaccination in two Gambian villages: no need for a booster dose. *PLoS One*, *8*(3), e58029.

Mphahlele MJ, François G, Kew M, Hoosen A, Meheus A. (2002). Hepatitis B virus infection in eastern and southern Africa: epidemiology, clinical aspects and prevention. *South Afr J Epidemiol Infect,* 17(l), 1-66.

Negero A, Sisay Z, Medhin G. (2001). Prevalence of Hepatitis B surface antigen (HBsAg) among visitors of Shashemene General Hospital voluntary counseling and testing center. *BMC Research Notes,* 4, 35.

Norouzirad R, Shakurnia AH, Assarehzadegan MA, Serajian A, Khabazkhoob M. (2014). Serum Levels of Anti-Hepatitis B Surface Antibody among Vaccinated Population Aged 1 to 18 Years in Ahvaz City Southwest of Iran. *Hepat Mon,* 14(1), 1-5.

Peto TJ, Mendy ME, Lowe Y, Webb EL, Whittle HC and Hall AJ. (2014). Efficacy and effectiveness of infant vaccination against chronic hepatitis B in the Gambia Hepatitis Intervention Study (1986–90) and in the nationwide immunization program. *BMC Infect Dis,* 14, 7.

Pontisso P, Vidalino L, Quarta S, Gatta A. (2008). Biological and clinical implications of HBV infection in peripheral blood mononuclear cells. *Autoimmun Rev,* 8, 13-17.

Poovorawan Y, Chongsrisawat V, Theamboonlers A, Leroux-Roels G, Crasta PD and Hardt K. (2012). Persistence and immune memory to hepatitis B vaccine 20 years after primary vaccination of Thai infants, born to HBsAg and HBeAg positive mothers. *Human Vacc & Immuno,* 8(7), 896–904.

Qin B, Zhou G, Dong X. (2016). Epidemiology, Prevention and Control of Hepatitis B virus Infection in China. *RRJMB,* 5 (2), 115-127.

Qu C, Chen T, Fan C, Zhan Q, Wang Y, Lu J, Lu LL, Ni Z, Huang F, Yao H Zhu J. (2014). Efficacy of neonatal HBV vaccination on liver cancer and other liver diseases over 30-year follow-up of the Qidong hepatitis B intervention study: a cluster randomized controlled trial. *PLoS Med*, *11*(12), e1001774.

Qureshi H, Arif A, Riaz K, Alam SE, Ahmed W and Mujeeb SA. (2009). Determination of risk factors for hepatitis B and C in male patients suffering from chronic hepatitis. *BMC Research Notes,* 2, 212.

Rafizade B, Kazemi AN, Amir Moghadami HR, Moosavi Nasab N. (2004). Survey of Anti-HBs Serum Level in vaccinated 7-9 Year-old Children in Zanjan City. *ZUMS J,* 12(47), 49-55.

Resti M, Difrancesco G, Azzari G, Rossi ME, Vierucci A. (1993). Anti-HBs and immunological memory to HBV vaccine: implication for booster timing. *Vaccine,* 11, 1079-1082.

Rezaei M, Nooripoor S, Ghorbani R, Ramezanshams F, Mamishi S, Mahmoudi S. (2014). Seroprotection after hepatitis B vaccination in children aged 1 to 15 years in central province of Iran, Semnan. *J PMH,* 55, 1-3.

Romanò L, Paladini S, Galli C, Raimondo G, Pollicino T, and Zanetti AR. (2015). Hepatitis B vaccination Are escape mutant viruses a matter of concern? *Human Vacc & Immuno,* 11(1), 53–57.

Salama II, Sami SM, Said ZNA, El-Sayed MH, El Etreby LA, Rabah TM, Elmosalami DM, Hamid ATA, Salama SI, Mohsen AMA Emam HM. (2015). Effectiveness of hepatitis B virus vaccination program in Egypt: Multicenter national project. *World J hepa*, *7*(22), 2418.

Schönberger K, Riedel C, Rückinger S, Mansmann U, Jilg W, Kries RV. (2013). Determinants of long term protection after hepatitis B vaccination in infancy: A meta-analysis. *Pediatr Infect Dis J,* 32(4), 307-13.

Seeger C, Mason WS. (2006). 36 hepadna virus. New York: Cold Spring Harbor Monograph Archive, 729-744.

Shaaban FA, Hassanin AI, Samy SM, Salama SI and Said ZN. (2007). Long term immunity to hepatitis B among a sample of fully vaccinated children in Cairo, Egypt. *East Med Helth J,* 13(4), 750-757.

Su H, Zhang Y, Xu D, Wang B, Zhang L, Li D, Xiao D, Li F, Zhang J, Yan Y. (2013). Occult hepatitis B virus infection in anti-HBs-positive infants born to HBsAg-positive mothers in China. *PloS one*, *8*(8), e70768.

Sugunan AP, Bhattacharya H, Bhattacharya D, Mandal A, Ghosal SR, Rao RC Mandal AK. (2014). Impact of hepatitis B immunization among the Nicobarese tribe-antibody titres & seroprotection five years after vaccination. *Indian J Med Res*, *139*(3), 427.

Tajiri K, Shimizu Y. (2015). Unsolved problems and future perspectives of hepatitis B virus vaccination. *World J Gastroenterol,* 21(23), 7074-7083.

Tatematsu K, Tanaka Y, Kurbanov F, Sugauchi F, Mano S, Maeshiro T, Nakayoshi T, Wakuta M, Miyakawa Y, Mizokami M. (2009). A genetic variant of hepatitis B virus divergent from known human and ape genotypes isolated from a Japanese patient and provisionally assigned to new genotype J. *J Virol,* 83, 10538–10547.

Torbenson M, Thomas DL. (2002). Occult hepatitis B. *Lancet Infect Dis* 2: 479-486

Uddin AI, Pervin M, Munna S, Noor R. (2014). Study of risk factors related to HBsAg reactivity among outdoor patients in Dhaka Medical College and Hospital, Bangladesh. *American J Biomed and Life Science,* 2(1), 18-21.

Upreti SR, Gurung S, Patel M, Dixit SM, Krause LK, Shakya G, Wannemuehler K, Rajbhandari R, Bohara R, Schluter WW. (2014). Prevalence of chronic hepatitis B virus infection before and after implementation of a hepatitis B vaccination program among children in Nepal. *Vaccine*, *32*(34), 4304-4309.

Van Damme P, Van Herck K. (2007). A review of the long-term protection after hepatitis A and B vaccination. *Travel Med Infect Dis,* 5, 79-84.

Sande MVD, Waight P, Mendy M, Rayco-Solon P, Hutt P, Fulford T, Doherty C, McConkey SJ, Jeffries D, Hall AJ, Whittle HC. (2006). Long-term protection against carriage of hepatitis B virus after infant vaccination. *J of infect dis*, *193*(11), 1528-1535.

Van der Sande MA, Waight PA, Mendy M, Zaman S, Kaye S, Sam O, Kahn A, Jeffries D, Akum AA, Hall AJ, Bah E. (2007). Long-term protection against HBV chronic carriage of Gambian adolescents vaccinated in infancy and immune response in HBV booster trial in adolescence. *PLoS One*, *2*(8), e753.

Vardas E, Mathai M, Blaauw D, McAnerney J, Coppin A and Sim J. (1999). Pre immunization Epidemiology of Hepatitis B Virus Infection in South African Children. *J Med Virol,* 58(2), 111–115.

West DJ, Calandra GB. (1996). Vaccine induced immunological memory for hepatitis B surface antigen: implication for policy on booster vaccination. *Vaccine* 14(11), 1019-1026.

WHO. (2014). Ethiopia country office. Strengthening Routine Immunization in Ethiopia Progress in 2014. Available on URL:http//www.afro.who.int. Accessed on July 3 2015.

WHO. (2002). Global Alert and Response. Hepatitis B. Prevention and treatment. Available on URL: http://www.who.int. Accessed on July 2, 2015.

WHO. (2006). Global immunization coverage. www.who.int/immunization news room Global immunization data. PDF .

WHO. (2015). Guidelines for the prevention, care and treatment of persons with chronic hepatitis B infection. www.who.int. Accessed on March 15, 2017.

WHO. (2013). Media Centre. Hepatitis B. Available on URL: http://www.who.int. Accessed on June 21 2015.

Williams IT, Goldstein ST, Tufa J, Tauillii S, Margolis HS, Mahoney FJ. (2003). Long term antibody response to hepatitis B vaccination beginning at birth and to subsequent booster vaccination. *Pediatr Infect Dis J,* 22, 157-163.

Wright MD, Teresa L. (2006). Introduction to chronic hepatitis B infection. *Am J Gastroenterol*, *101*, S1-S6.

Wu S, Hwang LY, Goodman KJ, and Beasley RP. (1999). Hepatitis B Vaccination in High-Risk Infants: 10-Year Follow-Up. *The J Infect Dis,* 179, 1319-1325.

Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, Huang Y, Qi Y, Peng B, Wang H, Fu L. (2012). Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *elife*, *1*, e00049.

Yang SG, Wang B, Chen P, Yu CB, Deng M, Yao J, Zhu CX, Ren JJ, Wu W, Ju B, Shen JF. (2012). Effectiveness of HBV vaccination in infants and prediction of HBV prevalence trend under new vaccination plan: findings of a large-scale investigation. *PloS one*, *7*(10), e47808.

Yazdanpanah B, Safari M, Yazdanpanah S. (2010). Persistence of HBV Vaccine's Protection and Response to Hepatitis B Booster Immunization in 5- to 7-Year-Old Children in the Kohgiloyeh and Boyerahmad Province, Iran. *Hepat Mon,* 10(1), 17–21.

Zanetti AR, Mariano A, Romanò L, D'Amelio R, Chironna M, Coppola RC, Cuccia M, Mangione R, Marrone F, Negrone FS, Parlato A. (2005). Long-term immunogenicity of hepatitis B vaccination and policy for booster: an Italian multicentre study. *Lancet*, *366*(9494), 1379-1384.

Zanetti AR, Van Damme P, Shouval D. (2008). The global impact of vaccination against hepatitis B: a historical overview. *Vaccine,* 26(49), 6266-6273.

11. Annex

Annex I Information sheet for participants' family

Dear Research Participant's family

My name is Seifegebriel Teshome. I'm an Assistant Lecturer in Addis Ababa University, Department of Microbiology, Immunology and parasitology. I'm doing my MSc research on "The level of antibody against HBV infection after HBV vaccination and Sero prevalence of HBV in children Addis Ababa, Ethiopia".

The purpose of this questionnaire is to get some relevant information on the research. The main purpose of this research is to determine Hepatitis B infection and antibody level against HBV after hepatitis B vaccination in children. The findings of this research are believed to be useful to assess the Sero-prevalence of HBV and the effect of HBV vaccine in children which will help to control the disease in the country.

The duration of the study is not more than five months and there will be a compensation for the time and energy you spend with us. We will take 4ml of blood from your child and which only have a local pain but by participating on this research you and your child will not be affected by any means.

Therefore, the information and responses obtained from you are very important for this research to meet its prime objective. The study can be successfully accomplished only when you answer all the items honestly, frankly, and genuinely. The information and responses obtained from you will be kept confidential and used only for the purpose of this research. You can give your informed consent freely and voluntarily and you have full right to refuse giving blood sample from your child or to withdraw from participation in study at any stage. The results of the research study may be published; no individual responses will be reported. Your assistance with this research would be much appreciated.

With Regards,

Seifegebriel Teshome

የጥናቱ ተሳታፊዎች የመረጃ ቅጽ

ውድ የጥናቱ ተሳታፊ ወላጆች

ስሜ ሰይፈገብርኤል ተሾመ ይባላል፡፡ በአ.አ.ዩ ጤና ሣይንስ ኮሌጅ በማይክሮባዮሎጂ አሚኖሎጂና ፓራሲቶሎጂ ት/ት ክፍል ረዳት ሌክቸረር ነኝ፡፡ የሁለተኛ ዲግሪ ጥናቴንም በህጻናት ላይ የሄፐታይተስ ቢ የሚባለውን የጉበት ቫይረስ ስርጭት እና ይህን ቫይረስ ለመከላከል በሚሰጠው ክትባት የህጻናቱ ቫይረሱን የመከላከል አቅም ምን ላይ እንደደረሰ መለካት በሚል አየሰራሁ ነው፡፡ የዚህ ጥናት አላማም ቫይረሱ በህጻናቱ ላይ ያለውን ስርጭት ማወቅና ክትባቱ ያመጣውን ለውጥ መለካት ነው፡፡

የዚህ ጥናት ውጤትም በሽታውን ለመከላከል የሚደረገውን ጥረት በማገዝ የክትባቱን ውጤታማነት በመለካት መፍተሄ ማስቀመጥ ይሆናል፡፡ ይህ ጥናት የሚወሰደው ጊዜ ከአምስት ወር የማይበልጥ ሲሆን በጥናቱ ላይ ሲሳተፉ ለሚያጠፉት ጊዜና ጉልበት ተገቢውን ማካካሻ የምናደርግ ይሆናል፡፡ በዚህ ጥናት ላይ ከልጅዎ 4 ሚ.ሊ የድም ናሙና የሚያስፈልግ ሲሆን መርፌ ሲወጋ ከሚኖረው ህመም ውጪ ሌላ ጉዳት በእርስዎም ሆነ በልጅዎ ላይ የሚደርስ ነገር አይኖርም፡፡

ስለዚህም ከእርስዎ የሚወሰደው ማንኛውም መረጃ ለጥናቱ ስኬት አስፈላጊ ሲሆን ጥናቱ ሊሳካ የሚችለውም የእርስዎ ቀና ትብብር፤ ታማኝነትና ፈቃደኝነት ባለበት ሁኔታ ፎርሙን ሲሞሉልን ነው፡፡ ከእርስዎ የምንወስደው ማንኛውም መረጃ በሙሉ ምስጢራዊነቱ የተጠበቀና ለጥናቱ አላማ ብቻ የሚውል ነው፡፡ በዚህ ጥናት ሲሳተፉም በማንኛውም ጊዜ ጥናቱን የማቁዋረጥ እንዲሁም ከልጅዎ ላይ ናሙና ያለመስጠት መብትዎ የተጠበቀ ነው፡፡ የጥናቱ ውጤትም ሊታተም የሚችል ሲሆን እርስዎ ለጥናቱ መሳካት የሚያደርጉት ድጋፍ ሁሉ እጅጉን ይበረታታል፡፡

ከምስጋና ጋር

ሰይፈገብርኤል ተሾመ

ማይክሮባዮሎጂ፤ኢሚኖሎጂና ፓራሲቶሎጂ ት/ት ክፍል

ጤና ሳይንስ ኮሌጅ፤አዲስ አበባ ዩኒቨርሲቲ

አዲስ አበባ

ስልክ 09 10 10 46 99

Annex II Informed Consent form

Please Give Your Informed Consent Below.

I have read the information regarding this research and I have been informed about all aspects of the study. I understand that there will be no effect if I agree to participate in this study and give permission to the researcher to take blood sample from my child in understanding that the data obtained will be confidential and I and my child will not be identified in any way. I was also assured that I have full right to withdraw from this study anytime

I _____ give my informed consent to participate in the above study and permit the researcher to take blood sample from my child.

Signature: _____ Date: _____

የፈቃደኝነት መጠየቂያ ቅጽ

ጥናቱን በተመለከተ ያሉትን መረጃዎች በሙሉ አንብቤያለሁ፡፡ በዚህ ጥናት በመሳተፌ በኔም ሆነ በልጄ ላይ የሚደርስ ጉዳት እንደማይኖር በመረዳቴ ከልጄ ላይ የደም ናሙና እንዲወሰድ ስፈቅድ እና ቃለመጠይቁን ስሞላ ምስጢራዊነቱ እንደተጠበቀ እንደሚሆን እኔም ሆንኩ ልጄ የምንታወቅበት ሁኔታ እንደማይኖር ተገንዝቤያለሁ፡፡ በማንኛውም ጊዜ ጥናቱን የማቁዋረጥ መብቴም እንደተጠበቀ አረጋግጫለሁ፡፡

እኔ_____ከላይ በተጠቀሰው ጥናት ለመሳተፍና ከልጄ ላይ የደም ናሙና አንዲወሰድ በመፍቀድ ተስማምቻለሁ፡፡

ፊርማ_____ ቀን_____

Annex III Questioner in English

1. Code No._____
2. Sub city_____
3. Sex of the child i) Female ii) Male
4. Age of the child_____
5. Weight_____
6. Mother's age at child's birth_____
7. Place of birth of the child_____
8. What is your occupation? _____
9. What is your educational status?

i) below certificate ii) certificate iii) diploma iv) degree and above

10. How many children do you have?

i) one ii) two iii) three iv) > four

11. If you have more than one child, how many of them are vaccinated?

i) all of them ii) only one iii) more than one

12. Number of vaccination

i) once ii) twice iii) three times iv) None

13. How much time would it take to travel from your home to the vaccination center?

i) < 30min ii) 30-60 min iii) more than an hour

14. Where did you born your child?

i) home ii) private hospital iii) public hospital iv) health center

15. What is the family's overall monthly income?

i) <1000Birr ii) 1000-2000Birr iii) 2001-3000Birr iv) >3000Birr

16. Is there anyone in the family who is affected by HBV?

i) Yes ii) no

17. Was a child being chronically ill before?

i) Yes ii) no

18. If your answer is yes can you tell me the disease? _____

Thank you

ቃለ መጠይቅ

1. የኮድ ቁጥር_____
2. ክፍለ ከተማ_____
3. የህጻኑ ጾታ ሀ) ወንድ ለ)ሴት
4. የህጻኑ እድሜ_____
5. ክብደት_____
6. ህጻኑ ሲወለድ የነበረው የእናት እድሜ_____
7. ህጻኑ የተወለደበት ቦታ_____
8. የሚሰሩት ስራ ምንድን ነው?_____
9. የእርስዎ የትምህርት ደረጃ?

ሀ)ከሰርተፍኬት በታች ለ)ሰርተፍኬት ሐ)ዲፕሎማ መ)ዲግሪና ከዚያ በላይ

10. ስንት ልጆች አሎት?

ሀ) አንድ ለ)ሁለት ሐ)ሦስት መ) ዐራትና ከዚያ በላይ

11. ከአንድ በላይ ልጆች ካለዎት ከነዚህ ውስጥ ስንቶቹ ተከትበዋል?

ሀ)ሁሉም ለ) አንዱ ብቻ ሐ) ሁለትና ከዚያ በላይ

12. ህጻኑ ስንት ጊዜ ተከትቡዋል?

ሀ አንድ ጊዜ ለ)ሁለት ጊዜ ሐ) ሦስት ጊዜ መ) አልተከተበም

13. ከቤትዎ እሰከ ክትባት መስጪያ ቦታ ድረስ ምን ያህል ጊዜ ይፈጅቦት ነበር?

ሀ) ከ30 ደቂቃ በታች ለ) ከ30-60 ደቂቃ ሐ) ከአንድ ሰአት በላይ

14. ልጆትን የት ነበር የወለዱት?

ሀ) ቤት ውስጥ ለ) በግል ሆስፒታል ሐ) በመንግስት ሆስፒታል መ)በጤና ጣቢያ

15. አጠቃላይ የቤተሰቡ ወርሃዊ ገቢ ስነት ይሆናል?

ሀ) ከ1000 ብር በታች ለ) ከ1000-2000 ብር ሐ)ከ2001-3000ብር መ)ከ3000ብር በላይ

16. ከቤተሰብዎ በጉበት ህመም የተጠቃ አለን?

ሀ) አዎ ለ)የለም

17. ልጅዎ ከዚህ በፊት ከባድ ህመም ታሞ ያውቃልን?

ሀ) አዎ ለ) የለም

18. መልስዎአዎከሆነየታመመውንህመምቢነግሩኝ-

_____

እናመሰግናለን::

Annex IV: laboratory protocols

A. ELISA procedure for detection of anti HBsAg

1. Bring all reagents and specimens to room temperature (+20 to +30°C) before assay. Adjust water bath or incubator to +37±1°C.
2. Prepare Conjugate Working Solution (R7a + R7b), Working Diluted Substrate Solution (R8 + R9) and Diluted Washing Solution (diluted R2).
3. Remove the microplate frame and strips (R1) from their protective bag. Remove strips not needed for the assay and replace them with labeled Null Strips, as necessary.
4. Dilute specimens, calibrators and controls 3:4 in the Specimen Diluent (Specimens, Calibrators and Controls may be diluted in-well (Add 25 µl of Specimen Diluent R6 to each well first, followed by 75 µl of specimen or control within 15 minutes, then mix gently by a minimum of 2 aspirations to avoid foaming).

5. Add directly, without prior washing of the plate, and in succession depending on the method

Anti-HBs Negative Control (C0) in well A1,

10 mIU/ml Calibrator (C1) in wells B1 and C1,

100 mIU/ml Calibrator-Positive Control (C2) in well D1,

400 mIU/ml Calibrator (C3) in well E1,

1000 mIU/ml Calibrator (C4) in well F1,

Samples in wells G1, H1, etc.

6. Cover, if it is possible, the wells with adhesive film by pressing over the whole surface to ensure tightness.
7. Incubate the plate for 60 ± 5 minutes at 37 ± 2°C.
8. Remove the adhesive film. Aspirate the contents of all wells into a liquid waste container and add immediately a minimum of 0.375 ml of Washing Solution to each well. Soak each well for 30 to 60 seconds between each wash cycle. Aspirate again. Repeat the washing step 4 times (minimum of 5 washes). The residual volume must be lower than 10 µl (if necessary blot the plate by turning it upside down on absorbent paper).
9. If an automatic washer is used, follow the same procedure.
10. Add quickly 100 µl of the Conjugate Working Solution (Buffer with HBsAg (human ad and ay subtypes) coated 2.5 ml with peroxydase and protein stabilizers + Buffer with calf

serum and protein stabilizers) to each well. Cover, if it is possible, the wells with a new adhesive film and incubate for 60 ± 5 minutes at 37°C ± 1°C.

11. Remove the adhesive film. Aspirate the contents of all wells into a liquid waste container and add immediately a minimum of 0.375 ml of Washing Solution to each well. Soak each well for 30 to 60 seconds between each wash cycle. Aspirate again. Repeat the washing step 4 times (minimum of 5 washes). The residual volume must be lower than 10 µl (if necessary blot the plate by turning it upside down on absorbent paper).
12. If an automatic washer is used, follow the same procedure.
13. Add quickly 100 µl of the Working Diluted Substrate Solution (Solution containing Tetramethyl Benzidine (TMB) + Citric acid and Sodium acetate solution pH 4.0 60 ml containing H_2O_2 (0.015 %) and DMSO (4%)) to each well. Allow the reaction to develop in the dark for 30 ± 5 minutes at room temperature (18 - 30°C). Do not use adhesive film during this incubation.
14. Add 100µl Stopping Solution (1 N sulfuric acid solution) by using the same sequence and rate of distribution as for the Working Diluted Substrate Solution. Homogenize the reaction mixture.
15. Carefully wipe the plate bottom. At least 4 minutes after stopping solution addition and within 30 minutes of stopping the reaction, read at the optical density at 450/620-700 nm and 405/620- 700 nm using a plate reader.

CALCULATION AND INTERPRETATION OF THE RESULTS

12. Calculate the mean absorbance of the 10 mIU/ml Calibrator

13. Calculate the cut off value

The mean absorbance of the 10 mIU/ml Calibrator is the Cutoff Value for the assay.

14. Validation of the results

For Anti-HBs Negative Control

The measured absorbance value must be greater than 0.000 and less than or equal to 0.070

For Positive Control

The measured absorbance value must be greater than or equal to 0.400 (ODC2 ≥ 0.400).

For Negative Control and Positive Control, if any one of the above criteria is not met for qualitative and quantitative method, the assay is invalid and must be repeated.

For 10 mIU/ml Calibrator

The measured absorbance value must be greater than or equal to 0.050 and less than or equal to 0.200. Each measured absorbance values must be greater than or equal to 1.5 the OD of the absorbance value of the Negative Control.

15. Interpretation of the results

Specimens with absorbance values greater than or equal to the cutoff value are considered reactive.

Specimens with absorbance values less than the cutoff value are considered non-reactive.

The absorbance value of each sample was compared with the absorbance value of different concentrations of controls and categorized under their concentration value.

B. ELISA procedure for detection of HBsAg

1. Bring all reagents and specimens to room temperature (+20 to +30°C) before assay. Adjust water bath or incubator to +37±1°C.
2. Prepare the diluted washing solution (dilute1:20 with distilled water)
3. Prepare the conjugate R6+R7 working solution (refer to chapter 8).
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 and reclose it.
5. Distribute in the wells in the following order (advisable plate distribution) :
   Wells A1, B1, C1 and D1: 100 µl of negative control (R3)
   Well E1: 100 µl of positive control (R4)
   Well F1, G1, H1...etc: 100 µl of unknown sample.

Depending on the used system, it is possible to modify the position of controls or the order of distribution.

6. Quickly dispense 50 µl of conjugate solution (Tris HCl buffer pH 7.4 containing BSA, Tween®20, bovine immunoglobulins and mouse immunoglobulins with sample addition control reagent + Mouse Monoclonal anti-HBs antibodies and Goat polyclonal anti-HBs antibodies bound to the peroxidase. Lyophilized) 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 and incubate for 1 hour and 30 • ± 5 minutes at 37 ± 1°C.
8. Remove the adhesive film, empty all wells by aspiration and wash a minimum of 5 times. The residual volume must be lower than 10 µl (if necessary, dry the strips by turning them upside down on absorbent paper).
9. Quickly dispense into each well 100 µl of prepared development solution (Citric acid and Sodium acetate solution pH 4.0 containing H_2O_2 (0.015%) and DMSO (4%) + Solution containing tetramethyl benzidine (TMB)), freshly prepared before use. Allow the reaction to develop in the dark for 30 ±5 minutes at room temperature (18 - 30°C). Do not use adhesive film during this incubation.
10. Add 100 µl stopping solution (1N sulphuric acid solution) by using the same sequence and rate of distribution as for the substrate solution. Homogenize the reaction mixture.
11. Carefully wipe the 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.
12. Check for agreement between the spectrophotometric and visual readings and against the plate and sample distribution and identification plans.

CALCULATION AND INTERPRETATION OF THE RESULTS

1) Calculate mean optical density of negative control

2) Calculate the cut-off value

For each method, the cut-off value is equal to: mean OD of negative controls + 0.050

3) Test validity conditions

All the values of the negative control should be lower or equal to 0.080 unit of optical density.

The positive control value should be over or equal to 1.000.

If one negative control value does not respect this norm or is superior to 40% compared to the mean value of the negative controls disregard and recalculate the mean using the three remaining values. Only one value may be eliminated in this way.

In case of very low background for the negative control (average value of negative control below 0.010 OD) do not use these rejection criteria's for negative control.

The test must be redone if all control values are out of these norms.

4) Calculation of ratio sample

For each sample, calculate the ratio:

$$\text{Ratio} = \frac{\text{OD sample}}{\text{Cut-off value}}$$

5) Interpretation of the results

Samples with ratio values lower than 1 are considered to be negative

Results just below the cut-off value (sample ratio between 0.9 and 1) should however, be interpreted with caution.

Samples with ratio values equal to or greater than 1 are considered to be initially positive

C. ELISA procedure for detection of Anti HBc

1. Bring all reagents and specimens to room temperature (+20 to +30°C) before assay. Adjust water bath or incubator to +37±1°C.
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 :

   4.1 200 µl of diluent (R6) into each well

   4.2 20 µl of negative control serum (R3) in A1, B1

   20 l of positive control serum (R4) in C1, D1, E1

   20 µl of the first sample in F1 if this well is not used as a reagent control for the sample addition monitoring

   20 µl of the second sample in G1, etc ...Depending on the utilized system, it is possible to modify the position of the controls.
5. Homogenize the reaction mixture by a minimum of 3 aspirations with the 20 µl pipette or by shaking the microplate after the pipetting step.
6. Cover the wells with adhesive film by pressing over the whole surface to ensure tightness.
7. 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
8. Remove the adhesive film. Aspirate the contents of all wells into a liquid waste container and add a minimum of 0.370 ml of washing solution to each well. Aspirate again. Repeat

the washing step three times (4 washes). The residual volume must be lower than 10 µl (if necessary, blot the microplate by turning it upside down on absorbent paper). If an automatic washing device is used, follow the same operating cycle.

9. Distribute quickly 200 µl of the conjugate solution (Mouse biotinilated monoclonal antibodies against capsid HCV antigen + Mouse antibody directed against human IgG/peroxidase and streptavidine/peroxidase) into all wells. The conjugate must be shaken gently before use.
10. Cover with new adhesive film and incubate for 60 min ± 5 min at 37°C ± 1°C
11. Remove the adhesive film, empty all wells by aspiration and wash 4 times as previously described. The residual volume must be lower than 10 µl (if necessary, blot the microplate by turning it upside down on absorbent paper).
12. Prepare the substrate solution (see section 8, reagent R8 + R9).
13. Quickly dispense into each well 100µl of prepared development solution (Citric acid and Sodium acetate solution + Solution containing tetramethyl benzidine (TMB)), freshly prepared before use. Allow the reaction to develop in the dark for 30 ± 5 minutes at room temperature (18 - 30°C). Do not use adhesive film during this incubation.

    N.B.: The distribution of the development solution, which is colored pink, can be visually controlled at this step of the manipulation: There is a clear difference of coloration between empty well and well containing the pink substrate solution.
14. Add 100 µl stopping solution (1 N sulfuric acid solution) by using the same sequence and rate of distribution as for the substrate solution. Homogenize the reaction mixture.

    N.B.: The distribution of the stopping solution, which is not colored, can be visually controlled at this step of the manipulation. After the addition of the stopping solution the pink coloration of the substrate disappears (for the negative samples) or turns from blue to yellow (for the positive samples).
15. Carefully wipe the plate bottom. **At least 4 minutes after stopping solution addition** and within 30 minutes of stopping the reaction, read the optical density at 450/620-700 nm using a plate reader.
16. Before recording the results, check the correlation between the reading and the microplate and sample distribution and identification plan.

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.

1. Calculate the mean of the absorbance values for the positive control serum

2. Calculation of the cut-off value (Vs)

$$Vs = \frac{\text{Mean of OD R4}}{5}$$

The validation criteria are 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.

Interpretation of the results

Samples with an optical density less than the cut-off value are considered to be negative

Samples with an optical density higher than, or equal to, the cut-off value are considered to be Initially positive and must be retested in duplicate before the final interpretation.

However, results just below the cut-off value Vs -10% < OD should be interpreted with care (it is advised to retest the corresponding samples in duplicate when the utilized systems and laboratory procedures allow it).

For initial reactive or doubtful (0.9<ratio<1) samples, after retesting, the sample is considered to be positive if at least one of the both measurements is positive, i.e. higher than, or equal to, the cut-off value. The sample is considered to be negative if both values are less than the cut-off value.

Annex V: Interpretation of Hepatitis B panel results

Tests	Results	Interpretation
HBsAg **anti-HBc** **anti-HBs**	negative negative negative	susceptible
HBsAg **anti-HBc** **anti-HBs**	negative negative positive with ≥10mIU/mL	immune due to vaccination
HBsAg **anti-HBc** **anti-HBs**	negative positive positive	immune due to natural infection
HBsAg **anti-HBc** **IgM anti-HBc** **anti-HBs**	positive positive positive negative	acutely infected
HBsAg **anti-HBc** **IgM anti-HBc** **anti-HBs**	positive positive negative negative	chronically infected
HBsAg **anti-HBc** **anti-HBs**	negative positive negative	four interpretations possible ✓ previous infection ✓ occult infection ✓ immune failure ✓ chronic infection