SERO-PREVALENCE OF HEPATITIS B VIRUS INFECTION AND SERO-PROTECTION OF HEPATITIS B VACCINE AMONG CHILDREN IN JIMMA TOWN, SOUTHWEST ETHIOPIA

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ABSTRACT

Background: *Hepatitis B virus (HBV) is the leading cause of viral hepatitis and about 240 million people worldwide are chronic carrier. The virus was known widely prevalent in Ethiopia and has several serious complications. The vaccines used for the virus have an outstanding record of safety and effectiveness; however, about 10% of the vaccinees still remain susceptible to HBV, especially those vaccinees were from infected mothers. The disease burden among children and sero-protection of HBV vaccine is mainly unknown in the study area. Therefore, this study assessed the sero-prevalence of HBV infection and sero-protection of HBV vaccine among children in Jimma town.*

Methods: *Community based cross-sectional study was conducted among 900 children of 5 to 9 years of age from June to December, 2016. Simple random sampling technique was employed to recruit study participants by proportional allocations into different Kebeles of Jimma town. Data was collected by using pretested questionnaire and about 3-5ml of blood sample was collected and tested for HBsAg, anti-HBc and anti-HBs by using ELISA (Bio-rad, Monolisa, Lacquote, France) technique. Data were analysed using chi-square and logistic regression test.*

Result: *This study revealed that the HBsAg and anti-HBc prevalence among all participants were 3.5% and 3.8%, respectively. The prevalence of HBsAg among vaccinated and non-vaccinated was 2.1% and 7.0% respectively. Anti-HBc positivity was 1.1% and 6.2% among vaccinated and non-vaccinated children. The sero-protection of HBV vaccine among fully vaccinated children showed that 71.6% against chronic HBsAg carriage whereas 83.4% against total HBV infection. It was also found that 58.4% of vaccinated children retained protective level of HB surface antibodies, ≥ 10 mIU/ml with the mean of 50 mIU/ml. Multiple logistic regression revealed that lack of vaccination (AOR =2.788, P < 0.029), child who were born at home (AOR= 3.211, P < 0.009) and hospital admission (AOR= 7.122, P <0.001) were the significant predicting variables for HBsAg positivity.*

Conclusion: *The sero-prevalence of hepatitis B infection is high among children who have not been vaccinated for the virus. Hepatitis B vaccine has contributed to the reduction of the infection in this endemic area, though further efforts are required to improve timely vaccination and its coverage. The prevalence of protective anti-HBs is low among fully vaccinated children, hence, it is better to include the monovalent birth dose of the vaccine and conduct further studies to evaluate underlining cuases for wanning of serum anti-HBs level.*

Keywords*: Hepatitis B virus, Vaccine efficacy, Sero-prevalence, Children, Vaccination*

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LIST OF ACRONYMS\ ABBREVIATIONS

Ab: ---------- Antibody
cccDNA---------- Covalently closed circular DNA
CHB: ---------- Chronic Hepatitis B infection
DNA: ---------- Deoxyribonucleic Acid
DPT: ---------- *Diphtheria, Pertussis*, and *Tetanus*
ds: ---------- Double-stranded
ELISA: ---------- Enzyme Linked Immune Sorbent Assay
EPI ---------- Expanded Program on Immunization
GAVI ---------- Global Alliance for Vaccines and Immunization
GMT ---------- Geometric mean
HBcAb: ---------- Hepatitis B Core Antibody
HBcAg: ---------- Hepatitis B Core Antigen
HBeAg: ---------- Hepatitis B e Antigen/pre-core antigen
HBIG: ---------- Hepatitis B Immunoglobulin
HBsAb: ---------- Hepatitis B Surface Antibody
HBsAg: ---------- Hepatitis B Surface Antigen
HBV: ---------- Hepatitis B Virus
HCC: ---------- Hepatocellular Carcinoma.
HIV: ---------- Human Immunodeficiency Virus
IU ---------- International Unit
mRNA---------- Messenger RNA
MTCT: ---------- Mother to child transmission
ORF: ---------- Open Reading Frame
pgRNA ---------- Pregenomic RNA
rcDNA ---------- Relaxed circular DNA
RNA---------- Ribonucleic Acid
SOP: ---------- Standard Operational Procedure
SSA: ---------- Sub Saharan Africa
WHO: ---------- World Health Organization

CHAPTER ONE: - INTRODUCTION

1.1. Background

Hepatitis B virus (HBV) is a member of the genus *orthohepadnavirus* and classified under family Hepadnaviridae and it has a relaxed circular partially double-stranded DNA (rcDNA) of approximately 3200 nucleotides [1]. The epidemiology of HBV infection is geographically diverse in the world with population practice, age and mode of acquisition and likelihood of progression to chronic infection mutually interdependent [2].

Hepatitis B virus infection can produce either asymptomatic or symptomatic infection and the average incubation period ranges between 1 and 4 months. The infection is known to lead to chronic infection especially if acquired in the perinatal period and in early childhood**.** Chronic hepatitis B infection in children, with HBsAg positivity for at least six months, may be sub-divided according to the level of HBV replication and the strength of the host immune reactivity into high replicative (immune tolerance) phase, immune clearance phase, inactive HBsAg carrier stage, low replicative phase and reactivation phase [3].

Chronic carrier status represents an elevated risk for chronic hepatitis, fulminant hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC). Both the severity of clinical disease and the viral clearance correlate with the cellular immune response to various viral proteins [4]. The virus genotype-specific pathogenesis may contribute to heterogeneous clinical outcomes in chronic hepatitis and also mutations in specific regions of HBV genome could be responsible for unwanted clinical outcomes of chronic infection [5; 6].

In highly endemic areas, HBV is most commonly spread from mother to child at birth, or from person to person in early childhood. Mother to child transmission (MTCT) may occur prenatally, during delivery, or early post-partum. Mother to child transmission has been found to be a primary reason for vaccination failure [7]. Certain types of behaviors and factors increase the risk for contracting HBV infection such as in medical procedure, traditional practices and epidemiologic incidence. For example ear piercing and tattooing children in highly endemic areas, infants born from infected mothers and hospital admission have been shown as factors associated for acquiring the virus [8-10].

One of the greatest achievements in the prevention of hepatitis was the development of the hepatitis B vaccine in 1982. Both plasma-derived and recombinant vaccines contain HBsAg and elicit the development of specific antibodies (anti-HBs), which alone are sufficient to confer protective immunity [11]. The vaccine is safe and 95 % effective against all genotypes [12]. Since 1991, World Health Organization (WHO) has recommended universal hepatitis vaccination of children and high-risk groups to reduce new infections and minimize the progression to cirrhosis and hepatocellular carcinoma. The recommendation was that all infants to receive their birth dose of Hepatitis B vaccine as soon as possible after birth, preferably within 24 hours [13].

The persistence of anti-HBs antibody above the protection level (> 10 IU/ml of serum) is the main goal of vaccination but it declines by age. The persistence of anti-HBs over time is correlated with the peak level of anti-HBs immediately achieved after primary immunization. In other words, the higher the vaccine-induced anti-HBs concentration after the primary vaccination course, the longer the antibodies will persist [14]. Despite HBV immunization, around 10% of the infants born from HBV carrier mothers still become chronically infected [15]. Several factors was explained such as inappropriate vaccine storage conditions, administration not following the recommendations, immune-suppression, occult HBV infection, hospital admission, surgical operation, blood transfusion and vaccine escape mutants have been found to be associated with a lower rate of vaccine response [16,17].

In Ethiopia, the pentavalent vaccine currently on administration has included hepatitis B virus vaccine that has been introduced since 2007. In 2014, this vaccine campaign has led to coverage of 87 % of all infants nationally [18]. The vaccine is a recombinant DNA vaccines of HBV surface protein, along with *Diphtheria, Pertussis, Tetanus* (DPT) and *Haemophilus influenza* type b which is given at 6, 10, and 14 weeks of age after delivery according to Ethiopian Expanded Program on Immunization (EPI) and funded by Global Alliance for Vaccines and Immunization (GAVI) [19].

1.2. Statement of the Problem

Hepatitis B virus is the leading cause of viral hepatitis and about 2 billion people worldwide have been infected [20] and it accounts annually for 786, 000 deaths from cirrhosis, liver failure, and HCC worldwide. Despite the availability of an effective vaccine, HBV infection remains a major health problem worldwide with estimates of nearly 240 million chronic surface antigen (HBsAg) carriers [21]. Approximately 45% of the world's populations live in regions of high endemicity, defined as areas where at least 8% of the population are positive for HBsAg such as Southeast Asia and Sub-Saharan Africa (SSA) [22].

Chronically infected persons are at increased lifetime risk for cirrhosis and HCC. Among the etiologies, hepatitis B virus infection accounts for 30 % of cirrhosis and 45 % of hepatocellular carcinoma [23; 24]. In high prevalence areas, HBV infection in infancy is very common, particularly acquired from the carrier mothers at birth. The high carrier rate and the high rate of perinatal infection appear to be the main mechanism for maintaining the high prevalence rate in some developing countries [25]. Following acute HBV infection, the risk of developing chronic infection varies inversely with age: 90% for perinatal infection, 25–35% for infection at age 1–5 years and less than 10% for adults [26]**.** Once chronic hepatitis is established 15 to 40% evolve to liver cirrhosis and HCC [27].

Africa has the second largest number of chronic carriers after Asia and is considered a region of high endemicity. However, the exact burden of HBV in Africa is difficult to assess due to irregularities in the availability of testing system, inaccurate records and under-reporting. The estimated HBsAg sero-prevalence were reported to be ranging from 6 to 20% [12]. About 50 million peoples are carriers of the virus, while 25% of these are at risk for dying from the illness and in some African regions, 90% of children have been infected and 20% have become chronic carriers [28; 29]. Moreover in Africa, infection is acquired during early childhood, and the chronicity rate ranges from 82% in infants less than 6 months old to 15% in children between 2 and 3 years of age. Most of these carriers are able to clear the infection around 20 years of age. Therefore, the age of transmission inversely correlates with the duration of infection, which is most likely because the immature host immune system in children is less capable of clearing infection [30].

In Ethiopia, similarly in other African countries, there is lack of nationwide representative data on hepatitis B infections. Hence, it is difficult to present or predict the prevalence and related mortality rates accurately associated to the virus. The regional estimates have shown wide geographic and socioeconomic variation in hepatitis B prevalence, ranging from 5.7 % to 10.9% [31-33]. The virus was widely prevalent among hepatic diseased cases and one study also found that at least one of the hepatitis markers was found in 78 % of patients with hepatocellular carcinoma, 86 % of chronic hepatitis cases, and 88 % of cirrhotic patients in Ethiopia [34]. It accounts for 12 % of hospital admissions and 31 % of deaths in Ethiopian hospitals [35].

One study conducted among Ethiopian full term women and their newborn in selected health facilities of Addis Ababa, found that HBsAg prevalence was 3.0 % and 2.3%, respectively [36], which indicated utero transmission as an important route for its acquisition. This may be one of important factors for vaccine failure.

The hepatitis B vaccine is the mainstay of hepatitis B prevention and it has proved to be safe and highly effective in reducing the incidence of carrier rate and HBV-related mortality at a global scale [37]. World Health Organization recommends that all infants receive HBV vaccine as soon as possible after birth, preferably within 24 hrs and followed by 2 or 3 further monovalent or multivalent vaccines given as part of the standard infant vaccination schedule. Birth-dose HBIg is not currently recommended by the WHO and is not likely to be feasible in the near future due to logistical and costing limitations in developing nations such as Africa [10].

Hepatitis B vaccination has proved to be safe and highly effective in reducing the incidence of carrier rate, however, currently after many years of starting the HBV vaccination program, there are 10% of the vaccinees still remain susceptible to HBV, especially those vaccinees were from infected mothers [15].

There is inadequacy of evidence on the magnitude of HBV infection among children and no existing evidence on the sero-protection of HBV vaccination in this part of the country after its commencement. The aim of this study was to assess the current magnitude of HBV infection and its sero-protection among vaccinated versus non-vaccinated children in Jimma town, Southwest Ethiopia.

CHAPTER TWO: LITERATURE REVIEW

2.1. General structure of HBV

Hepatitis B virus belongs to the family Hepadnaviridae and it is composed of the envelope, core, DNA genome and viral polymerase. The whole virion has a size of 42-45 nm in diameter and the spherical form (Dane particle) has two-layered shells [1]. The outer shell is the envelope protein referred to as hepatitis B surface (HBs) protein and the inner shell is a core protein referred to as the hepatitis B core protein in which viral polymerase and the HBV genome is enclosed. The peculiar feature of HBV is the great excess of envelope material found in the circulation consisting of both small spheres and rods with an average width of 22 nm [38]. The HBcAg is the nucleocapsid that encloses the viral DNA and HBeAg is a circulating peptide derived from the core gene and then modified and exported from the liver cells, serve as a marker of active viral replication [39].

2.2. Genetic organization and replication

Hepatitis B virus is one of the smallest DNA virus and it has a partially double-stranded with the complete minus (-) strand and the incomplete (+) strand, circular DNA genome of ~3200 base pairs [40]. This compact genome contains four partly or completely overlapping open reading frames (ORFs): precore/core (preC/C) that encodes the e antigen (HBeAg) and core protein (HBcAg); P for polymerase (reverse transcriptase), PreS1/PreS2/S for surface proteins and X for a transcriptional trans-activator protein and required for HBV replication both in vitro and in vivo [41] as shown figure 1.

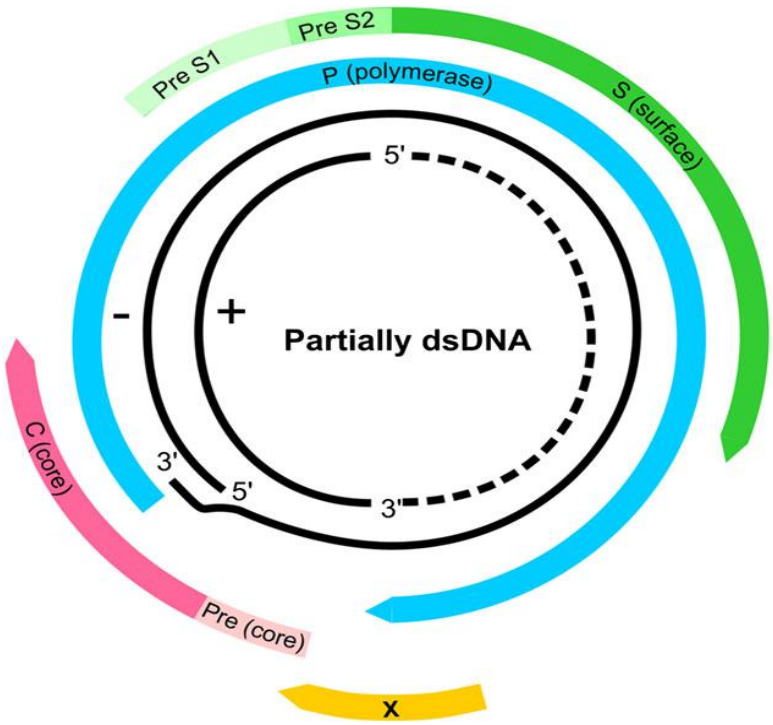


Figure 1 The genome of hepatitis B virus (HBV) **[Adoapted from reference 41]**

HBV enters the hepatocyte through the sodium-taurocholate co-transporting polypeptide (NTCP) receptor [42] and is uncoated in the cytoplasm (Fig. 2). Core particles are transported to the nucleus and the single-stranded gaps in the relaxed circular DNA (rcDNA) are repaired either through (+) strand extension by the HBV polymerase or through repair activity of host proteins, and covalently closed circular DNA (cccDNA) is formed as a nucleosome bound mini-chromosome in the nucleus and which serves as the template for all viral transcripts [43].

The viral ORFs are encoded in distinct capped and polyadenylated RNAs that can be divided into genomic, which is act as messenger (mRNAs) for precore, core, and polymerase and sub-genomic transcripts used as templates for HBV proteins. The pregenomic RNA (pgRNA) is the template for HBV replication and is reverse transcribed to generate the HBV DNA genome for new progeny [44]. The result is a pool of cccDNA that contains a fluctuating number of copies (typically less than 10) of cccDNA per cell and suggested half-life of a single cccDNA molecule is between 33 and 57 days [45].

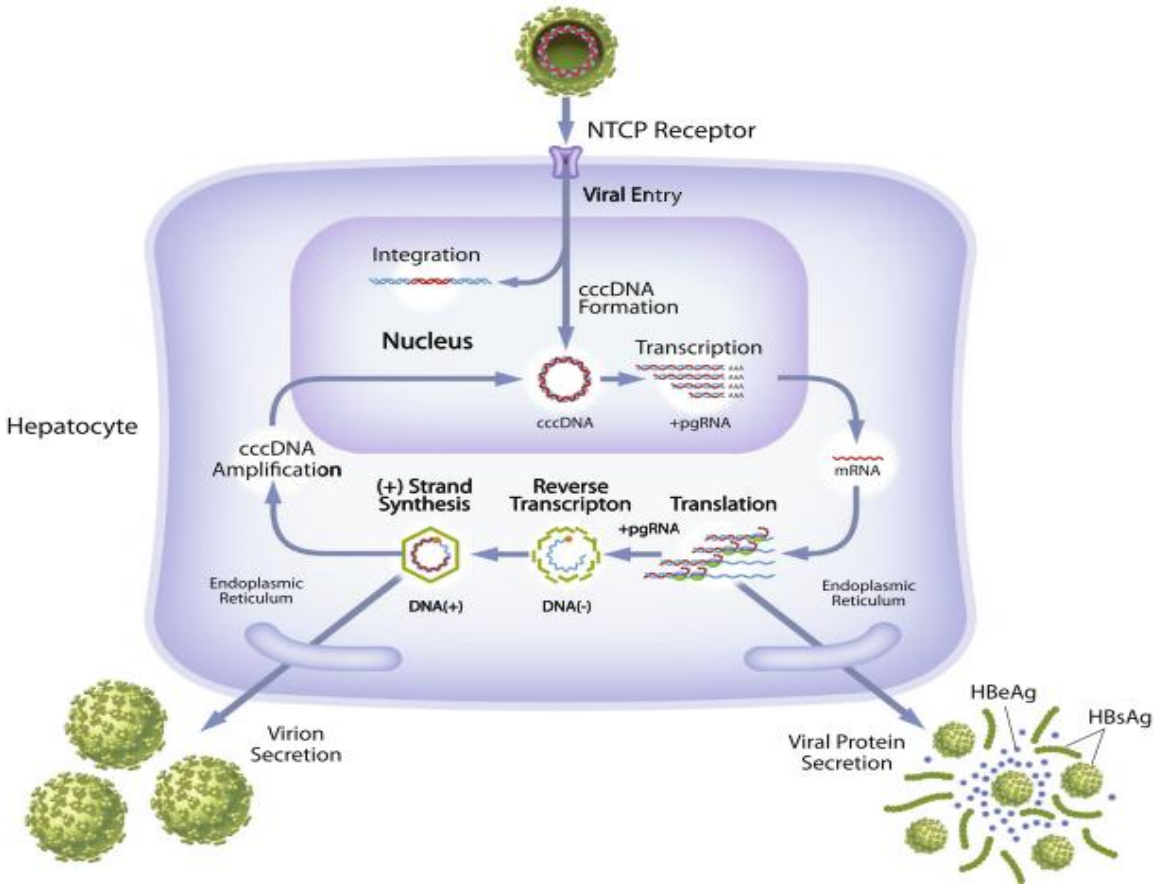


Figure 2 Schematic of the HBV life cycle **[Taken from reference 42].**

2.3. Natural history of HBV infection in children

Liver damage in chronic hepatitis B results mainly from the direct interaction between the host's immune system and HBV infected hepatocytes. The antiviral cytokines, such as interferon alpha, beta, and gamma as well as tumor necrosis factor alpha (TNF-a), have been implicated as the major contributors to viral clearance, whereas destruction of infected hepatocytes by cytotoxic T lymphocytes contributes to both viral clearance and the development of liver disease [46].

Hepatitis B virus infection can produce either asymptomatic or symptomatic infection and the incubation period ranges from 6 weeks to 6 months, and development of clinical manifestations is highly age dependent. Newborns generally do not develop any clinical signs or symptoms while acute infection produces typical illness in only 5 to 15% of children 1 to 5 years of age [47] with symptoms such as yellowing of the skin and eyes (jaundice), dark urine, extreme fatigue, nausea, vomiting and abdominal pain [12].

Development of chronic infection, following acute hepatitis B, requires the expression of HBeAg and thus the risk of developing chronic hepatitis is >90% in newborns of HBeAg-positive mothers. This is probably because of the immature immune system of the infant and/or the fetus developing immune tolerance as a result of the trans-placental crossing of either the virion or HBeAg, the only HBV antigen that can cross the placenta and which induces a specific unresponsiveness of helper T cells to both HBcAg and HBeAg [48-50].

Chronic HBV infection acquired perinatally or in early infancy is broadly divided into four phases in children based on the level of viral replication and the immune response (Figure 3). The high replicative phase, low inflammatory phase is HBsAg-positive, HBeAg-positive, with high HBV DNA levels (>2×10^5IU/ml). The immune clearance or reactive phase is the second phase, characterized by fluctuating ALT and HBV DNA levels and ending with spontaneous HBeAg loss. HBeAg seroconversion is accompanied by elevated ALT and decreased HBV DNA levels [51]. The third phase is HBeAg negative chronic hepatitis phase where necroinflammation persists with high or fluctuating ALT levels and immune clearance is ineffective. Viral loads are moderate to high and liver disease is progressive. The fourth one is low replicative phase, which is characterized by the absence of HBeAg, anti-HBe positivity, normal ALT and low or undetectable HBV DNA levels. Two additional phases, the reactivation phase and the HBsAg loss or occult phase, have been described in the natural history of HBV infection [52] but these are infrequent in children.

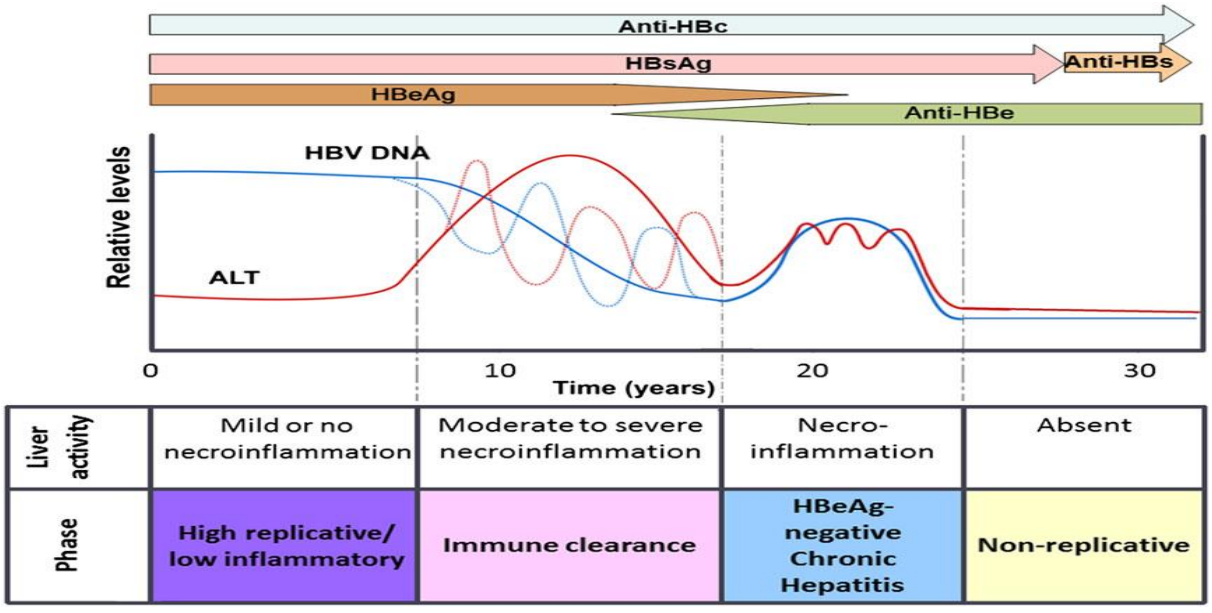


Figure 3 Natural history of infection of hepatitis B virus in children [Adoapted from ref. 52].

The major complications of chronic HBV infection (CHB) are cirrhosis and HCC. The overall risk of developing cirrhosis during the lifetime of patients with CHB is 15–40%. The risk of HCC in patients with cirrhosis is estimated to be 2–5%; however, it varies according to the geographical area and may be influenced by the presence of HBsAg, HBeAg, certain mutations and levels of HBV DNA >20,000 IU/ml [53]. The HBV replication cycle is not directly cytopathic and host immune responses against viral antigens is considered the main cause of hepatocellular injury [54]. However, several lines of evidence indicate that a certain number of HBV genetic variants, apparently provided with higher pathogenicity, may emerge during the course of the infection under endogenous (immunity) and/or exogenous (immunoprophylaxis and antiviral therapies) selection pressures [55].

Laboratory diagnosis of hepatitis B infection focuses on the detection of the HBsAg and acute HBV infection is characterized by the presence of HBsAg and IgM HBcAg. During the initial phase of infection, patients are also seropositive for HBeAg. Chronic infection is characterized by the persistence of HBsAg for at least 6 months (with or without concurrent HBeAg) and persistence of HBsAg is the principal marker of risk for developing chronic liver disease and liver cancer (hepatocellular carcinoma) later in life [12].

2.4. Epidemiology

Two of the 7.3 billion world population have been exposed to HBV and about 240 million individuals are estimated to be chronically infected with this virus [20]. According to the homogeneity of virus sequences, at least 10 HBV genotypes (A to J) were identified and several subtypes have been defined by divergence in the entire HBV genomic sequences, respectively, >8% for genotypes and 4–8% for subtypes. Except for the newly identified genotypes I and J, the geographic and ethnic distributions of HBV genotypes and subtypes are well known [56; 57].

According to 2012 global HBV disease burden estimates between 1990 and 2005, the global prevalence of chronic HBV infection varies widely, from low (< 2%) in Tropical and Central Latin America, North America and Western Europe, to low intermediate (2–4%) in South Asia, North Africa and the Middle Eastern region, to high intermediate (5–7%) in southern parts of Africa and parts of Asia and high (> 8%) in all sub-Saharan African regions and parts of Asia Hepatitis B is highly endemic in developing regions with large population such as in all sub-Saharan African regions, East Asia and, to a lesser extent, in Oceania and Andean Latin America [21] as indicated in figure 4.

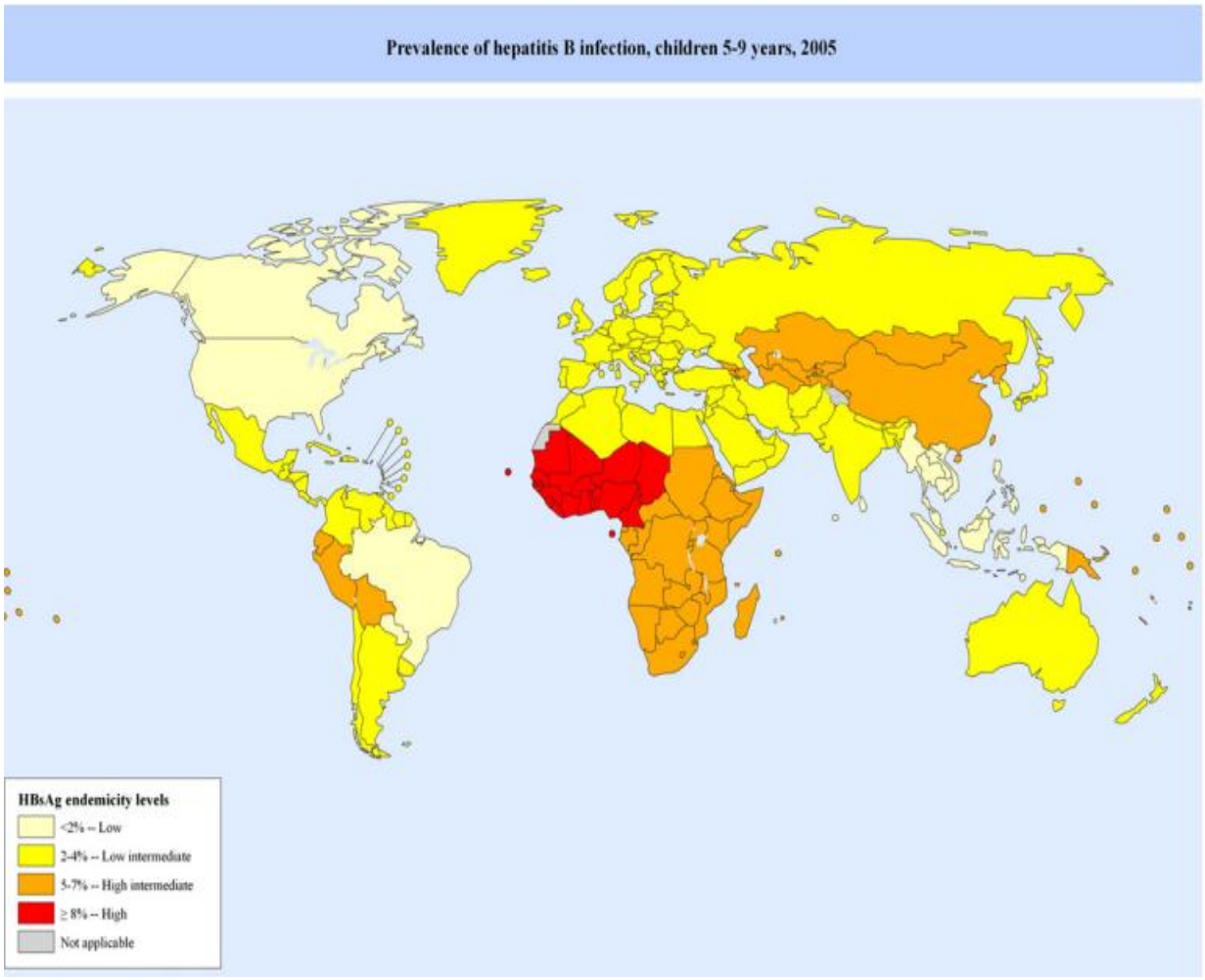


Figure 4 Prevalence of HBV infection among children 5-9 years 2005 [**Taken from reference 21**]

According to 2012 global HBV disease burden estimates the prevalence in high income countries of North America and European regions was low and declined among both sexes between 1990 and 2005. On the other hand, in central and eastern Europe the most affected age group was those who were younger than 9 years old. In contrast, the strongest reduction in prevalence of HBsAg of 1.2–1.4% among the young age groups of 0–14 year children in 2005 was recorded in South East Asia. This decrease is attributable to the introduction of effective vaccination programs [21].

Worldwide, once hepatitis B vaccination has become implemented in early childhood, a subsequent significant decrease of chronic HBV infection was observed. In areas less endemic for hepatitis B, the HBsAg carriage rate even reached to zero [58]. Several studies in different parts of the world have shown decreasing HBV infection after implementation of universal HBV vaccination among children and the prevalence and vaccine efficacy varies accordingly.

Assessment of vaccine efficacy conducted in Alaska found 50% HBV vaccine efficacy with GMT 13.8 mIU/ml after 30 years of primary vaccination but the titer was decrease steadily by time [59]. A study conducted in East Java, Indonesia, among 185 pre-school vaccinated children showed the overall prevalence of 3.2% for anti-HBc but none of them positive for HBsAg. In this study 26.5% of the participants had protective levels of anti-HBs (≥10 mIU/ml) and the titer was decreased with age [60]. A study of HBV infection after a decade of hepatitis B vaccination in an indigenous tribe of Andaman, Nicobar Islands of India found that 2.4% and 9.5% prevalences of HBsAg among vaccinated and non-vaccinated individuals respectively. However, 72.8% vaccinated children had retained protective levels of anti-HBs titer [61].

A study from Anhui Province (China) in 2006 has found 2.0% HBsAg positivity among 0-14 years of vaccinated children and increased with age and common in male gender was reported [62]. Another study from Northwest China found 2.5% HBsAg positivity and 14.1% anti-HBc in 5-9 years children and common in male gender was reported [63]. The risk factors for acquisition of HBV found that older age, rural residence, birth at home and under vaccination were reported from china and in this study 88.3% HBV vaccine efficacy against HBV infection reported [64]. A cross-sectional study conducted in Guangdong Province (China) revealed the prevalence of HBsAg and anti-HBc among vaccinated and non-vaccinated children were found to be 1.99% and 5.56% and 3.28% and 5.56%, respectively [65]. The prevalence of HBsAg and anti-HBc was 0.8% and 2.6% as reported from Central China, respectively. In this study factors like no history of vaccination, born at home and living in a rural area were reported to be significantly associated with HBV infection [10].

Moreover, a study conducted in Colombia among children revealed 6.2% overall prevalence HBV infection and 1.1% HBsAg positivity. In this study rural area and those born other than health institution were reported to be significantly associated factors for the acquisition of HBV [66]. A community based study in Dhaka; Bangladesh indicated HBsAg positivity significantly higher in females (40%) than in males (10%) (OR 5.75) [67]. As reported from Pakistan, associated factors like education less than the primary level, persons working in the government/ private sector, daily wage earners [68], uneducated children's mother, housewives, nose/ear piercing and lack of knowledge were significantly associated with HBV infection [9]. Another cross sectional study conducted in Sana'a, Yemen showed 54.8% of vaccinated children with age ranges of <1-10 years had protective level of anti-HBs titer in 2010 [69].

As Africa has the second largest number of chronic carriers after Asia, it is considered a region of high endemicity and the estimated HBsAg sero-prevalence ranges from 6-20% [12]. According to 2012 HBV disease burden estimate between 1990 and 2005, there was an increase in CHB infections among younger age groups (<14 yrs.) with age-specific prevalence of 8–9% in Southern sub-Saharan Africa. On the other hand, in Eastern sub-Saharan African countries, an increase in the prevalence of CHB was observed among youngest ages, whereas in the central sub-Saharan Africa parts, there was a corresponding decrease in prevalence was noted [21].

A comparative cross-sectional study conducted in rural Nigeria revealed that 4.6% and 1.3% HBsAg positivity among non-vaccinated and vaccinated children, respectively [70]. An assessments of the efficacy and effectiveness of infant vaccination against chronic hepatitis B in Gambia from 1986–90 found that the prevalence of HBsAg was 0.8% (2/255), 12.4% (59/475) and 17.9% (4/23) among vaccinated, non-vaccinated and partially vaccinated individuals, respectively, with 94% vaccine efficacy against HBV infection [71] and another study among 113 vaccinated individuals of 1 to 29 years of ages in Gambia from 2008–2009, the result showed 10.2% anti-HBc and 11.5% HBsAg overall positivity and HBV vaccine efficacy of 95.1% against chronic infection and 85.4% against total HBV infection [72]. Similarly, a retrospective study in age ranging from 1 to 15 years of 96 Ivorian children found 24% anti-HBc and 17.4% HBsAg positivity [73]. The risk for acquisition of HBV infection among 5-9 years was associated with being born in a non-health facility reported from central Lao People's [74].

Moreover, a cross-sectional study by Abushady and his colleagues, evaluated HBV vaccine efficacy in 600 Egyptian children aged 2-13 years after 12 years of the start of the vaccination program. They found that their anti-HBs levels were meaningfully high with vaccine efficacy of 83% in children 4-13years with 0.81% anti-HBc and 2.04% HBsAg carriage [75]. A similar study from 2010 to 2013 among children aged from 9 months to 16 years in Egypt found that 57.2% overall sero-protection rate and no significant difference of anti-HBs concentrations by gender. According to this study, history of hospital admission, surgical operation and blood transfusion significantly lowered sero-protective rates of the children [17]. In a community based survey conducted in Northern Uganda showed that 21.9% prevalence of HBsAg among 1 to 14 years of children and anti-HBc antibody positivity of 48% as marker of lifetime exposure [76]. A study from Sudan indicated that 39.9% anti-HBc and 21.3% HBsAg positivity and vaccinated children had reduced rates of exposure compared to non-vaccinated [77].

2.5. Prevention and treatment

Strategies to fight HBV infection comprise treating the chronically infected patients, interrupting the route of transmission and immunizing susceptible individuals. Among them, vaccination is the most effective by preventing individuals from contracting HBV infection [78]. Chronic hepatitis B infection can be treated with drugs, including oral antiviral agents. Treatment can slow the progression of cirrhosis, reduce incidence of liver cancer and improve long term survival. World Health Organization recommends the use of oral treatments - tenofovir or entecavir, because these are the most potent drugs to suppress hepatitis B virus and rarely lead to drug resistance as compared with other drugs [12]. In most people, however, the treatment does not cure hepatitis B infection; therefore, most people who start HBV treatment must continue it for life. Treatment using interferon injections may shorten treatment duration, but its use is less feasible in low-resource settings due to high cost and significant adverse effects requiring careful monitoring [12].

2.5.1. HBV vaccine

The first HBV vaccine was prepared from the plasma of asymptomatic carriers of HBV in the form of purified inactivated HBsAg particles [79]. Plasma derived HBV vaccines have been demonstrated to be highly immunogenic, efficacious and safe. However, the use of this vaccine was dropped due to concern regarding the safety of a human blood-derived products, the inconsistency of a source of raw viral particles and the availability of new recombinant vaccines produced from yeast transfected with vector plasmid with DNA sequence coding for the production of soluble r-HBsAg proteins that are easily purified from the yeast proteins [80]. The r-HBsAg vaccinations induce neutralizing antibodies (anti-HBs) that are directed mainly towards the “a” determinant of HBsAg in all HBV genotypes [81].

The hepatitis B vaccine is the mainstay of hepatitis B prevention. World Health Organization recommends that all infants receive HBV vaccine as soon as possible after birth, preferably within 24 hrs and followed by 2 or 3 doses to complete the primary series. The complete vaccine series induces protective antibody levels in more than 95% of infants, children and young adults. Protection lasts at least 20 yrs and is probably life-long, thus WHO does not recommend booster vaccination for persons who have completed the 3 dose schedule [12].

The vaccine has an excellent record of safety and effectiveness. Since 1982, over 1 billion doses of hepatitis B vaccine have been used worldwide. In many countries where between 8–15% of children used to become chronically infected with the hepatitis B virus, vaccination has reduced the rate of chronic infection to less than 1% among immunized children [12]. The success of this vaccine was first demonstrated in Taiwan, which was one of the first countries to implement universal vaccination. The HBsAg carrier rates in children decreased from 10% in 1984 to < 1% in 2004, with a concomitant 68% decrease of acute liver failure in infants younger than 12 months, and a 75% reduction of HCC in children aged 6 to 14 years [82]. HBsAg prevalence decreased dramatically after the implementation of yeast-derived r-HBsAg vaccine for 12 years for children in HBV endemic areas in China, with no need for booster immunization [83].

The efficacy of HBV vaccination of infants in preventing HBV transmission has also been confirmed in multiple studies conducted in Africa, with reported efficacy od >83% against HBV infection and sero-protection rate of >57.2% [71, 72, 75, 17]. Factors shown to reduce HBV vaccine efficacy in infants include time since vaccination, male gender, history of hospital admission (OR 1.3), surgical operation (OR 1.4) and blood transfusion (OR 1.5), incomplete vaccine regimen were independent risk factors for breakthrough HBV infection.

Active and passive immunoprophylaxis in children were not showm to prevent MTCT in 10% of children [15]. The reasons for this outcome may be due to vaccination failure, occult infection or immune-suppression. If the maternal HBV load is >9 log10copies/ml, nearly 30% of the infants are infected [84; 85]. Vaccination failure often as a result of incomplete or delayed vaccination, high HBV DNA levels in mothers leading to in utero transmission, vaccine escape mutations or low response to vaccine and/or waning anti-HBs levels [85; 86]

In Ethiopia, feed-back on the coverage rate of vaccination and its sero-protection in the community has been ignored for a long period due to various reasons. In addition, there is no information on the prevalence of HBV infection among children in our study area. Hence, this study attempts to fill this gap and also considers additional factors like mother's educational level, marital status and occupation if they have a significant association or not

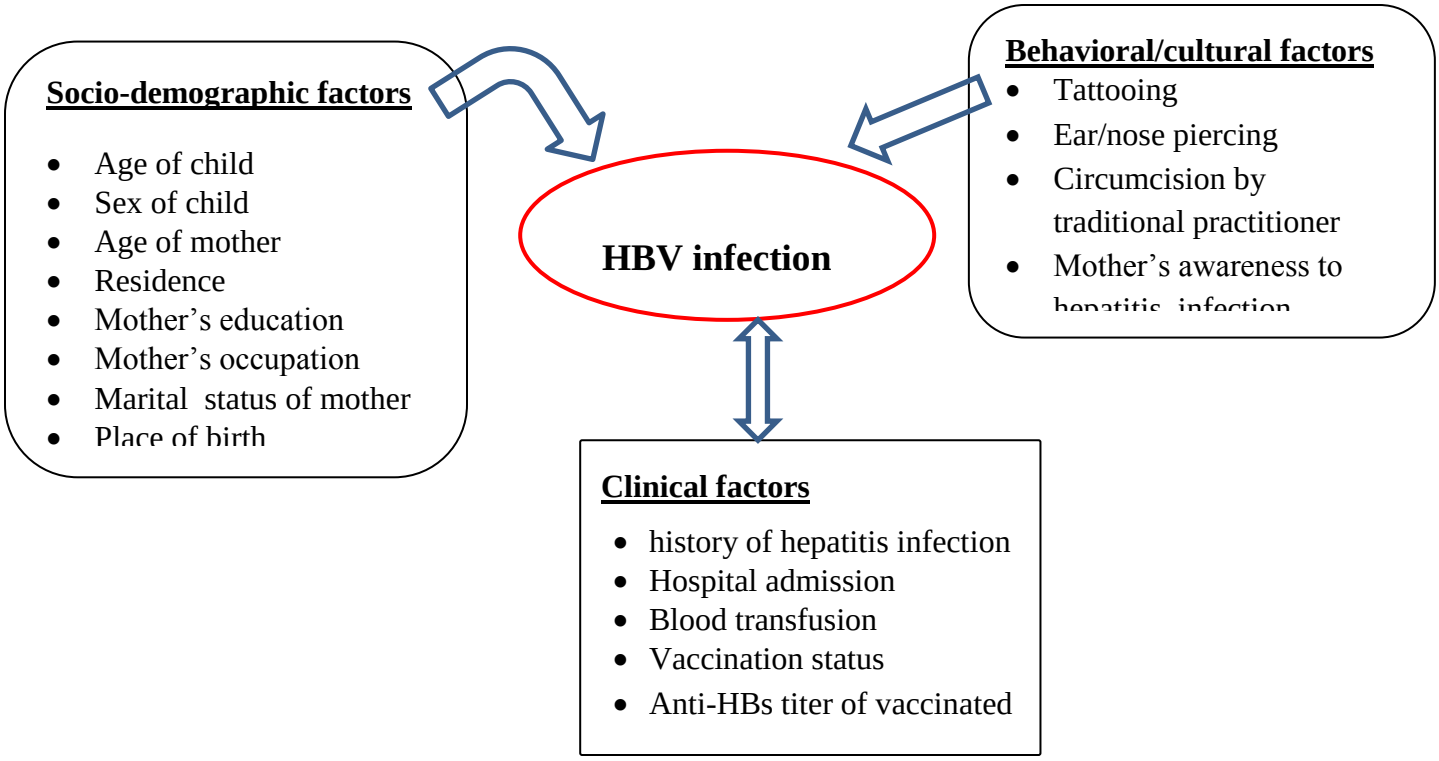


Figure 5- Conceptual frame work adapted from literature

2.6. Significance of the Study

The implementation of universal vaccinations against HBV has significantly reduced the prevalence of HBsAg-positive individuals and aimed to activate immune-system in order to produce enough concentration of anti-HBs in serum. Serum HBsAg was considered as a marker of chronic HBV infection whereas anti-HBs levels of ≥10 IU/L was associated with protective immunity level. Therefore the vaccinated children especially living in HBV endemic areas should be carefully monitored for vaccine response and HBV infection. Hepatitis B virus is regarded as a serious public health issue in Ethiopia, however disease burden and vaccine efficacy among children is not well known.

The outcomes of the findings can help in the evidence- based information about the protection level of the vaccine for the policy makers at the national and local health bureaus. Furthermore, information on current prevalence of HBV infection in the most vulnerable group vis a vis Ethiopian children. The findings will also help in targeting interventions to prevent, treat, or mitigate the impact of HBV infections among children of the study place and it can be used as a base line data for further investigations.

2.7. Research Question

The research question of this study was:

- What is the sero-prevalence of Hepatitis B virus infection and likely associated factors for infection to occur as well as the extent of HBV vaccine induced antibodies response and its sero-protection among children of 5 to 9 years of age in Jimma town, Southwest Ethiopia?

CHAPTER THREE: - OBJECTIVES

3.1. General Objective

Assess the sero-prevalence of hepatitis B virus infection and sero-protection of HBV vaccine among children in Jimma town, Southwest Ethiopia.

3.2. Specific Objectives

- To determine the sero-prevalence of HBV infection among HBV vaccine vaccinated and non-vaccinated children of 5 to 9 years old.
- To identify the significantly associated risk factors for acquisition of HBV infection among children of 5 to 9 years old.
- To determine the prevalence of protective anti-HBs level among vaccinated children of 5-9 years old.
- To identify the sero-protection of HBV vaccine in 5 to 9 years vaccinated children in the study area.

CHAPTER FOUR: - MATERIALS AND METHODS

4.1. Study Setting

The study was done in the Jimma town of Southwestern Ethiopia which is located in the Jimma zone, Oromia region. It is located at 356 kms from Addis Ababa, capital city of Ethiopia. The town has mean altitude of 1780 meters above sea level and it is the largest town in Southwestern Ethiopia characterized by tropical climate of heavy rainfall and warm climate having a mean annual temperature and rainfall of 24.9 ^{0}C and 800-2500 mm^{3}, respectively [87].

According to the data from the town health department during the study period, the total population was estimated to be 194,139. Out of these male is 98,907 and female 95,233. The town undertakes its administrative duties and responsibility with one municipality at the top and under this, three sub cities, and 13 urban Kebeles as well as 4 newly merged surrounding Kebeles. According to the town health department data the Primary health services of the town are provided by 2 hospitals and 4 health centers and additionally the town also has 44 private clinics of all types, 21 pharmacies and 31 drug stores which improve the health services of the town. Currently the health services of the town are supported by 53 Health Extension Workers.

4.2. Study Design and Period

A community based cross-sectional study design was carried out in Jimma town, southwest Ethiopia from June, 2016 to December, 2016.

4.3. Source Population:

All children living in Jimma town were considered as source population.

4.4. Study Population.

All children aged between 5 to 9 years and living in Jimma town were considered as study population

4.5. Study Unit

Randomly selected children with recommended age groups from each household which fulfill the inclusion criteria were included in the study

4.6. Eligibility Criteria

4.6.1. Inclusion Criteria

Those children aged between 5-9 years who lived at least for six months in the study area and whose parent's had been willing to participate were included in the study.

4.6.2. Exclusion Criteria

Severely ill child and children < 5 years and greater than 9 years old were excluded.

4.7. Sample Size Determination

The following standard single population proportion formula were used.

$$N = \frac{(z\alpha/2)^2}{W^2} P(1 - P)$$

where ,

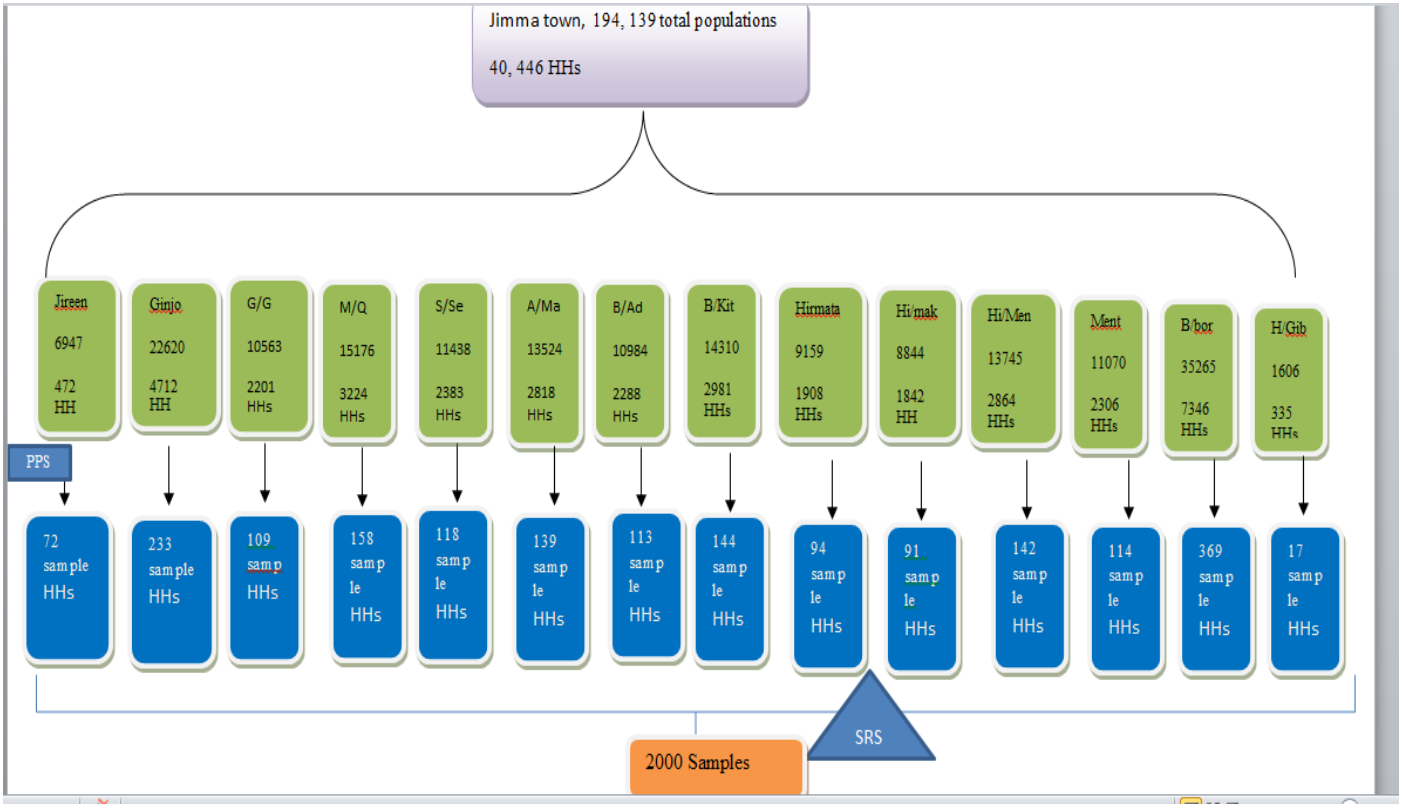
- **N** = initial sample size obtained by using the above estimation formula
- $\mathbf{Z}\alpha_{/2}^{\mathbf{2}}$ = is confidence level, i.e. 1.96 at 95% confidence level.
- **P** = is the 7% prevalence of HBV in general population from Addis Ababa[88]
- **W** = is margin of error to be tolerated 2%
- **Nf** = final sample size

$$\text{Therefore, } N = \frac{Z\text{-}\alpha/2)^2 P(1-P)}{W^2} = \frac{\mathbf{1.96^2 * (0.07)(0.93)}}{\mathbf{(0.02)^2}} = \mathbf{627}$$

The final sample size calculated by adding 15% non-response rate is Nf =722; however, 2000 children and their respective mothers recruited for the national hepatitis project by simple random sampling technique from all Kebeles of Jimma town which is higher than the calculated sample size. In this study, a total of 900 children aged between 5 and 9 years were randomly selected from the total included study participants.

4.8. Sampling Procedure

The target population comprised local residents' aged 5–9 years children and their respective mothers who were living at all Kebeles of Jimma town. There were 17 Kebeles in Jimma town including the rural and urban Kebeles were selected for this study. Proportional allocation of study participants was done based on the total population in each Kebele and a list of households which have a child with the anticipated age group was developed by using surveyors and health extension workers of the Kebele. From the prepared list, simple random sampling technique was employed to select households and only one child was recruited per each household at the time of the survey.



G/G Ginjo Gudoru, M/Q- Manadara Qochi, S/Se- Seto Semero, A/Ma- Awoytu Mandara, B/Ad Bossa Addis, B/Kit- Bossa Kitto, H/Mek Hirmata Markato, H/Men- Hirmata mentina, B/bor-Bocho Boree

Figure 6 Diagrammatic presentation of sampling procedure of households of Jimma town From June to December, 2016

4.9. Study Variables

4.9.1. Dependent Variable

- Hepatitis B virus infection
- Sero-protection

4.9.2. Independent Variables

- Age of child
- Sex of child
- Residence
- Age of the child mother
- Marital status of the mother
- Occupation of mother
- Educational status of mothers
- Place of birth
- Mother's awareness of viral hepatitis
- Ear / Nose piercing of child
- Tattooing of child
- Circumcision by traditional practitioner
- Vaccination status of a child
- Anti-HBs titer of vaccinated children
- History of hospital admission of child
- Family history of hepatitis infection
- History of blood transfusion of a child

4.10. Operational Definitions

HBV infection: - Samples which tested positive for either HBsAg or Anti-HBc antibody.

Sero-protection: - The presence of anti-HBs alone with the concentrations of 10 milli-International Units per ml (mIU/ml) or higher in serum.

Vaccination status: -

- **Vaccinated**: those who had received three doses of HBV vaccine in compliance with Ethiopian EPI schedule [18].
- **Partially vaccinated:** those children who received one or two doses of HBV vaccine,
- **Non-vaccinated**: those whose records indicated they had not been vaccinated against hepatitis B virus and
- **Unknown**: - those study participants who had no any recorded information at home or health facility.

Rural: - Those are rural Kebeles recently merged with Jimma town.

Kebele: - the smallest administrative unit in Ethiopian political system where these Kebele in Jimma town has known number of households (inhabitants) and administration.

4.11. Data Collection

4.11.1. Socio-demographic and Clinical data

A house-to-house survey was performed by face-to-face interview with sampled mothers. Socio-demographic, cultural (behavioral) and clinical data were included in the developed structured questionnaire. The detail content of the questionnaire was adapted from similar studies and developed in English, and then translated into local languages including Afan Oromo and Amharic then back-translated into English (appended at Annex-III).

For those enrolled children, vaccination against HBV and number of doses was assessed and recorded from their home immunization certificate (yellow card), or if not available, the mothers or guardians were asked about the child's vaccination status and counter checked from the nearby health facility. Otherwise in the absence of the vaccination card and the name of the child from original EPI registration logbook were recorded as *unknown*. Study participants were grouped according to their vaccination status into *vaccinated* if all the three vaccine injections were received according to the vaccination card in compliance with the Ethiopian EPI vaccination schedule. *Partially vaccinated* if a child had received one or two doses of HBV vaccine was received; and *non-vaccinated* if a child had not received the vaccine at all and *unknown* if no information about its vaccination status was identified.

4.11.2. Laboratory data

A total volume of 3 to 5 milliliter (ml) of blood samples were collected from each study participants aseptically. All acceptable precautions were followed while approaching children for sample collection. All collected samples from different Kebeles of Jimma town were transported to Jimma University Microbiology laboratory at the end of the day by using cold box with ice packs (at 4^0C). The blood sample was processed to separate serum and aliquoted into labeled sterile eppendorf tube and stored at -80 ^{0}C (Thermo Fisher scientific, USA) until the actual serologic analysis was done.

4.12. Serological Assay

Detection of HBV markers was performed at the Jimma blood bank laboratory, located to near to Jimma University, Ethiopia. A detailed laboratory testing protocol was established before testing, including the retesting of specimens with inconsistent results. All of the serum samples were tested using commercially available an Enzyme Linked ImmunoSorbent assay (ELISA) for HBsAg (Bio-Rad kit, Monolisa™, HBsAg ULTRA, La coquette, France,); for Anti-HBc (using a Monolisa ™ Anti-HBc PLUS, Bio-Rad, La coquette, France) and Anti HBs quantitatively measured (using Monolisa™ Anti-HBs PLUS, Bio-Rad, La coquette, France). Quadratic standard curve was generated with absorbance's of each test run by using immunoglobulin anti-HBs standard sets (Bio-Rad, Monolisa™, La coquette, France) in a software Microplate Manager (Bio_Rad Microplate Manager ™ version 4). The lower limit of detection for the anti-HBs assay was defined as ≥ 2 mIU/ml and serum titers of anti-HBs that were 10.0 mIU/ml of serum or greater were considered as protective for vaccinated children. The tests were carried out and interpreted in accordance with manufacturer's instructions. Sample identification plan were prepared for all ELISA test run according to the test protocol and annexed at VII whereas, the laboratory procedures and company's procedures were also annexed at Annex-IV and Annex-V.

4.13. Quality Control

To ensure the quality of data, pre-testing of questionnaire, standardization of procedures and providing training for data collectors and periodic supervision was conducted. Data collectors selected based on ability to speak the local language and team members of the survey were recruited from the same Kebeles that were under survey and the Kebele health extension worker and Kebele health development army leader were included, to implement the survey more smoothly. Every questionnaire was crosschecked daily for completeness and consistency and ELISA kits were checked for appropriate storage conditions and its expiry date.

Internal positive and negative controls were included in each assay run and Standard operation procedures (SOPs) and manufacturer instructions were strictly followed. At the beginning of the ELISA tests some of the test runs (around 13 runs) were performed under the supervision of senior expert from blood bank staff his name is Ababaw Tiruneh, and at a time we both only run either child or mam samples to maintain the quality of our test results. Duplicate test was done for those results which showed pattern on the microplate. For quantitative anti-HBs analysis, the team completely relied on the company's anti-HBs standard sets, which includes negative control (C0), 10mIU/ml (C1), 100mIU/ml (C2), 400mIU/ml (C3) and 1000mIU/ml (C4) (BIO-RAD, Monolisa™, La coquette, France) to generate quadratic standard curve.

4.14. Data management and Statistical analysis

All of the questionnaires were coded during data collection with each collected specimens to identify with initials of the Kebele so as to protect mess up. Completed questionnaires were brought and the data were partially double entered into EpiData version 3.1, checked for consistency and accuracy and finally exported to SPSS version 20 software package for analysis. The data was analyzed by using descriptive statistics like frequency tables, appropriate summary tables, crosstabs and graphs were made to present study results. Chi-square test, logistic regression test and odds ratios were used to see whether there is statistically significant association between dependent and independent variables. Variable in Bivariate analysis with P-value of < 0.25 was taken as candidates for multivariate analysis. Those independent variables which show significant association were reported by using P- value and odds ratios and with 95% CI and P- value less than 0.05 was considered as statistically significant association.

4.15. Ethical Considerations

Ethical approval was obtained from Institutional Review Board (IRB), Institue of Health, Jimma University and Armauer Hansen Research Institute ethics committee. Permission to conduct the research was granted by Jimma town Health Department. And formal letters were obtained from each health centers to their catchments and it was provided to Kebeles chair person. Similarly, written informed consent was obtained from parents or guardians of all children who participated in this study after clear discussion or explanation was made on the purpose of the study. And also assent was made for participated children.

Those children with low titers of anti-HBs and non-vaccinated, the Ethiopian Federal Ministry of Health ongoing program on EPI will be approached to provide HBV vaccine and those study participants with sero-positive for HBV markers, it is on process for molecular confirmation and then after cases will be linked to Jimma University Specialized hospital for follow up and providing appropriate information on the prevention of further transmission within the family members.

4.16. Dissemination of the Study findings

The finding of this study was submitted and will be presented to School of Medical Laboratory Sciences. Furthermore, it will also be submitted to Armauer Hansen Research Institute. Besides, part or all of the finding will also be presented in different seminars and workshops as well as shared to Federal Ministry of Health and regional health bureau and efforts will be made to publish the paper on international reputable journal.

CHAPTER FIVE: - RESULTS

5.1. Characteristics of study participants

From the total of 2000 children to their mothers pairs, 92% were completed the questionnaires and blood samples collection. The mother to child pairs was collected for national hepatitis project led by AHRI. A total of 900 serum samples of randomly selected hepatitis B vaccinated and vaccine status unknown children as well as all of non-vaccinated and partially vaccinated children were included for this study and 4 study participants were removed from analysis due to inadequate serum samples. The baseline characteristics of the 900 children and their respective paired mothers were summarized in Table 1.

The mean and median age of the mothers were 29.74 $\pm$ 6.11 and 28 years, respectively; and the mean and median ages of the children were 6.64 $\pm$1.22 and 7 years, respectively with age ranged between 5–9 years. Among all participants, 472 (52.7%) were females and a total of 470 (52.5%) were born at home helped by traditionally trained birth attendants and out of the total 380 (42.4%) were vaccinated with three doses of HBV vaccine, 227 (25.3%) were non-vaccinated and the remaining 28 and 261 were registered as partially vaccinated or unknown status respectively as shown in Table 1.

Table 1 Socio-demographic characteristics of children and mothers in mother-children pair in Jimma town, from June-December, 2016

Socio-demo variables	Category	Frequency (n=900)	Valid Percent
Residence	Urban	786	87.3
	Rural	114	12.7
Marital status	Married	822	91.3
	Widowed	33	3.7
	Divorced	45	5
Educational level	Illiterate	359	39.9
	Read and write	10	1.1
	Primary (grade 1-6)	235	26.2
	Junior (7& 8)	136	15.1
	Secondary (9-10)	102	11.3
	Preparatory (11-12)	24	2.7
	Diploma & above	34	3.7
Occupation	Employed (GO/NGO)	32	3.6
	House wife	832	92.4
	Daily labor	12	1.3
	Self-employee	17	2
	Others	7	0.7
Child's place of birth	Home	470	52.2
	Health institution	430	47.8
Child sex	Male	428	47.5
	Female	472	52.5
Age of child	5	247	27.4
	6	148	16.4
	7	239	26.5
	8	241	26.9
	9	25	2.8
Total		900	100

5.2. Distribution of HBsAg and Anti-HBc by socio-demographic variables

The overall prevalence of HBsAg among all study participants was 31/896 (3.5%) with 95% CI of (2.3% - 4.7%) and the prevalence of HBc antibody was 34/896 (3.8%), (95% CI 2.6% - 5.1%). Among 31 HBsAg positive participants 28/786 (3.6%) were urban, 23/609 (2.8%) were aged group 5-7 years old. The prevalence of HBsAg was statistically significant among children who were born at home (4.9%) than those born at health institution (1.9%) ($\chi^2 = 6.08$, $P < 0.014$) and HBsAg positivity was significantly higher in children who were born from illiterate mothers 23/359 (6.4%) ($P < 0.001$) as indicated in Table 2. Other socio-demographic characteristics such as residence of children, sex and age group were not significantly associated with HBsAg carriage of the child.

The prevalence of hepatitis B core antibody was higher in those children sampled from urban than rural Kebeles (4.1% vs 1.8%) and the anti-HBc positivity was increased among aged group 7-9 years (5.2% vs 3.1%). None of socio-demographic characteristics were statistically significant as shown in Table 2.

Table 2 The distribution of children with HBsAg and Anti-HBc positivity by socio-demographic variables using χ^2 tests

Variables		**HBsAg**			**P**	**Anti-HBc**			**P**
		Negative	Positive	Total		Negative	Positive	Total	
Residence	Urban	758	28(3.6%)	786	0.65	754	32 (4.1%)	786	0.247
	Rural	107	3 (2.7%)	110		108	2 (1.8%)	110	
Child sex	Male	409	15(3.5%)	424	0.9	410	14(3.3%)	424	0.464
	Female	456	16(3.4%)	472		452	20(4.2%)	472	
Place of birth	Home	447	23(4.9%)	470	0.014	452	18 (3.8%)	470	
	Health Inst.	418	8 (1.9%)	426		410	16 (3.8%)	426	0.954
Age group	5-7 Years	586	23(3.8%)	609	0.450	590	19 (3.1%)	609	
	7.1-9 Years	279	8(2.8%)	287		272	15 (5.2%)	287	0.124
Mom educational level	Illiterate	336	23(6.4%)	359	< 0.001	342	17(4.7%)	359	0.228
	Primary above	529	8(1.5%)	537		520	17(3.2%)	537	
Total		865	31(3.5%)	896		862	34(3.8%)	896	

5.3. Distribution of HBsAg and Anti-HBc by clinical and cultural variables

When considering the clinical and cultural variables, the HBsAg positivity was significantly higher in non-vaccinated than fully vaccinated children (16/227 (7.0%) vs 8/380 (2.1%) with $P < 0.008$). The HBsAg positivity was significantly higher in those vaccinated children who had anti-HBs titer < 10 mIU/ml of serum than children with higher titer ($P < 0.05$). The prevalence of HBsAg was significantly higher in those children who had history of hospital admission ($P < 0.001$) and family history of hepatitis infection ($P < 0.042$) as indicated in Table 3.

The total number of HBV exposure or anti-HBc positivity was statistically significant in partially vaccinated 3/28 (10.7%) than fully vaccinated children 4/380 (1.1%) with ($P < 0.001$). The total HBV infection (anti-HBc) was statistically associated with those children who had history of hospital admission ($P < 0.002$) and family history of hepatitis infection ($P < 0.001$) as shown in Table 3.

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Table 3 The distribution of children with HBsAg and Anti-HBc positivity by clinical and cultural variables using χ^2 tests

Clinical or cultural Variables	Category	HBsAg			P	Anti-HBc			P
		Negative	Positive	Total		Negative	Positive	Total	
Vaccination Status	Vaccinated	372	8 (2.1%)	380	0.008	376	4 (1.1%)	380	0.001
	Non-vaccinated	211	16(7.0%)	227		213	14 (6.2%)	227	
	Partially Vaccinated	27	1(3.6%)	28		25	3 (10.7%)	28	
	Unknown status	255	6 (2.3%)	261		248	13 (5.0%)	261	
Anti-HBs Response	<10mIU/ml	152	6(3.8%)	158	0.05	158	0(0%)	158	0.09
	>10mIU/ml	220	2(0.9%)	222		220	2(1.8%)	222	
Hospital admission	No	646	6(0.9%)	652	< 0.001	635	17(2.6%)	652	0.002
	Yes	219	25(10.2%)	244		227	17(7%)	244	
Family history of hepatitis infection	No	861	30(3.4%)	891	0.042	859	32(3.6%)	891	< 0.001
	Yes	4	1(20%)	5		3	2(40%)	5	
Circumcision	No	527	20(3.7%)	547	0.908	526	21(3.8%)	547	0.977
	Yes	337	11(3.2%)	348		335	13(3.7%)	348	
Nose or ear piercing	No	617	20(3.1%)	637	0.411	611	26(4.1%)	637	0.481
	Yes	248	11(4.2%)	259		251	8(3.1%)	259	
Blood transfusion	No	864	31(3.5%)	895	0.85	861	34(3.8%)	895	0..84
	Yes	1	.0%0	1		1	0(0%)	1	
Total		865	31(3.5%)	896		862	34(3.8%)	896	

5.4. Immunization status and mean anti-HBs distribution by age and sex

This survey found that immunization records were not well kept; the HBV vaccination history for those with no written records was obtained by interviewing the child's mothers and cross-checked from nearby health facility. Written immunization records, both home immunization card and health facility registrations were available for 940 out of the total participants (51%) of national hepatitis project.

Levels of anti-HBs were quantified and titers of anti-HBs among vaccinated children with highest titer of 719.45 mIU/ml and the anti-HBs mean, geometric mean and median antibody titer among all vaccinated children were 50, 14.2 and 11.5 mIU/ml respectively. Fifty of 380 (13.2%) children had anti-HBs levels above 100mIU/ml as indicated in fig 7. The mean serum anti-HBs titer among 5-7 years age group (52.87) were higher than 7-9 years age group (40.71) and it is also higher in female gender as indicated in fig 6.

5.5. Prevalence of protective anti-HBs by different variables

The immune response to HBV vaccine was assessed by quantifying anti-HB antibody levels among all vaccinated children. A total of 222 (58.4%) of the 380 children responded to the vaccine with anti-HBs antibody levels ≥10 mIU/ml, while 158 (41.6%) had non-protective anti-HBs antibodies level (<10 IU/ml). Of those children with protective antibody level 112/222 (50.5%) were females and 110/222 (49.5%) were males. About two third (65.6%) of vaccinated children with protective anti-HBs level were aged between 7 and 9 years. There were no significant differences in the prevalence rates of protective anti-HBs antibodies based on gender or age. Other socio-demographic or clinical or cultural variables were not significantly associated with protective level of anti-HBs as summarized in Table 4.

Table 4 The Prevalence of protective serum anti-HBs titer among vaccinated children according to socio-demographic/clinical/cultural variables from June-December, 2016.

Variables	Category	Anti-HBs titer		χ^2	P
		< 10mIU/ml	> 10mIU/ml		
Place of birth	Home	80 (46.2%)	93 (53.8%)	2.844	0.092
	Health inst.	78 (37.7%)	129 (62.3%)		
Age	5 years	55 (37.9%)	90 (62.1%)	3.395	0.494
	6 years	32 (48.5%)	34 (51.5%)		
	7years	41(44.1%)	52(55.9%)		
	8 years	27(38%)	44(62%)		
	9 years	3 (60%)	2(40%)		
Resident area	Urban	140(41.7%)	196 (58.3%)	0.009	0.924
	Rural	18(40.9%)	26 (59.1%)		
child's sex	Male	85(43.6%)	110(56.4%)	0.667	0.414
	Female	73(39.5%)	112(60.5%)		
Mother educational level	Illiterate	51(36.7%)	88(63.3%)	2.156	0.142
	Primary +	107(44.4%)	134(55.6%)		
Family history of hepatitis infection	No	156(41.4%)	221(58.6%)	0.784	0.376
	Yes	2(66.7%)	1(33.3%)		
Ear/Nose piercing	No	109(40.1%)	163(59.9%)	0.893	0.345
	Yes	49(45.4%)	59(54.6%)		
Hospital admission	No	120(42.0%)	166(58.0%)	0.068	0.794
	Yes	38(40.4%)	56(59.6%)		
Total		158 (41.6%)	222 (58.4%)		

5.6. Sero-protection of HBV vaccination

With regard to HBV sero-protection (sero-efficacy), the crude odds ratio for HBsAg positivity by comparing those fully vaccinated versus non-vaccinated was 0.284, suggesting 71.6% vaccine efficacy against chronic HBsAg carriage whereas for anti-HBc positivity was 0.162, implying 83.8% vaccine efficacy against total HBV infection as shown in Table 6 .It can be calculated by

$$\text{Sero-efficacy} = \frac{\text{AR non-vacc} - \text{AR vacc sample}}{\text{AR non-vacc}}$$

Where *ARnon-vacc: - Attacked rate among non-vaccinated children*

ARvacc: - Attacked rate among non-vaccinated children

Additionally the observed differences in the prevalence rates of anti-HBc between vaccinated and non-vaccinated was 1.1% vs 6.2%, P = 0.001 and the HBsAg positivity was 2.1% vs 7.0%, P < 0.008, suggesting HBV vaccination was successful in preventing the infection as indicated in Table 3.

Table 5 Efficacy of HBV vaccine against chronic infection and total HBV infection among all fully vaccinated in infancy after 5-9 years at Jimma town from June - December, 2016

HBV vaccine	HBsAg+ %	Odds ratio	95 % CI of OR	P	Anti-HBc + %	Odds ratio	95 % CI OR	P
Non-vaccinated	16/227 (7.0%)	1	-	0.004	14/227 (6.2%)	1	-	
Vaccinated	8/380 (2.1%)	0.284	0.119- 0.674		4/380 (1.1%)	0.162	0.053 -0.498	0.001

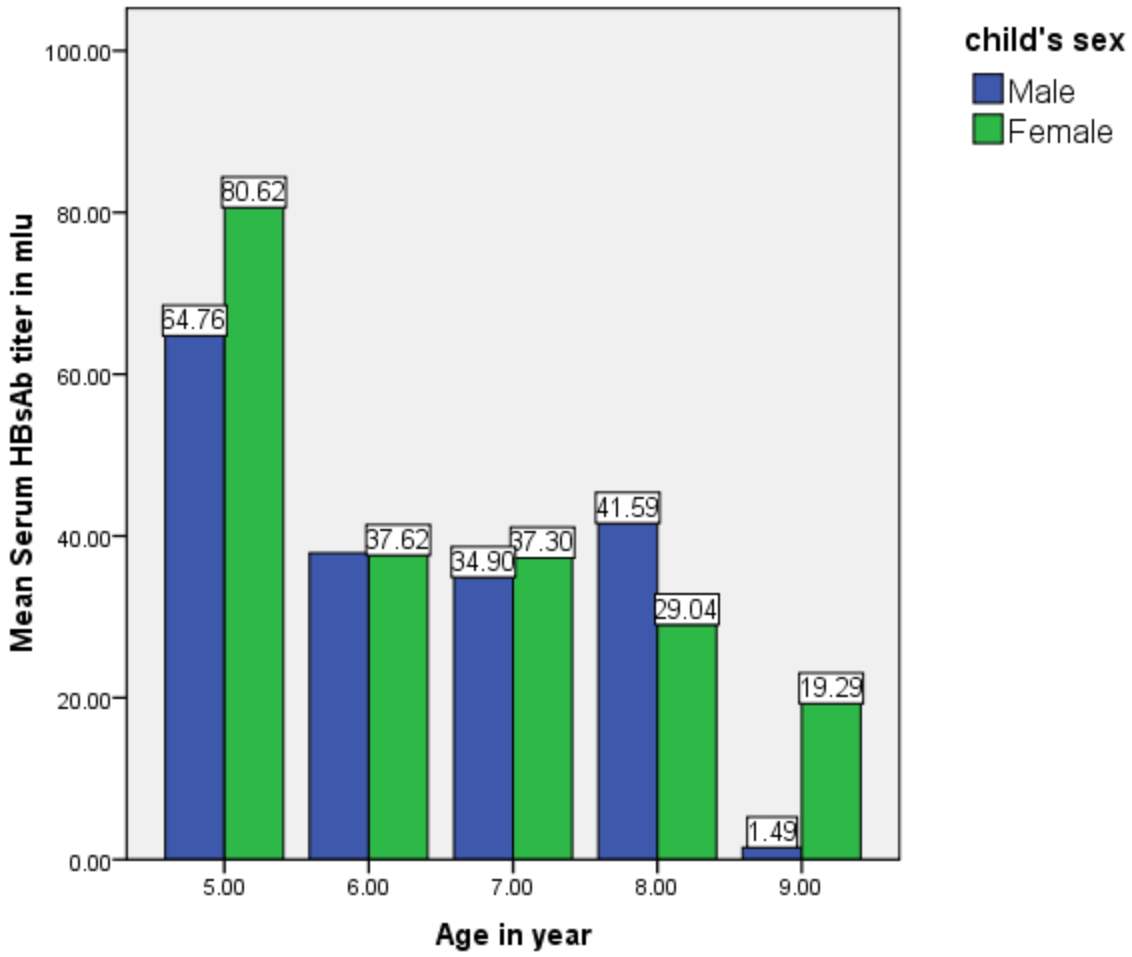

Figure 7 Distribution of mean and SE of anti-HBs titer among vaccinated children by age groups and sex

5.7. Bivariate logistic regression analyses of HBsAg positivity

Different associated risk factors within socio-demographic, cultural practices and clinical factors of study participants which might have association with prevalence of hepatitis B virus infection were analyzed. In bivariate analysis, a variety of independent variables such as lack of HBV vaccine vaccination (COR 3.526; $p < 0.004$), children who were born at home (COR 2.688; $p < 0.017$), hospital admission (COR 12.292; $p < 0.001$), family history of hepatitis infection (COR 7.175; $p < 0.082$), were significantly associated with hepatitis B surface antigen positivity (Table 6).

Table 6 Bivariate logistic regression analysis of HBsAg prevalence for predictor variables

Variables	Category	OR	P
Place birth	Health institution	1	
	Home	2.688	0.017
Vaccination status	Vaccinated	1	
	Non-vaccinated	3.526	0.004
Hospital admission	No	1	
	Yes	12.291	< 0.001
Family history of hepatitis infection	No	1	
	Yes	7.175	0.082
Residence	Urban	1	
	Rural	1.318	0.655
Educational level	Diploma and above	1	
	Illiterate	2.259	0.432
Awareness	Yes	1	
	No	0.983	0.985
Sex	Male	1	
	Female	1.045	0.904
Age	5-9 years	0.85	0.281
Ear/nose piercing	No	1	
	Yes	1.368	0.413

5.8. Multiple logistic regression analyses of HBsAg positivity

After adjusting for potential confounding effects in the multiple logistic regression analysis, hospital admission (AOR = 15.342; P <0.001), children who were born at home (AOR = 3.211, P < 0.009), lack of HBV vaccine vaccination (AOR = 2.788, P < 0.029) remained independent predictors of HBsAg sero-positivity as shown in Table 7 below.

Table 7 Multiple logistic analysis of HBsAg prevalence for candidate variables

Variables	Category	AOR	95% CI for AOR		P
			Lower	Upper	
Place birth	Health inst	1			
	Home	3.211	1.342	7.679	0.009
Vaccination	Vaccinated	1			
status	Non-vaccinated	2.788	1.112	6.990	0.029
Hospital	No	1			
admission	Yes	15.342	6.044	38.944	< 0.001
Family history of	No	1			
hepatitis infection	Yes	2.353	0.208	26.672	0.490

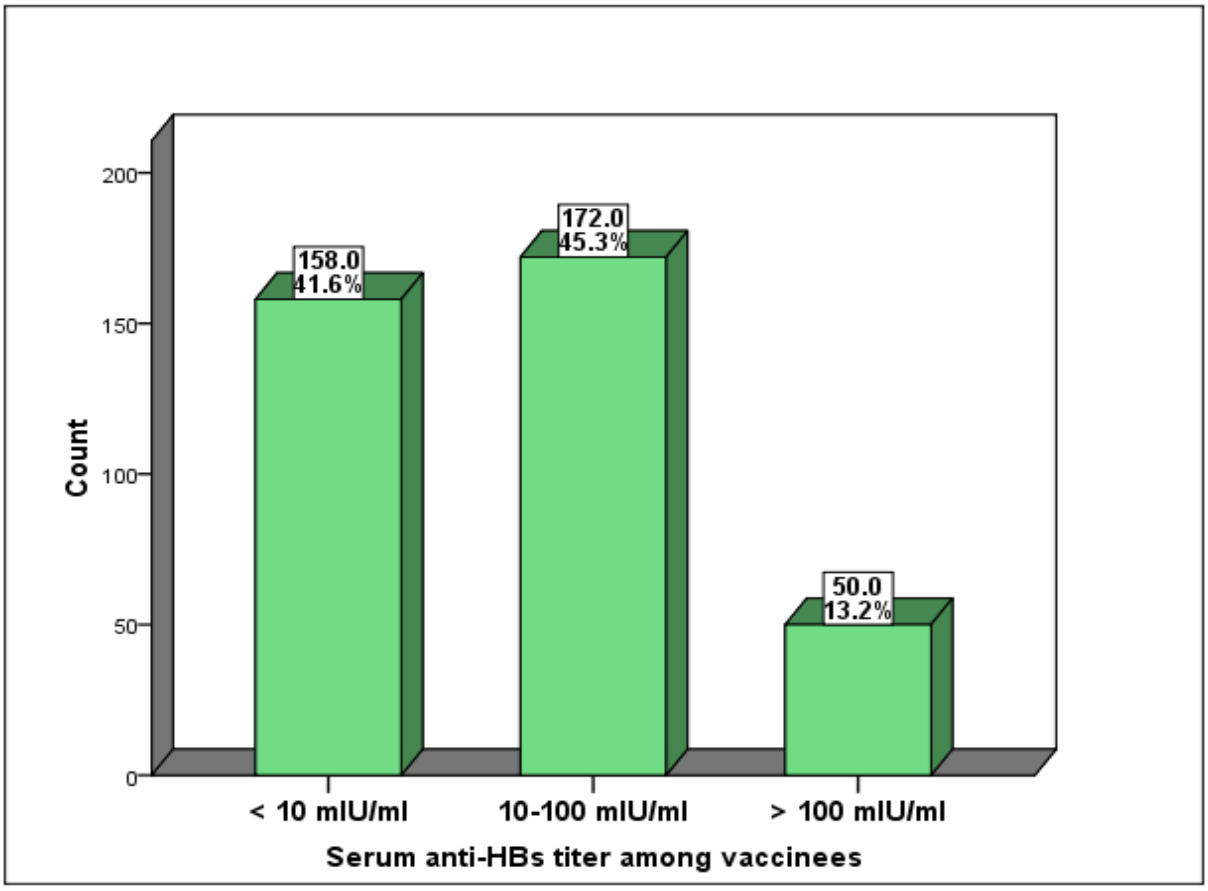

Figure 8 Serum levels of anti-HBs among vaccinees

CHAPTER SIX: - DISSCUSION

Hepatitis B virus infection is a public health problem and a major cause of morbidity and mortality particularly in developing countries. In highly endemic areas, infection occurs mainly in infancy and early childhood, with mother-to-child transmission accounting for more than half of chronic infections [21]. In Ethiopia, there is a lack of nationwide representative data on hepatitis B infections as well as in Jimma twon. In Ethiopia, the estimated prevalence ranges from 5.7 to 10.8% in general population as derived from institutional based studies [31-33]

In 1992, the World Health Organization (WHO) had recommended the integration of the hepatitis B vaccine into the national immunization programs of all highly endemic countries by 1995. However, Ethiopia introduced a universal immunization program against the virus for infants in early 2007 which was several years later than the recommended time by WHO [18]. In Ethiopia, feed-back on the coverage rate of vaccination and its efficacy in the community has been ignored for a long period due to various reasons. In addition, there is no information on the prevalence and associated factors of HBV infection as well as on HBV vaccine response rate among children in our study area and when further compiled, in Ethiopia. Therefore this study was carried out in response to this information gap.

In this study, the overall prevalence of HBsAg was 3.5% among all participants (n=896). This finding is consistent with previous study conducted in Karachi, Pakistan 3.3% [9] and Ivory Coast 4.2% [73]. However, this figure is higher than those reported from Northwest China [63] and Central Lao [74]. On the other hand it is lower than a study reported from Northern Uganda [76]. This could be explained by the fact that the socio-demographic features between study populations are different. It can also be explained by difference in HBV vaccine commencement, inclusion of birth dose of the vaccine and difference in herd immunity of the community.

This study revealed that the presence of anti-HBc is 3.8% in study participants which was comparable with previous study reported from Indonesia 3.2 % [60]. On the other hand high anti-HBc positivity was reported from other countries such as Colombia (6.2%), Gambia (10.2%), Ivory Coast (24%), Northern Uganda (48%) and Northwest China (14.1%) [66, 72, 73, 76, 63]. In contrast, the finding of this study was higher than report from Central china (2.6%) [10]. This could be due to variation in socio-demographic features or cultural practice between the study populations. It may be also explained by the difference in the period for the introduction of HBV vaccine, vaccine regimen and inclusion of birth dose of the vaccine.

In the present study, it was found that the frequency of HBsAg and anti-HBc positivity among the whole group of vaccinated children were 2.1% and 1.1%, respectively. The result of HBsAg positivity of this study was found to be consistent with previous study reported from Egypt (2.0%) [75], Nicobar, India (2.4%) [61] and Sana'a, Yemen (1.8%) [69]. This figure is lower than the previous findings reported from two villages of Gambia (11.5%) [72] and Ivory Cost (17.4%) [73]. On the other hand, it is higher than studies reported from Nigeria (1.3%) [70] and Gambia (0.8%) [71]. Beside to HBsAg prevalences, the anti-HBc positivity of vaccinated children was comparable with previous study from Egypt (0.81%) [75] although, it may be higher than finding reported from different location in Egypt, 0.36% [17]. In contrast, our finding is lower than study reported from Guangdong Province, China (3.28%) [65]. This difference may be due to difference in the management of cold chain system, administration and inclusion of birth dose of the vaccine. It can be also explained by presence vaccine scape mutants or variation in clinical or socio-demographic characteristics between study participants.

The present study found that the prevalence of HBsAg and anti-HBc among non-vaccinated children were 7.0% and 6.2%, respectively. This HBsAg prevalence of the non-vaccinated children was comparable to the previous study from Nicobar, India (9.5%) [61] and Gambia (12.4%) [71]. This figure is higher than studies reported from Northwest China [63] and Guangdong Province, China [65]. On the other hand, the anti-HBc positivity of this study is similar to a study reported from Guangdong Province, China (5.56%) [65]. However, it is lower than a study from Nicobar, India (11.9%) [61]. This difference may be due to variation in clinical or cultural practice between study participants and it may be explained by difference in herd immunity of the community.

According to this study, the HBsAg positivity is high in urban area and age group of 5-7years old as well as it is significantly higher among those children who had lower anti-HBs titer and children who were born from illiterate mothers. This finding is consistent with previous studies reported from Pakistan [68, 9]. However, our finding is inconsistent with studies reported from Henan, Anhui Province, China [62, 10], Dhaka, Bangladesh [67] and Colombia [66]. This difference may be explained by socio-demographic or cultural variation between study participants.

Hepatitis B vaccination of infants and children has been demonstrated to reduce the prevalence of HBsAg in many different populations that previously experienced high endemicity of HBV infection [71, 75,]. In this study, different HBV markers were obtained from vaccinated children. However, due to the lack of serological data either before or after vaccination among studied children, it is impossible to conclude whether these children were already infected at the time of vaccination or had been infected subsequently, in which they acquire.

In the present study, the sero-protection (sero-efficacy) against chronic HBV infection was 71.6% whereas 83.8% against total HBV infection after 5-9 years of primary vaccination. This vaccine efficacy is consistent with previous study reported from Egypt that have 83% vaccine efficacy after 12 years of HBV vaccine inclusion [75]. However, it is lower than two studies reported from Gambia (94%) and (95.1%) of HBV vaccine efficacy [71, 72]. The difference is explained by difference in HBV endemicity, inclusion of birth dose of HBV vaccine, management of cold chain system and administration of the vaccine.

Moreover, the efficacy of HBV vaccine in our finding was indicated by the reduced frequency of HBsAg positivity (AOR 3.526; $p < 0.004$) and anti-HBc positivity (COR 6.178, $P < 0.001$) among vaccinated children. Hence, hepatitis B vaccine was successful in preventing and limiting chronic carriage of HBV. The risk factor for the few vaccinated children who became HBsAg carriers may be associated with low anti-HBs antibody titer, where, this study found that 6 out of 8 HBsAg positive children had <10 mIU/ml serum anti-HBs titer. This kind of findings was also pointed out in several other long-term follow-up studies such as in Gambia [71], China [10] and Egypt [75]. The positive rate of HBsAg in children born after the introduction of the immunization program was much lower than in children born before the introduction of immunization program were reported.

Vaccine induced immunity against HBV provides protection against infection and against HBV disease progression. Protection against infection is associated with presence of antibody, which is directly related to the peak concentration of anti-HBs after primary vaccination. Protection against disease is associated with immune memory, which persists beyond the time when anti-HBs disappears [89]. On the other hand, after primary hepatitis B immunization, anti-HBs concentrations decline rapidly within the first year and more slowly thereafter. Children who respond to a primary 3-dose vaccination series, 15–50% have low or undetectable concentrations of anti-HBs after 5–15 years vaccination [90]. In the present study, 58.4% of the vaccinated children still retained protective level of hepatitis B surface antibodies after 5-9 years of primary infant immunization. This figure is similar with previous studies reported from Egypt (57.2%), Sana'a, Yemen (54.8%) and Alaska (50%) [17, 69, 59]. It is lower than studies reported from India (72.8%), Gambia (94%), (95.1%) and Egypt (83%) [61, 71, 72, 75]. On the other hand, this figure is higher than findings reported from Yemen (44.2%) and East Java, Indonesia (26.5%) [69, 60]. This difference in the findings could be attributed to different age groups, to the different degrees of exposure to natural boosters, or to differences in nutritional status and cold chain system of the vaccine.

The present study found that the protective antibodies level was higher in female gender, in children who were born at health institution and in age group 7-9 years old children. Our finding was inconsistent with a study reported from Yemen [69] and Egypt [75] and India [61]. In contrast to our finding, higher protective titer in male was reported from Yemen [69]. In this study, there was an inverse relationship observed between the mean titer of anti-HBs concentration and age, since there was decreased antibody titer with increasing age. It is consistent with many previous studies reported from Egypt [75], India [61], Yemen [69] and Alaska [59]. The reason may be related difference in immunological factors or may be explained by cultural or clinical or socio-demographic difference among study participants.

Among the vaccinated children of this study, 41.6% had low antibody level (< 10 mIU/ml), indicating a poor anti-HBs response after receiving a full course of vaccine as appeared in the result part. It can be deduced from this finding either that these vaccinated individuals were hypo-responsive to the immunization and that their antibodies may have waned rapidly over time, or that the vaccine was of poor quality. However, studies showed that protection is still maintained among vaccinees, even in HBV-endemic country despite waning or undetectable anti-HBs levels [91; 92]. Thus, WHO does not recommend booster vaccination for persons who have completed the three doses vaccination schedule and primarly responded [12].

In this study, we also assessed the associated factors for acquisition of hepatitis B virus infection. It is clear from our results that some of these factors are still important predictors of HBV infection after vaccine introduction. The hospital admission and the child's birth place were the most important independent variables identified. Children who were born at home were three times more likely to be infected with HBV than those who were born at health institution.

Other important predictor was lack of HBV vaccine vaccination; children who have not been vaccinated for hepatitis B virus were three times more likely to be infected with HBV than those who have been vaccinated for the virus. These finding was comparable with studies reported from Henan [62], China central Lao [74] and Egypt [17]. However, associated factors related to mother including rural area residence, educational level less than primary, daily wage earner and housewives as well as nose/ear piercing of the child were significantly associated with HBV infection as reported by other studies [10, 68, 9].

There were some limitations inthis study. Positive samples weren't retested due to shortage of extra ELISA kits and the risk of recall biases are the major limitation of this study. An other limitation of this study was positive samples not checked for viral genes.

CHAPTER SEVEN: - CONCLUSION AND RECOMMENDATION

7.1. Conclusion

The sero-prevalence of hepatitis B infection is high among children who have not been vaccinated for the virus, indicating that the infant HBV vaccination effectively prevented the transmission of the virus for a period of up to 10 years. The prevalence of protective anti-HBs level (> 10 mIU/ml) is low among fully vaccinated children after 5-9 years of primary infant immunization, implying inclusion of birth dose of the HBV vaccine necessary to increase its efficacy. There is significant number of chronic carriers among non-responder of the vaccine, implying the importance of evaluating primary response of the vaccine in children and its underlining causes. We also noted higher prevalence among children who were born at home, suggesting likely poor sanitation and high risk of contamination especially using sharp materials. The findings also highlight the need for infection prevention system in our health facilities.

7.2. Recommendation

- Hepatitis B vaccine has contributed to the reduction of the infection in this endemic area, though further efforts are required to improve a timely vaccination and its coverage for children. .
- Considering the coverage of HBV vaccine among 5-9 years children was low, it is better if re-vaccination strategy or catch-up vaccination policies can be developed
- There is significant numbers of children chronically infected with HBV in the study local, hence, health education on its modes of transmission and prevention should be promoted and strengthened in the public.
- The prevalence of protective anti-HBs level (> 10 mIU/ml) is low among fully vaccinated children after 5-9 years of primary infant immunization, hence, it is better to include the monovalent birth dose of the vaccine and conduct further studies to evaluate underlining cuases for wanning of serum anti-HBs level.
- Continued follow-up study is needed to assess the effectiveness of the vaccine in study area

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9. ANNEXES

ANNEX I: INFORMATION SHEET

1. English Version

Title of the project: Magnitude of hepatitis B virus infection, its associated factors and efficacy of hepatitis B vaccination among children in Jimma town, Southwest Ethiopia

Name of Principal Investigator: Rebie Kedir

Organization: Jimma University (School of Medical laboratory sciences)

Name of sponsor: AHRI **(FMOH)**

This information sheet is prepared for the parents of children and whose children will be involved in project entitled above. I am going to tell you about the whole process that will happen in the study and requesting you and your child to participate in the study voluntarily.

Description and Purpose of the study

Hepatitis B virus (HBV) is the leading cause of viral hepatitis and about 240 million people worldwide are chronic carrier. It is mostly acquired in endemic area either through perinatally or in early childhood. The virus was known also widely prevalent in Ethiopia and has several serious complications. The vaccines used for the virus have an outstanding record of safety and effectiveness; however, about 10% of the vaccinees still remain susceptible to HBV, especially those vaccinees were from infected mothers. The expected impact of infant immunization needs to be monitored and children should be carefully monitored for HBV infection and vaccine efficacy. The purpose of this study is to examine HBV vaccine protection and to determine the magnitude of HBV infections and its associated risk factors in children.

Procedures

If you are willing to participate in the study, you will be asked to sign a consent form and the following procedures will be done.

- Your socio demographic and your child's history of vaccination will be reviewed
- Possible associated risk factors for acquisition of HBV infection of you and your child will be reviewed
- You will provide us a maximum of 10 minutes interview
- A total of 3ml to 5 ml blood sample will be collected from your child
- The collected sample will be processed and tested for HBV markers at Jimma university specialized hospital laboratory, Jimma University

Risks and discomforts

There is no risk and discomfort in participating in this study. During all sample collection we will follow Standard operational procedures.

Benefits and Compensation

By participating in this study, there will not be direct financial benefit. If your child is HBV positive, he/she will be referred to health personel with respected Health center and Jimma university specialized hospital for further care and treatment. If he/she negative for HBV infection and not vaccinated, HBV vaccine will be given.

Confidentiality

All information that will be collected from the study subjects will be kept confidential. Any information about the participant will be stored in a file that will not bear a name on it, but only a number assigned to it instead.

Voluntary participation and withdrawal:

Your participation in this study is voluntary. You may decide not to participate or you may leave the study at any time. Your decision will not result in any penalty or loss of benefits to which you are entitled. Your decision will not put at risk at any present or future medical care or other benefits to which you otherwise entitled. You should ask the study investigators listed below any questions you may have about this research study. You may ask questions in the future if you do not understand something that is being done.

Use the following address for any question.

Mr. Rebie Kedir, Phone No +251 913189564, Email: rebiekedir11@gmail.com

Dr. Tesfaye Kassa, Phone No +251 9 31057195 Email: tesefaye.kassa@ju.edu.et

Mr LuleTeshager phone No +251 922783320 Email: lule_teshager2007@yahoo.com

Mr.Belayneh Dimah, Phone No +251 911743623, Email: belaydimah@gmail.com

1. Afan Oromo version

Duudha Infoormeeshinii afaan oromootiin

***Mata duree pirojektiicha**: :* Godina jimmaatti naannawa Dhaabbilee fayyaa filatamanitti gandoota jiran keessa daa'imman jiraatan kan umriin isanii waggaa 5-9 irratti tamsaasa dhukkuba tiruu (Heepaataayitas B) fi dandeettii dhoorguu taalaallii isaa beekuuf

Maqaa invasigaatara jalqabaa: Rabii'I Kadiir

Maqaa ispoonsaraa: AHRI **(FMOH**

Duudha informeeshina kun kan qophaayee.akkuma olitti ibsameen qorannoorratti kan hirmaatan matii ijoolleewwanii yookaan guddistoota (abbaa yokaan haadha) yoo ta'u walii galatti waa'ee qorannoo dhimma bekuu barbaannee fi waa'ee qorannoo ibsa walii gala kennuufi. kanatti aansine adeemsa qoranno kana keessatti ta'u siif ibsine fedhii hirmaannakee sigaaffana.

Ibsafi kaayyoo qoranno kana: dhukkubni tiruu (Heepaataayitas Bii) ummata addunyaa keenyaa kan biliyoona 2 ta'an kan galaafatee fi dhukkubicha talaaliidhaan ittisuun kan danda'amuu dha. Talaalliin biyya keenyatti waggaa 7 dura kan eegale yeroo ta'u dhukubichi garuu ammaas bali'inaan akka argamu ragaan ni ni ibsu. Dhukubichi irra caalatti xuxuqaa dhiigaa, wal qunnamtii saalaa, haadhaa gara ilmoottii fi karaa dhangalaa'aa adda addaa kan qaama keenya keessatti argamuun namarraa gara namaatti kan darbu yeroo ta'u sababani dhukkubichaa baayeedha. Kun ta'uus biyya keenyatti waa'ee babal'insa dhukubaa, sababa isaa fi dandeettii ittisa talaallii isaa beekuuf qorannoon qoratame hin jiru. Waan ta'eef kaayyoon guddaan qorannoo kanaa Godina jimmaatti daa'imman umriin isaanii waggaa 5-8 jiran irratti dhukkuba tiruu(heepaataayitas Bii) hanga isaatii fi amma taalaalli Heepaataayitas Bii kan kennamaa jiru dandeettii ittisinsa isaa qorachuudhaafi.

Adeemsa: qoranno armaan oliitti ibsame kanarratti hirmachuuf yoo fedhii qaabaattee gaaffii qorannoo kanaan walqabate fudhanna.

- Gaaffii daqiiqaa 15niif godhamaaf
- Galmeen talaallii ilmoo ishee ni ilaalama
- Ilmoo isheerraa dhiigni fudhatamee qorannoon ni godhama

wantoota walitti qabame laabaraatoorii yuunivarsitii jimmaatti qorannoon ni godhamaaf

Balaa gahuu danda'u: walii galatti dhiiga olitti dubbatame kana yeroo fudhatamutti dhukkubbii yeroo kan marfee dhiigni ittiin fudhatamuun dhufu malee rakkoon biraa akka hin qunnamne mirkanaayeera.

Faayidaa: qorannoo kanarratti ilmoon ishee hirmaachuuf maallaqni kennamuuf hin jiru. Hirmaattonni qorrannoo kana bu'aa qorannoorratti hundaa'uun yoo ilmoo isaanii keessatti Heepaataayitas Biin argame kunuunsaa fi yaalaminsa caaluuf gara hoospitaala yuunivarsitii jimmaatti ni ergamu. Yoo dhukkubicha hin qabnee fi hin talaalamin ta'an talaalliin Heepaataayitas Bii akka kennamuuf ni godhama.

Iccitii : infoormeeshina hirmaattota irra argame iccittiinsa kan eegame ta'a akka nama sadaffatti hindabarre akkasumas informeeshinni kun kan inni kusaamu koodiidhan ta'a.

Hirmaanna fedhiirratti hundaayeef yeroo fedhanitti addaan kutuu: hirmaannan qoranno kana fedhiirratti kan hundaayeedha. Yeroo feetanitti addan kutuuf mirga guutuu qabdu kanaaf murtoon keessan dhiibbaa tokkoyyuu isinirraan hin ga'u.

Gaaffii yoo qabaattan odeeffanno armaan gadii fayyadama

Waan isinii hin galle yoo jiraate ittigaafatamtoota qorannoo kana gaditti caqasaman gaaffachu ni dandeessu.

Obboo Rabii'I Kadiir Bilb +251-913189564, Email: rebiekedir11@gmail.com

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2. Amharic version

የጥናቱ ርዕስ፡ በጅማ ከተማ አድሜያቸዉ 5 እስከ 9 ዓመት በሞሳቸዉ ህፃናት ላይ የጉበተ በሽታ (ህፓታይተስ ቢ) ክስተት መጠን እና የክትባቱ መከላከል አቅም ማወቅ

የዋና ተመራማሪ ስም: ረቢዕ ከድር

የድርጅቱ ስም፡- አርማዎር ሀንሰን ምርምር ማዕከል (ጤና ፕበቃ ሚንስቴር)

ይህ የመረጃ ቅጽ የተዘጋጀዉ ከላይ በተጠቀሰው ጥናት ስሚሳተፉ የልጆች ወላጅ ወይም አሳዳጊ (አባት ወይም እናት)ሲሆን በአጠቃላይ በጥናቱ ውስጥ ልናካሂዳቸው ስለፈለግናቸው ጉዳዮች እና ስለጥናቱ ጠቅላላ ማብራርያ ለመስጠት ነዉ፡፡ በመሆኑ በጥናቱ የሚሳተፉት በራሳቸዉ ፍላጐት ብቻ መሆኑን በትህትና ገልፀንላቸዋል፡፡

ስለጥናቱ በጥቂቱ

የጉበት በሽታ (ህፓታይተስ ቢ) በአለማችን ወደ 2 ቢሊዮን የሚሆን ሕዝብ ያጠቃ ሲሆን እና በሽታዉ በክትባት መከላከል የሚቻል ነው፡፡ ክትባቱ በሀገራችን ሰባት ዓመት አከባቢ የተጀመረ ሲሆን በሽታዉ ግን አሁንም በስፋት እንደሚገኝ መረጃዎች ይጠቁማሉ:: በሽታው በአብዛኛው በደም ንኪኪ፣ በግብረስጋ ግንኙነት፣ ከእናት ወደ ልጅ እና በተለያዩ በሰዉነታችን ወስጥ በሚገኙ ፈሳሾች አማካይነት ከሠው ወደ ሰው የሚተላለፍ ሲሆን የበሽታ ተጉዳኝ ምክንያቶች በርካታ ናቸዉ፡፡ ይህ ቢሆንም በሀገራችን ስለ በሽታዉ ስፋት፣ ተጉዳኝ ምክንያቶች እና የክትባቱ መከላከል አቅም ለማወቅ በህፃናት ላይ የተጠና ጥናት የለም፡: ስለሆነም የዚህ ጥናት ዋና አላማ በጅማ ከተማ በሚኖሩ አድሜያቸዉ ከ5-9 ዓመት ባሉት ህፃናት ላይ ያለዉ የህፓታይተስ ቢ (የጉበት በሽታ) ክስተት መጠን እና አሁን በመሰጠት ላይ ያለዉን የህፓታይተስ ቢ ክትባት የመከላከል አቅም ለማጥናት ነዉ፡፡

የጥናቱ ሂደት ዝርዝር

በጥናቱ ለመሳተፍ ከተስማሙ የሚከተሉትን መረጃዎችና ናሙና እንወስዳለን፡

- የ15 ደቂቃ ቃለ መጠየቅ ይደረግላችዋል፡፡
- የልጅዎ የክትባት መዝገብ ይታያል::
- የልጅዎ ሕክምና መረጃ ይታያል፡፡
- ከልጅዎ የደም ናሙና ተወስዶ ምርመራ ይደረጋል ::

በተሰበሰበዉ ናሙና በጅማ ዩኒቨርሲቲ ሆስፒታል ላቦራቶሪ ከጥናቱ ጋር የተያያዙ የህፓታይተስ ቢ ምርመራ ይደረጋል፡፡

ስጋትና ጉዳት

በአጠቃላይ ከላይ የተጠቀሰዉን ናሙና በሚወሰድበት ጊዜ በናሙና መወሰጃ መርፌ አነስተኛ የሆነ ጊዜያዊ ህመም ወጪ ሌላ ምንም ችግር አንደማያጋጥም የተረጋገጠ ነዉ፡፡

ሊያስገኛቸው የሚችሉት ጥቅሞች

በዚህ ጥናት ውስጥ ልጅዎ በመሳተፉ በጥሬ ገንዘብ የሚደረግ የካሳ ክፍያ አይኖርም ፡፡ የጥናቱ ተሳታፊዎች በምርመራ ዉጤት ላይ በመመርኮዝ ህፓታይተስ ቢ በልጅዎ ከተገኘበት ለበለጠ ህክምና እና እንክብካቤ ወደ ጅማ ዩንቨርሲቲ ሆስፒታል እንድሄዱ ይደረጋል እና በሽታዉ የሌላቸዉና ያልተከተቡ ከሆነ የህፓታይተስ ቢ ክትባት እንድሰጣቸዉ ይደረጋል፡፡

የጥናቱ ምስጢራዊነት

ማንኛዉም በጥናቱ የተገኙ መረጃዎች ምስጢራዊነቱ የተጠበቀ ነዉ:: የጥናቱ መረጃዎች በሙሉ የተቀመጡት ለጥናቱ ተብሎ በሚሰጠው ስውር ቁጥር ሲሆን ጥናቱን ከሚያስከሄዱት ባለሙያዎች በስተቀር ማንም ሊያውቅ አይችልም፡፡ የጥናቱ ተሳታፊ ማንነት በሚገልጥ መልኩ የተዘጋጁውን መረጃ የጥናቱ ተሳታፊ በፊርማዉ የተረጋገጠ ፍቃድ ሳይሰጥ ይፋ አይደርግም:: ይህ ጥናት ሳይንሳዊ መረጃ እንደ መሆኑ መጠን በወረቀት ታትሞ ቢወጣ ወይንም በሚድያ ቢነገር የጥናቱ ተሳታፊ ስም በምንም መልኩ አይጠቀስም::

ያለመቀበል ወይም ጥሎ የመውጣት መብት

በዚህ ጥናት ዉስጥ የሚኖርዎት ተሳትፎ ሙሉ በሙሉ ፈቃደኝነት ላይ የተመሰረተ ሲሆን በማንኛውም ጊዜ ይህንን ጥናት የማቋረጥ መብታቸዉ ሙሉ በሙሉ የተጠበቀ ነዉ፡፡ በጥናቱ ባለመሳተፋቸዉ ወይም ከጥናት በመገለላቸዉ ምክንያት በአሁኑ ወይም የወደፊት የህክምና አርዳታ ላይ ተፅዕኖ አይኖርም፡፡

ጥያቄ ካለዎት

ስለ ጥናቱ ማንኛዉም ጥያቄ ወይም ቅሬታ ስኖራቸዉ የሚከተሉትን ስልኮች ወይም ኢሜል አድራሻ በመጠቀም የጥናቱን ባለቤቶች ማነጋገር ይችላሉ፡፡

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ANNEX II: - CONSENT FORM

A. English Version

Code number_____

I, the undersigned, have been informed about the study that plans to assess seroprevalence of HBV infection, its associated factors and HBV vaccine efficacy. I confirm that the objective of the study has been explained to me in the language I understand well and I am requested to my child to participate in this study.

I am also informed that all the information contained within the questionnaire is to be kept confidential. Moreover, I have also been well informed of my right to keep hold of information, decline to cooperate and make myself withdraw from the study.

It is, therefore, with full understanding of the situation that I gave the informed consent voluntarily to the researcher to collect 5ml of venous blood sample from my child for the investigation.

Name of participant _____

Signature _____

Date _____

Signature (Investigator) _____

B. Afan Oromo version

Lakkoofsa adda hirmaataa _____

Maqaa hirmaataa _____

Akkam bulten /akkam olten

Ani hirmaataan maqaan kiyya olitti ibsame Godina jimmaatti naannawa Dhaabbilee fayyaa filatamanitti gandoota jiran keessa daa'imman jiraatan kan umriin isanii waggaa 5-8 irratti tamsaasa dhukkuba tiruu (Heepaataayitas B) fi dandeettii dhoorguu taalaallii isaa beekuuf yaadame irratti afaan naaf galuun natti himameera ykn naaf ibsameera. Ragaan waa'ee fayyaatii fi naamunaa dhiigaas utuu rakkoo takka hin geessisne fuudhuun naaf galeera. Odeeffannoon qorannoo kana irraa argamu hunduu iccitiin akka kaa'amus irratti walii galleerra.qorannoo kana hirmaachuu yoon hin barbaadne ykn yoon addaan kute ,ammas ta'ee fulduraaf fayyadamummaa kiyyarratti rakkoo tokkoollee akka hin uumnee naaf himameera.

Nan barbaada…… hin barbaadu……

Maqaa hirmaataa…… mallattoo …… guyyaa…….

Maqaa qo'ataa…….. mallattoo…… guyyaa……

C. Amharic version

የተሳታፊው ልዩ መለያ ቁጥር _____

የተሳታፊው ስም _____

እኔ ስሜ ከላይ የተጠቀሰው ተሳታፊ በጅማ ከተማ አድሜያቸወ 5-9 ዓመት በሞላቸዉ ህፃናት ላይ የጉበተ በሽታ (ህፓታይተስ ቢ) ክስተት መጠን አና የክትባቱ መከላከል አቅም ማወቅ በታሰበው ምርምር ላይ በሚገባኝ ቋንቋ በቂ መረጃ አግኝቻለሁ፡፡ የህክምና መረጃና የደም ናሙና ምንም አይነት ጉዳት በማያደርስ መልኩ እንደሚወሰድ ተረድቻለሁ፡፡ በተጨማሪም የሚወሰዱ ማናቸውም መረጃዎች በሚስጢር እንደሚያዙ ተነግሮኛል፡፡ እንድሁም የሚጠየቀውን መረጃ ያለመስጠትና በጥናቱ ያለመሳተፍ መብት እንዳለኝ እንድሁም ከጥናቱ በማናቸውም ወቅት ራሴን ማግለል እንደምችል የተገለፀልኝ ሲሆን ይህንንም በማድረጌ ወደፊትም ሆነ አሁን የማገኛቸው የህክምና ግልጋሎቶች እንደማይጓደሉብኝ ተነግሮኛል፡፡

እስማማለሁ _____አልስማማም _____

የተሳታፊ ስም _____ፊርማ ---------- ቀን ----------

የተመራማሪ ስም ----------ፊርማ ---------- ቀን ----------

ANNEX III: QUESTIONNAIRE

Jimma University

Institute of health sciences

1. English version

Data collection tool: Assessment of hepatitis B virus infection, its associated factors and efficacy of hepatitis B vaccine among children in Jimma town, southwest Ethiopia

	Code : _____and lab ID_____	Date: _____	**Remark**
No	**Questions**	**Response category code**	
101.	Residence	☐ 1 Urban ☐ 2 Rural	
102.	What is your age?(mother) **Enter age in yrs**	_____	
103.	Marital Status	1. Married 2. Widowed 3. Divorced	
104.	What is your educational level?(mother)	☐ 1 Illiterate (cannot read and write) ☐ 2 Read and write ☐ 3 Primary (grade 1-6) ☐ 4 Junior (7&8) ☐ 5 Secondary (9-12) and above	
105.	What is your occupation? -(mother)	☐ 1 Employed (GO/NGO) ☐ 2 House wife ☐ 3 daily labor ☐ 4 Self-employee/merchant ☐ 5 Others	

106.	Sex of your child	male ☐ 0 female ☐ 1	
107.	Age of your child (enter in year)	----------	
108.	Place of birth (the child in question)	Home ☐ 0 Health institution ☐ 1	
109.	Do you know viral hepatitis infection	No ☐ 0 Yes ☐ 1	
110.	Do you know their route of transmission (mother)	No ☐ 0 Yes ☐ 1	
111.	Does your child in question vaccinated for anything in the past?	No ☐ 0 Yes ☐ 1	
112.	Does your child have vaccination certificate?	No ☐ 0 Yes ☐ 1	
113.	Date of immunization	----------	
114.	Number of doses of vaccine	----------	
115.	If a child has no certificate ask mother that the child completes his/her vaccination program	No ☐ 0 Yes ☐ 1	
116.	In which health facility he/she taken his/her vaccination	----------	
117.	If the child is male, is he circumcised by traditional practitioner?	No ☐ 0 Yes ☐ 1	
118.	Does your child in question have any history of tattooing?	No ☐ 0 Yes ☐ 1	

119.	Does your child have any history of body or ear or nose piercing?	No ☐ 0 Yes ☐ 1	
120.	Does your child have any history of hospital admission?	No ☐ 0 Yes ☐ 1	
121.	Does your child have any history of blood transfusion?	No ☐ 0 Yes ☐ 1	
122.	Is there any family member with known viral infection?	Father 1 ☐ Mother 2 ☐ Other 3 ☐ None 4 ☐	
123.	Do you know about H3IV /AIDS?	No ☐ 0 Yes ☐ 1	
124.	Have ever your child tested?	No ☐ 0 Yes ☐ 1	
125.	Result of your child HIV test	positive ☐ 0 negative ☐ 1	

2. Afan Oromo Version

Kuweeshinarii Ragaan ittiin funaanamu: Godina jimmaatti naannawa Dhaabbilee fayyaa filatamanitti gandoota jiran keessa daa'imman jiraatan kan umriin isanii waggaa 5-8 irratti tamsaasa dhukkuba tiruu (Heepaataayitas B) fi dandeettii dhoorguu taalaallii isaa beekuuf

	Lakk addaa:_____fi Lakk addaa Laaboraatoorii_____	Guyyaa: _____	**Remark**
Lakk.	**Gaaffii**	**gosaa deebii coodiidhan**	
101.	Jiraataa	☐ 1 magaalaa ☐ 2 baadiyyaa	
102.	Umriin kee meeqaa? Lakkoofsaan	_____	
103.	Gaaila	1 gaaila keessa 2 hiikamtee 3 gaailaan adda bahanii	
104.	Sadarkaan barnoota keetii maalii?	☐ 1. Hin baranne (dubbisuuf barreessuu hin dandau) ☐ 2. dubbisuu fi barreeessuu ☐ 3. idilee(1 hanga 6) ☐ 4. sadarkaa 1ffaa giddu gala(7 hanga 8) ☐ 5. sadarkaa 2ffaa ((9 hanga 12)	
105.	Hojiin kee maalii?	☐ 1 hojjataa (mootummaa /mit moot.) ☐ 2 hojjattuu/taa mana keessaa ☐ 3 dafqaan bulaa ☐ 4 Dhuunfaa ☐ 5 gara biraa	
106.	Saala mucaa keetii	dh. ☐ 0	

		du ☐ 1	
107.	Umrii daa'ima keetii	----------	
108.	Eessatti dhalatte/te	mana ☐ 0 dhabbata fayyaa ☐ 1	
109.	Waa'ee vaayirasii dhukkuba tiruu dhageessee beektaa?	eeyyee 0 ☐ hin beeku ☐ 1	
110.	Karaa kamiin akka dhufu beekta? (Haadhaaf)	eeyye ☐ hin beeku ☐ 1	
111.	Mucaan kee amma duree talaallii fudhateeraa.ttii?	eeyyee ☐ 0 lakkii ☐ 1	
112.	Waraqaa ragaa talaallii agarsiisu ni qabaa?	eeyyee ☐ 0 lakkii ☐ 1	
113.	Guyyaa talaallii itti xumure	----------	
114.	Talaallii yeroo meeqaaf fudhate	----------	
115.	Mucaan raga kaardii yoo hin qabaanne haadha gaafadhu akka mucaan talaallii xumure	eeyyee ☐ 0 mitii ☐ 1	
116.	Buufata fayyaa kamitti yaalame	----------	
117.	Daa'imti dubara yoo taate, akka aadaatiin dhaqna xaharattee/ dhaqna qabaa/kittaannaa?	eyye e ☐0 mitii ☐ 1	
118.	Mucaan keessan kanaan dura qaama isaa/isii tumatee/ttee beekaa/tii	miti ☐ 0 eeyyee ☐ 1	
119.	Mucaan keessan kanaan dura gurra, qaama, funyaan uratee ni beekaa?	miti ☐ 0 eeyyee ☐ 1	

120.	Mucaan keessan kanaan dura hoospitaala ciisee ni beekaa?	miti ☐ 0 eeyyeen ☐ 1	
121.	Mucaan keessan kanaan dura dhiigni nama biraa kennameefii ni beekaa?	miti ☐ 0 eeyyeen ☐ 1	
122.	Maatii keessan keessa namni dhukkuba HBV qabame ni jiraa?	abbaaa 1 ☐ Haadha 2\☐ gara biraa 3 ☐ Injiruu 4	
123.	Waa'ee HIV /AIDS ni beekuu?	eeyye 0 ☐ lakki 1 ☐	
124.	Mucaan keessaan kanaa dura Yaalamee beekaas?	miti ☐ 0 eeyyee ☐ 1	
125.	Bu'aa qorannoo HIV mucaa keetii	poozativii ☐ 0 nagativii ☐ 1	

3. Amharic version

ጅማ ዩንቨረሲቲ

የጤና ሲንስቲትዩት

መረጃ መሰብሰቢያ ; በጅማ ከተማ አድሚያችወ 5 እስከ 9 ዓመት በሞሳችዉ ህፃናት ሳይ የጉበት በሽታ (ህፓታይተስ ቢ) ክስተት መጠን አና የክትባቱ መከላከል አቅም ማወቅ

	መስያ ቁጥር : _____እና ላቦራቶሪ መስያ_____	ቀን: _____	ምርመራ
ተ.ቁ	ጥያቄዎች	መስያ ቁጥር ምላሽ	
101	መኖሪያ ቦታ	1 ከተማ 2 ገጠር	
102	ዕድሜሽ ስንት ነዉ ?	በዓመት ብቻ ግለፅ ----------	
103	የጋብቻ ሁኔታ	1. ያገባች 2. ባል የሞተባት 3. ፈት	
104	የትምህርት ሁኔታ	1. መጻፍና ማንበብ የማትችል 2. መጻፍና ማንበብ የምትችል 3. አንደኛ ደረጃ (ከ1-6 ክፍል የተማረች) 4. መለስተኛ (ከ7-8 ክፍል የተማረች) 5. ሁለተኛ ደረጃና ከዚያ በላይ የተማረች	
105	የሥራ ሁኔታ?	1 የመንግስት ሠራተኛ/መንግስታዊ ባልሆነ ድርጅት ዉስጥ የምትሰራ 2 የቤት እመቤት 3 የቀን ሠራተኛ 4 በግል የሚተዳደር 5 ሌላ (ይገለጽ) --------	

6	የልጅዎ ጾታ	0 ውንድ 1 ሴት	
7	የልጅዎ እድሜ	----------	
8	ደኽንን ልጅ የት ተገላገልሽ?	0 በቤት 1 በጤና ተቃም	
9	የጉበት በሽታ ያዉቃሉ ወይ?	0. አይደለም 1. አዎ	
10	የጉበት በሽታ በምን እንደሚመጣ ያዉቃሉወይ ? (ለእናት)	0. አይደለም 1. አዎ	
11	ደኽንን ልጅ ከዚህ በፊት ክትባት ተከትባል?	0. አይደለም 1. አዎ	
12	የክትባት ምስክር ካርድ አለዉ?	0. አይደለም 1. አዎ	
13	ክትባት የጨረሰበት ቀን	----------	
14	ክትባቱ ለስንት ጊዜ ወሰደ?	----------	
15	የክትባት ምስክር ካርድ ከሌለዉ ልጅዎ ክትባት ጨርሳል ወይ?	0. አይደለም 1. አዎ	
16	ክትባተቱ የት ተከተበ?	----------	

17	ልጅዎ ወንድ ከሆነ ተገርዞ ያወቃል ወይ?	0. አይደለም 1. አዎ	
18	ልጅዎ ከዚህ በፊት ንቅሳት ተነቅሶ ያወቃል ወይ?	0. አይደለም 1. አዎ	
19	ልጅዎ ከዚህ በፊት ጆሮ፣ ሰዉነት፣አፍንጫ ተበስቶ ያወቃል ወይ?	0. አይደለም 1. አዎ	
20	ልጅዎ ከዚህ በፊት ሆስፒታል ተኝቶ ያወቃል ወይ?	0. አይደለም 1. አዎ	
21	ልጅዎ ከዚህ በፊት የሴላ ሰዉ ደም ተሰጥቶት ያወቃል ወይ?	0. አይደለም 1. አዎ	
22	በቤተሰብ ዉስጥ በጉበት በሽታ የተያዘ ሰዉ አለወይ ? (ለእናት)	1.አባት 2.እናት 3.ሌሎች 4.የለም	
23	ስለ ኤች አይ ቪ ኤድስ ሰምቶ ያወቃሉ?	0. አይደለም 1. አዎ	
24	ልጅዎትን አስመርምሮ ያወቃሉ ወይ?	0. አይደለም 1. አዎ	
25	ለጥያቄ ቁጥር 132 አዎ ካሉ ዉጤቱ እንዴት ነበር?	0. አልተገኘበትም 1. ተገኝቶበታል 2. አይታወቅም	

ANNEX IV: LABORATORY PROCEDURES

1. Monolisa™ Anti-HBc PLUS

Procedure

1. Carefully define the sample distribution and identification plan.
2. Prepare the wash solution to working strength (20X).
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
   4.3.20 µl of positive control serum (R4) in C1, D1, E1
5. 20 µl of the first sample in F1 if this well is not used as a reagent control for the sample addition monitoring
6. Cover the wells with adhesive film
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 into all wells. The conjugate must be shaken gently before use. NB: The conjugate is coloured green.
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
12. Quickly dispense into each well 100µl of prepared development solution (R8+R9. Allow the reaction to develop in the dark for 30 ± 5 minutes RT. Do not use adhesive film
13. Add 100 µl stopping solution (R10) by using the same sequence and rate of distribution as for the substrate solution. Homogenize the reaction mixture.
14. 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.

2. Monolisa ^{Tm} Anti-HBs PLUS

Procedure

1. Carefully define the sample distribution and identification plan.
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.
4. Dilute specimens, calibrators and controls 3:4 in the Specimen Diluent R6, following one of the two procedures here below :
   4.1. 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.
   5.1. **Qualitative determination**
   - Anti-HBs Negative Control (C0) in well A1,
   - 10 mIU/ml Calibrator (C1) in wells B1, C1 and D1,
   - 100 mIU/ml Calibrator-Positive Control (C2) in well E1,
   - Samples in wells F1, G1, etc.

   5.2. **Quantitative determination**
   - 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
7. Incubate the plate for 60 ± 5 minutes at 37 ± 2°C.
8. Aspirate the contents of all wells into a liquid waste container. Repeat the washing step 4 times (minimum of 5 washes). The residual volume must be lower than 10 µl
9. If an automatic washer is used, follow the same procedure.
10. Add quickly 100 µl of the Conjugate Working Solution (R7a + R7b) to each well. Cover and incubate for 60 ± 5 minutes at 37°C ± 1°C.
11. Aspirate the contents of all wells and wash. Repeat the washing step 4 times.
12. Add quickly 100 µl of the Working Diluted Substrate Solution (R8 + R9) to each well. Allow the reaction to develop in the dark for 30 ± 5 minutes at RT. Do not use adhesive film
13. Add 100µl Stopping Solution (R10). Homogenize the reaction mixture.
14. 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.
    Before recording the results, check the correspondence between the reading and the microplate and sample distribution and identification plan.

3. **Monolisa™ HBs Ag ULTRA**

Procedures

1. Carefully establish the sample distribution and identification plan.
2. Prepare the diluted washing solution (20X)
3. Prepare the conjugate R6+R7 working solution
4. Take out R1 plate from the protective packing the support frame
5. Distribute in the wells in the following order (advisable plate distribution) :

   5.1. Wells A1, B1, C1 and D1: 100 µl of negative control (R3)

   5.2. Well E1: 100 µl of positive control (R4)

   5.3. Well F1: 100 µl of the first unknown sample if this well is not used as control (optional)

   5.4. Wells G1, H1...etc.: 100 µl of unknown sample.
6. Quickly dispense 50 µl of conjugate solution (R6 + R7) into all wells, the conjugate solution must be shaken before use. Homogenize the reaction mixture.
7. Cover the plate with new adhesive film and incubate for 1hour 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.
9. Quickly dispense into each well 100 µl of prepared development solution (R8+R9), allow the reaction to develop in the dark for 30 + 5 minutes at RT. Do not use adhesive film.
10. Add 100 µl stopping solution (R10) 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.

    Check for agreement between the spectrophotometric and visual readings and against the plate and sample distribution and identification plans

ANNEX V: CAMPANY'S PROCEDURES

A. Monolisa™ HBs Ag ULTRA

1 plate - 96 tests	72346
5 plates - 480 tests	72348

KIT FOR THE DETECTION OF THE SURFACE ANTIGEN OF THE HEPATITIS B IN HUMAN SERUM OR PLASMA BY THE ENZYME IMMUNOASSAY TECHNIQUE

INTENDED USE

Monolisa™ HBs Ag ULTRA assay is a one-step enzyme immunoassay technique of the "sandwich" type for the detection of the surface antigen of the Hepatitis B virus (HBs Ag) in the human serum or plasma.

PRINCIPLE OF THE Monolisa™ HBs Ag ULTRA

Monolisa™ HBs Ag ULTRA assay is a one-step enzyme immunoassay based on the principle of the "sandwich" type using monoclonal antibodies and polyclonal anibodies selected for their ability to bind themselves to the various subtypes of HBs Ag now recognized by the WHO and the most part of variant HBV strains. The Monolisa™ HBs Ag ULTRA solid phase is coated with monoclonal antibodies.The Monolisa™ HBs Ag ULTRA conjugates are based upon the use of monoclonal antibodies from mouse and polyclonal antibody from goat against the HBs Ag.These antibodies are bound to the peroxidase.

CONTENTS OF THE Monolisa™ HBs Ag ULTRA

All reagents are exclusively for *in vitro* diagnostic use.

LABEL	NATURE OF THE REAGENTS	PRESENTATION	
		72346	**72348**
R1	**MICROPLATE :** 12 strips of 8 wells each, coated with monoclonal anti-HBs antibodies (mouse)	1 plate	5 plates
R2	**CONCENTRATED WASHING SOLUTION (20X** Tris NaCl buffer pH 7.4 Preservative : ProClinTM 300 (0.04%)	1 vial 70ml	1 vial 135ml
R3	**NEGATIVE CONTROL** Tris HCl buffer containing BSA Preservative : ProClinTM 300 (0.1 %)	2 vials 2 x 2.5 ml	2 vials 2 x 2.5 ml
R4	**POSITIVE CONTROL (HUMAN)** Tris HCl buffer containing BSA with addition of mixture of purified HBs Ag from ad and ay subtypes Preservative : ProClinTM 300 (0.1 %)	1 vial 2.5 ml	1 vial 2.5 ml
R6	**CONJUGATE DILUENT** Tris HCl buffer pH 7.4 containing BSA, TweenR 20, bovine immunoglobulins and mouse immunoglobulins with sample addition control reagent Preservatives : ProClinTM 300 (0,1%), Ciprofloxacine (10 µg /ml)	1 vial 8 ml l	2 vials 2 x 18 ml
R7	**CONJUGATE** Mouse Monoclonal anti-HBs antibodies and Goat polyclonal anti-HBs antibodies bound to the peroxidase. Lyophilized.	1 vial sqf 8 ml	2 vials sqf 2 x 18 ml
R8	**SUBSTRATE BUFFER** Citric acid and Sodium acetate solution pH 4.0 containing H2O2 (0.015%) and DMSO (4%)	1 vial 60 ml	2 vials 2 x 60 ml
R9	**CHROMOGEN PINK COLOURED** Solution containing tetramethyl benzidine (TMB)	1 vial 5 ml	2 vials 2 x 5 ml
R10	**STOPPING SOLUTION** 1N sulphuric acid solution	1 vial 28 ml	3 vials 3 x 28 ml

PRECAUTIONS

The reliability of the results depends on correct implementation of the following Good Laboratory Practices:

- The name of the test, as well as a specific identification number for the test, are written on the frame of each microtiterplate. This specific identification number is stated on each strip too.

Monolisa® HBs Ag ULTRA: Specific ID number = 51

Verify the specific identification number before any use. If the identification number is missing, or different from the stated number corresponding to the assay to be tested, the strip should not be used.

- Do not use expired reagents.
- Do not mix reagents from different lots within a given test run.

REMARK: For washing solution (R2, label identification : 20X coloured green), peroxidase substrate buffer (R8, label identification : TMB buf, coloured blue), chromogen (R9, label identification : TMB 11X, coloured purple) and stopping solution (R10, label identification : 1N coloured red), it is possible to use other lots than those contained in the kit, provided the same lot is used within a given test run. These reagents can be used with some other products of our company. In addition, the wash solution (R2, label identification : 20X coloured green) can be mixed with the 2 other wash solutions included in various Bio-Rad Reagent kits (R2, label identifications : 10X coloured blue or 10X coloured orange) when properly reconstituted, provided only one mixture is used within a given test run.

Contact our technical service for detailed information.

Before use, it is necessary to wait 30 minutes for the reagents to stabilise to room temperature and one hour for diluted wash buffer R2.

- Carefully reconstitute the reagents avoiding any contamination.
- Do not carry out the test in the presence of reactive vapours (acid, alkaline, aldehyde vapours) or dust that could alter the enzyme activity of the conjugates.
- Use glassware thoroughly washed and rinsed with deionized water or preferably, disposable material.
- Do not allow the microplate to dry between the end of the washing operation and the reagent distribution.

- The enzyme reaction is very sensitive to metal ions. Consequently, do not allow any metal element to come into contact with the various conjugate or substrate solutions.
- The development solution (substrate buffer + chromogen) must be coloured pink. The modification of this pink colour within a few minutes after reconstitution indicates that the reagent cannot be used and must be replaced. Preparation of the development solution can be made in a clean disposable single use plastic tray or glass container that has first been pre-washed with 1N HCl and rinsed thoroughly with distilled water and dried. This reagent must be stored in the dark.
- Use a new distribution tip for each sample.
- Well washing is a critical step in this procedure: respect the recommended number of washing cycles and make sure that all wells are completely filled and then completely emptied. Incorrect washing may lead to inaccurate results.
- Never use the same container to distribute conjugate and development solution.
- Check the pipettes and other equipment for accuracy and correct operation.
- Do not change the assay procedure.

HEALTH AND SAFETY INSTRUCTIONS

All the reagents included in the kit are intended for "*in vitro* diagnostic use".

- Wear disposable gloves when handling reagents and samples and thoroughly wash your hands after handling them.
- Do not pipette by mouth.
- The positive control R4 contains purified HBs Ag from subtypes ad and ay prepared with negative human plasma for anti-HCV, anti-HIV1 and anti-HIV2 antibodies and inactivated by warming.
- Because no known test method can offer complete assurance that infectious agents are absent, handle reagents and patient samples as if capable of transmitting infectious disease.
- Any equipment directly in contact with specimens and reagents as well as the washing solutions should be considered as contaminated products and treated as such.
- Avoid spilling samples or solutions containing samples.
- Spills must be rinsed with bleach diluted at 10%. If the contaminating fluid is an acid, spills must be initially neutralized with sodium bicarbonate and dried with absorbent paper. The material used for cleaning must be discarded in a contaminated residue container.

- Samples and reagent of human origin, as well as, contaminated material and products must be discarded after decontamination: - either by immersion in bleach at a final concentration of 5% of sodium hypochlorite (1 volume of bleach for 10 volumes of contaminated fluid or water) for 30 minutes. - or by autoclaving at 121°C for 2 hours minimum. Autoclaving is the best method to inactivate the HIV and the HBV viruses.
- Do not forget to neutralise and/or autoclave the solutions or washing wastes or any fluid containing biological samples before discarding them into the sink.
- The Safety Data Sheet is available upon request.
- Chemicals should be handled and disposed of in accordance with Good Laboratory Practices.
- Avoid any contact of the substrate buffer, the chromogen and the stopping solution with the skin and mucosa (toxicity, irritation or burn hazard).
- Some reagents contain ProClin™ 300 (0.04%, 0.1% and/or 0.5%)

Xi Irritant

R43: may cause sensitisation by skin contact.

S28-37: After contact with skin, wash immediately with plenty of soap and water. Wear suitable gloves.

• The Safety Data Sheet is available upon request.

PREPARATION OF THE REAGENTS

NOTE: Before use, allow reagents to reach room temperature (18-30°C).

1) Ready for use reagents

- ✓ **Reagent 1 (R1) : Microplate**

Each frame support containing 12 strips is wrapped in a sealed foil bag. Cut the bag using scissors or a scalpel 0.5 to 1 cm above the sealing. Open the bag and take out the frame. Put the unused strips back into the bag. Close the bag carefully and put it back into storage at +2-8°C.

- ✓ **Reagent 3 (R3) : Negative control**
- ✓ **Reagent 4 (R4) : Positive control**
- ✓ **Reagent 10 (R10) : Stopping solution**

2) Reagents to reconstitute

- ➢ **Concentrated washing solution (20X) : Reagent 2 (R2)**

Dilute 1:20 in distilled water to obtain the ready-for-use washing solution. Prepare 800 ml for one plate of 12 strips.

- ➢ **Conjugate working solution (R6 + R7)**

Gently tap the vial of the lyophilized conjugate (R7) on the work-bench to remove any substance from the rubber cap.

Carefully remove the cap and pour the content of a conjugate diluent vial (R6) into the lyophilized conjugate vial (R7).

Put the cap on and let stand for 10 minutes while gently shaking and inverting from time to time to ease dissolution.

- ➢ **Enzyme development solution : Reagent 8 (R8) + Reagent 9 (R9)**

Dilute 1:11 the chromogen (R9) in the Substrate Buffer (R8) (ex : 1 ml reagent R9+10 ml reagent R8). Stability is for 6 hours in the dark once prepared.

STORAGE CONDITIONS - SHELF LIFE

The kit should be stored at +2-8°C. When stored at this temperature, each reagent contained in the kit can be used until the expiry date mentioned on the package (except for specific instructions).

R1: After the vacuum-sealed bag has been opened, the microwell strips stored at +2-8°C in the carefully resealed bag can be used for 1 month.

R2: The diluted washing solution can be stored at +2-30°C during 2 weeks. The concentrated washing solution (R2) can be stored at +2- 30°C.

R6 + R7: After the reconstitution, the reagents can be used for 1 month if stored at +2-8°C and 8 hours if stored at room temperature (18-30°C).

R8 + R9: After the reconstitution, the reagent stored in the dark can be used for 6 hours at room temperature (18-30°C).

10 - COLLECTION AND HANDLING OF SPECIMENS

Collect a blood sample according to the current practices. The test should be performed on undiluted serum or plasma (collected with EDTA, heparin, citrate, ACD-based anticoagulants). Separate the serum or plasma from the clot or red cells as soon as possible to avoid any haemolysis. Extensive haemolysis may affect test performance. Specimens with observable particulate matter should be clarified by centrifugation prior testing. Suspended fibrin particles or aggregates may yield falsely positive results.

The specimens can be stored at +2-8°C if screening is performed within 7 days or they may be deep-frozen at -20°C for several months.

Avoid repeated freeze/thaw cycles. Samples that have been frozen and defrozen more than 3 times cannot be used. If the specimens are to be shipped, they must be packaged in accordance with the regulations in force regarding the transport of aetiological agents.

DO NOT USE CONTAMINATED, HYPERLIPAEMIC OR HYPEHAEMOLYSED SERA OR PLASMA.

REMARK: Samples containing up to 90 g/l albumin, 100 mg/l bilirubin, lipemic samples containing up to the equivalent of 36 g/l triglyceride, and hemolyzed samples containing up to 1 g/l hemoglobin do not affect the results.

11 - ASSAY PROCEDURE

Strictly follow the proposed procedure.

Use the negative (R3) and (R4) positive controls for each series of determinations to validate the test results.

1.Carefully establish the sample distribution and identification plan.

2.Prepare the diluted washing solution (refer to chapter 8).

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: 100 µl of the first unknown sample if this well is not used as control well for the validation of the sample and conjugate deposition (optional)
- Wells 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.

NB: The sample distribution can be visually controlled at this step of the manipulation: there is a difference of coloration between empty well and well with sample (Refer to section 14 for automatic verification).

6.Quickly dispense 50 µl of conjugate solution (R6 + R7) into all wells, the conjugate solution must be shaken before use. Homogenize the reaction mixture.

NB: The sample distribution can also be visually controlled at this step of the manipulation, as well as the conjugate distribution : The conjugate solution (R6+R7), which is coloured red, can be visually controlled at this step of the manipulation. (refer to section 14 for automatic verification).

7.When possible, cover the plate with new adhesive film and incubate for 1hour 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 (R8+R9), 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 coloured pink, can be visually controlled at this step of the manipulation : There is a clear difference of colouration between empty well and well containing the pink substrate solution. (refer to section 14 for automatic verification : SPECTROPHOTOMETRIC VERIFICATION OF SAMPLE AND REAGENT PIPETING)

10. Add 100 µl stopping solution (R10) 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 coloured, can be visually controlled at this step of the manipulation. After the addition of the stopping solution the pink colouration of the substrate disappears (for the negative samples) or turns from blue to yellow (for the positive samples).

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) Calculation of the negative control mean optical density : OD R3

Example

Negative control R3 OD

1 0.030

2 0.031

3 0.032

4 0.027

Total R3 OD = 0.120

Total R3 OD / 4 = 0.030 = mean OD R3

2) Calculation of the cut-off value

For each method, the cut-off value is equal to : OD R3 + 0.050

Example: OD R3 = 0.030

Cut-off value = 0.030 + 0.050 = 0.080

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 (OD R4) 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 (OD R3), 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 R3 (average value of negative control below 0.010 OD) do not use these rejection criterias for R3 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:

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

Interpretation of the results

Samples with ratio values lower than 1 are considered to be negative by the Monolisa™ HBs Ag ULTRA.

- Results just below the cut-off value (sample ratio between 0.9 and 1) should however, be interpreted with caution. It is advisable to retest in duplicate the corresponding samples when the systems and laboratory procedures permit.
- Samples with ratio values equal to or greater than 1 are considered to be initially positive by the Monolisa™
- HBs Ag ULTRA. They should be retested in duplicate before final interpretation. If after retesting of a sample, the ratio values of the 2 duplicates are less than 1, the initial result is non-repeatable and the sample is declared to be negative with the Monolisa™ HBs Ag ULTRA.
- For initial reactive or doubtful (0.9 < ratio < 1) samples, if after retesting the ratio values of at least one of the 2 duplicates is equal to or greater than 1, the initial result is repeatable and the sample is declared to be positive with the Monolisa™ HBs Ag ULTRA test, subject to the limitations of the procedure, described below.
- The samples which have been retested twice and found negative with Monolisa™ HBs Ag ULTRA test, but with one value near the cut-off value (ratio between 0.9 and 1) should be considered with care. It is advised to retest the patient with another method or on another sample.
- In case of very low optical density for tested samples (negative OD) and when the presence of samples as well as of reagent is controlled, the results can be interpretated as negative.
- To verify the specificity of the reaction, every positive result in accordance with the interpretation criterias of Monolisa™ HBs Ag ULTRA should be confirmed by a neutralisation method of the HBs Ag.

Non repeatable reactions are often caused by:

- Inadequate microplate washing,
- Contamination of negative samples by serum or plasma with a high HBs Ag concentration,
- Contamination of the substrate solution by oxidising agents (bleach, metal ions, etc...),

LIMITS OF THE TEST

- A negative result indicates that the tested sample does not contain detectable HBs Ag with Monolisa™ HBs Ag ULTRA test. However because very low titer of HBs Ag could not be detected, such a result does not prelude the possibility of exposure to an infection by the hepatitis B virus.
- In addition, several authors have reported in the literature cases of viral hepatitis B (acute or chronic) where in viral DNA is detectable in the absence of the surface antigen (HBs Ag negative patients). These abnormal profiles, though rare, are the consequence of possible genetic mutations, either at the S and pre-S gene level (preventing recognition of the Ag by some immunological reagents) or, usually, at the X and pol gene level, inducing weak viral replication. Testing additional markers (HBs Ag-specific antibody or, if possible, amplified viral DNA) is recommended for the final diagnosis of the infection, in those very particular cases.
- To verify the specificity of the reaction, every positive result (in accordance with the interpretation criterias of Monolisa™ HBs Ag ULTRA) should be confirmed by a neutralisation method of the HBs Ag (test Monolisa™ HBs Ag confirmation for example, code number 72208)
- The colorimetric method for the samples, conjugate and development solution deposition verification does not allow to verify the accuracy of the dispensed volumes. This method only shows the presence of sample, conjugate and development solution into wells. The rate of wrong answers with this method is closely linked to the accuracy of the utilized system (cumulated coefficient of variation of dispensing and reading over 10% significantly decrease the quality of the verification).
- In case of very poor washing efficiency after the conjugate incubation, the automatic verification of the development solution pipetting (by reading OD of wells at 490 nm) may provide wrong results with OD above 0.100 in the absence of development solution.

B. Monolisa™ Anti-HBc PLUS

1 plate - 96 tests	72315
5 plates - 480 tests	72316

DETECTION KIT FOR ANTIBODIES TO NUCLEOCAPSID ANTIGEN (CORE) OF THE HEPATITIS B VIRUS IN HUMAN SERUM OR PLASMA BY ENZYME IMMUNOASSAY

2 - PRINCIPLE OF THE TEST

Monolisa™ Anti-HBc PLUS is an enzyme immunoassay (indirect ELISA type) for the simultaneous detection of total antibodies to hepatitis B virus core in human serum or plasma.

Monolisa™ Anti-HBc PLUS is based upon the use of a solid phase prepared with recombinant HBc antigen.

Steps of the manipulation:

a. The sera to be tested and the control sera are added to the wells. If antibodies to HBc are present, they will bind to the antigens fixed on the solid phase.
b. The peroxidase-labeled antibodies to human IgG and IgM are added after a washing step. They in turn bind to the specific antibodies captured on the solid phase.
c. After removal of the unbound enzymatic conjugate, the antigen-antibody complex is revealed by addition of substrate.
d. After the reaction has been stopped, the absorbance values are read using a spectrophotometer at 450/620-700 nm. The absorbance measured for a sample allows the presence or absence of antibodies to HBc to be determined. The colour intensity is proportional to the quantity of anti-HBc antibodies bound on the solid phase.

Composition of the kit

LABEL	REAGENT COMPOSITION	PRESENTATION	
		72315	**72316**
R1	**MICROPLATE :** 12 strips of 8 wells coated with purified recombinant antigen (expressed in *E.Coli*)	1 microplate	5 microplates
R2	**CONCENTRATED WASHING SOLUTION (20X)** Tris NaCl buffer, pH 7,4	1 vial 70 ml	1 vial 235 ml
	Preservative : ProClinTM 300 (0.04%)		
R3	**NEGATIVE CONTROL SERUM** Human serum negative for anti-HBc antibodies Preservative : Sodium Azide (0.1%)	1 vial 1.5 ml	1 vial 3 ml
R4	**POSITIVE CONTROL SERUM** Human serum containing anti-HBc antibodies Photochemicaly inactivated. Preservative : Sodium Azide (0.1%)	1 vial 1.5 ml	1 vial 3 ml
R6	**SAMPLE DILUENT** : PBS buffer with a coloured control for sample deposition (purple) Preservative : ProClin™ (0.1%) and Ciprofloxacin 10 µg/ml.	1 vial 30 ml	2 vials 2 x 60 ml
R7	**CONJUGATE** Peroxidase-labelled goat antibody directed against human IgG and IgM (green) Preservative : ProClin™ (0.1%) and Ciprofloxacin 10 µg/ml	1 vial 30 ml	2 vials 2 x 60 ml
R8	**PEROXIDASE SUBSTRATE BUFFER** Citric acid and Sodium acetate solution pH 4.0 containing H2O2 (0.015 %) and DMSO (4%)	1 vial 60ml	2 vials 2 x 60 ml
R9	**CHROMOGEN** Solution containing tetramethylbenzidine (TMB).	1 vial 5 ml	2 vials 2 x 5 ml
R10	**STOPPING SOLUTION** 1 N sulfuric acid solution	1 vial 28 ml	3 vials 3 x 28 ml

SAMPLES

Collect a blood sample according to the usual practice.

The test shall be performed on serum or plasma (collected in EDTA, heparin, citrate, ACD, CPD and CPDA-based anticoagulants). Extract it as soon as possible to avoid haemolysis. A severe haemolysis may alter the performance of the test. Samples containing aggregates must be clarified by centrifugation prior to testing. Suspended fibrin aggregates or particles may produce false positive results.

The samples should be stored at +2-8°C if the screening is carried out within 7 days or deep-frozen at -20°C. Avoid repeated freezing/thawing. If the samples are to be shipped, pack them in accordance with the regulations regarding the transport of etiologic agents, transport them preferably frozen.

DO NOT USE CONTAMINATED, HYPERLIPEAMIC OR HYPERHAEMOLYSED SERA.

REMARK: Samples containing up to 30 g/l albumin, 200 mg/l bilirubin, lipemic samples containing up to the equivalent of 36 g/l triolein, and hemolyzed samples containing up to 5 g/l hemoglobin do not affect the results.

RECONSTITUTION OF THE REAGENTS - VALIDITY - STORAGE

Before using the reagents of the Monolisa™ Anti-HBc PLUS kit, allow them to stabilize at room temperature (18-30°C) for 30 minutes.

1) Ready-for-use reagents

• HBc Ag microplate (R1)

Each frame support containing 12 strips is wrapped in a sealed foil bag. Cut the bag using scissors or a scalpel 0.5 to 1 cm above the sealing. Open the bag and take out the frame. Put the unused strips back into the bag. Close the bag carefully and put it back into storage at +2-8°C.

Negative control serum (R3)

• Positive control serum (R4)

• Sample diluent (R6)

Invert gently to homogenize before use.

• Conjugate (R7)

Invert gently to homogenize before use.

2) Reagents to be reconstituted

I. Washing solution (20X concentrate) : R2

Dilute 1:20 in distilled water to obtain the ready-for-use washing solution. Prepare 800 ml for one plate of 12 strips.

II. Working diluted substrate solution (R8 + R9)

Dilute reagent (R9) 1 :11 using reagent R8 (example : 1 ml of R9 reagent in 10 ml of R8 reagent).

10 ml are necessary and sufficient for 1 to 12 strips.

3) Validity

The kit should be stored at +2-8°C. When stored at this temperature, each reagent contained in the kit can be used until the expiry date mentioned on the package (except for specific instructions).

- **R1 :** After the vacuum-sealed bag has been opened, the microwell strips stored at +2-8°C in the carefully resealed bag can be used for 1 month.
- **R2 :** The diluted washing solution can be stored at +2-30°C during 2 weeks. The concentrated washing solution (R2) can be stored at +2-30°C.
- **R8 + R9 :** After the reconstitution, the reagent stored in the dark can be used for 6 hours at room temperature (18-30°C)

- METHOD

• Strictly follow the protocol.

• Use negative and positive control sera for each test, in order to validate the test quality.

• Apply Good Laboratory Practice.

Two methods are available with Monolisa™ Anti-HBc PLUS :

	METHOD 1	**METHOD 2**
Sample incubation	37 ± 1°C	40 ± 1°C
	30 ± 5 min water-bath dry incubator	
Conjugate incubation	37 ± 1°C	40 ± 1°C
	60 ± 5 min water-bath dry incubator	
Enzymatic revelation	30 ± 5 min room temperature 18 - 30°C (in the dark)	

a. Carefully define the sample distribution and identification plan.

b. Prepare the wash solution to working strength.

c. Remove the microplate frame and ready to use strips (R1) from their protective bag.

d. Add quickly, directly and in succession :

- 200 µl of diluent (R6) into each well
- 20 µl of negative control serum (R3) in A1, B1
- 20 µl of positive control serum (R4) in C1, D1, E1
- 20 µl of the first sample in F1 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.

Homogenize the reaction mixture by a minimum of 3 aspirations with the 20 µl pipette or by shaking the microplate after the pipetting step.

- It is also possible to dispense 220 µl of a sample previously diluted to 1:11.
- If the sample distribution is over 10 min, it is recommended to distribute the negative and positive controls after the samples to be tested.

NB: After the samples distribution, the purple diluent turns blue.

It is possible to verify the presence of the samples in the wells by spectrophotometric reading at 620 nm (refer to section 11 SPECTROPHOTOMETRIC VERIFICATION OF SAMPLE AND REAGENT PIPETING).

e. Cover the wells with adhesive film by pressing over the whole surface to ensure tightness.

f. Incubate the microplate in a thermostat-controlled water-bath or in a dry microplate incubator for:

- Method 1 : 30 min ± 5 min at 37°C ± 1°C
- Method 2 : 30 min ± 5 min at 40°C ± 1°C

g. 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.

h. Distribute quickly 200 µl of the conjugate solution into all wells. The conjugate must be shaken gently before use.

NB: The conjugate is coloured green.

It is possible to verify the presence of conjugate in the wells by spectrophotometric reading at 450 nm. (Refer to section 11 SPECTROPHOTOMETRIC VERIFICATION OF SAMPLE AND REAGENT PIPETING)

i. Cover with new adhesive film and incubate for :
   - Method 1 : 60 min ± 5 min at 37°C ± 1°C
   - Method 2 : 60 min ± 5 min at 40°C ± 1°C

j. 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).

k. Prepare the substrate solution (see section 8, reagent R8 + R9).

l. Quickly dispense into each well 100µl of prepared development solution (R8+R9), freshly prepared before use. Allow the reaction to develop in the dark for 30 ± 5 minutes at room temperature (18- 30°C). Do not use adhesive film during this incubation.

N.B.: The distribution of the development solution, which is coloured pink, can be visually controlled at this step of the manipulation: There is a clear difference of colouration between empty well and well containing the pink substrate solution. (Refer to section 11 for automatic verification

m. Add 100 µl stopping solution (R10) by using the same sequence and rate of distribution as for thesubstrate solution. Homogenize the reaction mixture.

N.B.: The distribution of the stopping solution, which is not coloured, can be visually controlled at this step of the manipulation. After the addition of the stopping solution the pink colouration of the substrate disappears (for the negative samples) or turns from blue to yellow (for the positive samples).

n. 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.

o. 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 (OD R4)

Example: Positive control R4

Well with positive control serum Optical density

C1 1.796

D1 1.802

E1 1.852

Total 5.450

$$\text{Mean of OD R4} = \frac{\text{Total optical density}}{3} = \frac{5.450}{3} = 1.817$$

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

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

Example : mean of OD R4 = 1.817

$$Vs = \frac{1.817}{5} = 0.363$$

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 with the Monolisa™ Anti-HBc PLUS test.

- Samples with an optical density higher than, or equal to, the cut-off value are considered to be initiallly positive with the Monolisa™ Anti-HBc PLUS test 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 with the Monolisa™ Anti-HBc PLUS test 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 with the Monolisa™ Anti-HBc PLUS test if both values are less than the cut-off value.

LIMITS OF THE TEST

A negative result indicates that the tested sample does not contain detectable antibodies anti-HBc with Monolisa™ Anti-HBc PLUS. However, such a result does not preclude the possibility of exposure to an Hepatitis B virus infection. The colorimetric method for the samples, conjugate and development solution deposition verification does not allow to verify the accuracy of the dispensed volumes. This method only shows the presence of sample, conjugate and development solution into wells. The rate of wrong answers with this method is closely linked to the accuracy of the utilized system (cumulated coefficient of variation of dispensing and reading over 10% significantly decrease the quality of the verification).

In case of very poor washing efficiency after the conjugate incubation, the automatic verification of the development solution pipetting (by reading OD of wells at 490 nm) may provide wrong results with OD above 0.100 in the absence of development solution. However this phenomenon has not been observed during evaluation on 939 tested samples.

C. Monolisa ^{Tm} Anti-HBs PLUS

72566

192 tests

ENZYME IMMUNOAS SAY (EIA) FOR THE DETECTION AND LEVEL DETERMINATION AF ANTIBODY TO HEPATITIS B SURFACE ANTIGEN (ANTI-HBs) IN HUMAN SERUM OR PLASMA

1 Intended use

Monolisa ^{Tm} Anti-HBs plus is an enzyme immunoassay intended for use in the qualitative and quantitative detection of total antibodies to hepatitis B surface antigen (anti-HBs) in human serum or plasma.

Principle of the procedure

In the assay procedure, patient specimens and controls are incubated with the antigen-coated microwells. If antibodies to HBs are present in a specimen and control, they bind to the antigen. Excess sample is removed by a wash step. The conjugate is then added to the microwells. The conjugate bind to any antigen-antibody complexes present in the microwells. Excess conjugate is removed by a wash step, and a chromogen/substrate solution is added to the microwells and allowed to incubate. If a sample contains anti-HBs, the bound enzyme (HRP) causes the coloration of tetramethyl-benzidine (TMB) in the chromogen solution which turns blue. The blue color turns yellow after the addition of a stopping solution. If a sample does not contain anti-HBs, the chromogen/substrate solution in the well remains colorless during substrate incubation, and after addition of the stopping solution.

The color intensity, measured spectrophotometrically, is proportional to the amount of anti-HBs present in the specimen. Absorbance values reading for patient specimens are compared to cutoff value determined by the 10mIu/mL calibrator.

Composition the kit

label	Nature of the reagents	presentation
R1	**Microplate:** 12 strips of 8 wells sensitized by a mixture of HBsAg, subtype ad and ay (human origin)	**2 microplates**
R2	**Concentrated washing solution (20x)** trisNaCl bufger PH 7.4 preservative agent : proclin ^{Tm} 300 (0.04%)	**1vial** **235ml**
CO	**ANTI-HBs NEGATIVE CONTROL** Buffer with fetal calf serum and protein stablizers preservative agent : proclin ^{Tm} 300 (0.5%)	**1 vial** **2.2ml**
C1	**10mIU/ml Calibrator** Buffer with anti-Hbs of human origin , fetal calf serum, protein stabilizer and sample indicator dye Preservative agent; proclin ^{Tm} 300 (0.5%)	**1 vial** **3ml**
C2	**100mIU/mlCalibrator –positive control** Buffer with anti-Hbs of human origin , fetal calf serum, protein stabilizer and sample indicator dye Preservative agent; proclin ^{Tm} 300 (0.5%)	**1 vial** **2.2ml**
C3	**400mIU/ml calibrator** Buffer with anti-Hbs of human origin , fetal calf serum, protein stabilizer and sample indicator dye Preservative agent; proclin ^{Tm} 300 (0.5%)	**1 vial** **2.2 ml**
C4	**1000mIU/ml Calibrator** Buffer with anti-Hbs of human origin , fetal calf serum, protein stabilizer and sample indicator dye Preservative agent; proclin ^{Tm} 300 (0.5%)	**1 vial** **2.2ml**
R6	**Specimen Diluent** Buffer with fetal calf serum, protein stabilizer and sample indicator dye Preservative agent; proclin ^{Tm} 300 (0.1%)	**1 vial** **27 ml**
R7a	**Concentrated conjugate (11x)** Buffer with HBsAg (human ad and ay subtypes) coated with peroxidase and protein stabilizers Preservative agent; proclin ^{Tm} 300 (0.5%)	**1 vial** **2.5 ml**
R7b	**Conjugate diluent** Buffer with calf serum protein stablizers Preservative agent; proclin ^{Tm} 300 (0.1%)	**1 vial** **25 ml**
R8	**Substrate Buffer** **Citric acid and sodium acetatesolution PH 4.0** **Containing H2O2 (0.015%) and DMSO (4%)**	**1 vial** **60ml**
R9	**Chromogen** **Solution containing tetramethyl Benzidine (TMB)**	**1 vial** **5ml**
R10	**Stopping solution** **1N sulfuric acid solution**	**1 vial** **28ml**

The controls are calibrated according to an internal reference, which is calibrated according to 1st IRP WHO 1977 reference.

PRECAUTIONS

The quality of results is dependent upon the following good laboratory practices:

- The name of the test, as well as a specific identification number for the test, are written on the frame of each microtiterplate. This specific identification number is stated on each strip too.

Monolisa™ Anti-HBs PLUS: Specific ID number = 63.

Verify the specific identification number before any use. If the identification number is missing, or different from the stated number corresponding to the assay to be tested, the strip should not be used.

- Do not use expired reagents.
- Do not mix reagents from different lots within a given test run.

REMARK: For washing solution (R2, label identification: 20X coloured green), peroxidase substrate buffer (R8, label identification: TMB buf, coloured blue), chromogen (R9, label identification: TMB 11X, coloured purple) and stopping solution (R10, label identification: 1N coloured red), it is possible to use other lots than those contained in the kit, provided the same lot is used within a given test run.

These reagents can be used with some other products of our company. In addition, the wash solution (R2, label identification: **20X coloured green**) can be mixed with the 2 other wash solutions included in various Bio-Rad Reagent kits (R2, label identifications: 10X coloured blue or 10X coloured orange) when properly reconstituted, provided only one mixture is used within a given test run. Contact our technical service for detailed information.

- Before use, wait for 30 minutes for the reagents to stabilize at room temperature.
- Carefully reconstitute the reagents avoiding any contamination.
- Do not carry out the test in the presence of reactive vapours (acid, alkaline, aldehyde vapours) or dust that could alter the enzymatic activity of the conjugate.
- Use glassware thoroughly washed and rinsed with deionized water or preferably, disposable material.
- Do not allow the microplate to dry between the end of the washing operation and the reagent distribution.

- The enzymatic reaction is very sensitive to any metals or metal ions. Consequently, no metal element must be allowed to come into contact with the various solutions that contain conjugate or substrate solution.
- The development solution (substrate buffer + chromogen) must be coloured pink. The modification of this pink colour within a few minutes after reconstitution indicates that the reagent cannot be used and must be replaced.
- Preparation of the development solution can be made in a clean disposable single use plastic tray or glass container that has first been pre-washed with 1N HCl and rinsed thoroughly with distilled water and dried. This reagent must be stored in the dark.
- Use a new distribution tip for each serum.
- Well washing is a critical step in this procedure : respect the recommended number of washing cycles and make sure that all wells are completely filled and then completely emptied. Incorrect washing may lead to inaccurate results.
- Never use the same container to distribute the conjugate and the development solution.

MATERIAL REQUIRED BUT NOT PROVIDED

- Distilled or deionized water.
- Sodium hypochlorite (bleach) and sodium bicarbonate.
- Automatic or semi-automatic adjustable or fixed pipettes capable of delivering 10 µl to 200 µl, 1 ml, 5 ml and 10 ml.
- Graduated cylinders of 25 ml, 100 ml and 1000 ml capacity.
- Container for contaminated residues.
- Water bath or Dry incubator, thermostatically set at 37°C ± 1°C.
- Automatic, semi-automatic or manual microplate washing system.
- Microplate reading device (equipped with 490, 450 and 620 nm filters).
- Absorbent paper.
- Disposable gloves.
- Clean polypropylene containers for TMB preparation.

PREPARATION OF REAGENTS

Before using the reagents of the Monolisa™ Anti-HBs PLUS assay kit, allow them to stabilize at room temperature for 30 minutes.

1) **Ready-for-use reagents**

   a. **Microplate (R1)**

Each frame support containing 12 strips is wrapped in a sealed bag. Cut the bag using scissors or a scalpel 0.5 to 1 cm above the sealing. Open the bag and take out the frame. Put the unused strips back into the bag. Close the bag carefully and put it back into storage at +2-8°C.

   b. **Specimen Diluent (R6)**

   c. **Anti-HBs Negative Control (C0)**

   d. **10 mIU/ml Calibrator (C1)**

   e. **100 mIU/ml Calibrator – Positive Control (C2)**

   f. **400 mIU/ml Calibrator (C3)**

   g. **1000 mIU/ml Calibrator (C4)**

Homogenize reagents before use by vortex or invert gently.

2) **Reagents to be reconstituted**

a) Concentrated Washing Solution (20X) : R2

Dilute 1:20 in distilled water to obtain the ready-for-use washing solution. Prepare 800 ml for one plate of 12 strips.

b) Conjugate working solution (R7a + R7b)

Bring Conjugate Diluent (R7b) to room temperature.

Invert Conjugate Diluent (R7b, colorless to pale straw) and Conjugate Concentrate (R7a, green) to mix before using.

Prepare a 1:11 dilution for each strip to be tested (example: add 100 µl of Conjugate Concentrate (R7a) to each 1 ml of Conjugate Diluent (R7b) in a clean, polypropylene tube). Use the following table as a guide. Mix well but gently to avoid foaming.

c) Working Conjugate Solution should be protected from light, both at room temperature and at +2-8°C.

Working Conjugate Solution should be green. It remains stable 8 hours at room temperature and 24 hours when stored at +2-8°C. Conjugate Solution can be prepared by pipetting the entire contents of the Conjugate Concentrate vial (R7a) into the Conjugate Diluent (R7b). Always mix working solution by inverting just prior to use. Return unused Conjugate Concentrate (R7a) to the refrigerator immediately after use. To avoid contamination of Conjugate, wear clean gloves and do not touch tips of pipettes.

Conjugate working solution preparation by strip

Number of Strips to be used	1	2	3	4	5	6	7	8	9	10	11	12*	24**
Amount of Concentrated Conjugate R7a (µl)	100	200	300	400	500	600	700	800	900	1000	1100	1200	2400
Amount of Conjugate Diluent R7b	1	2	3	4	5	6	7	8	9	10	11	12	24

** 1 Complete Plate ** 2 Complete Plates*

d) Working diluted substrate solution (R8 + R9)

- Bring Chromogen (R9) and Substrate Buffer (R8) to room temperature.
- Invert the Chromogen and Substrate Buffer to mix before using.
- Dilute Chromogen (R9) 1:11 using Substrate Buffer (R8) for each strip to be tested (example: add 1 ml of R9 reagent in 10 ml of R8 reagent). 10 ml are necessary and sufficient for 1 to 12 strips.
- Homogenize.
- Mix Working Diluted Substrate Solution gently prior to use. Wait 5 minutes before use. Working

- ❖ Diluted Substrate Solution should be used within 8 hours of preparation and kept in the dark at room temperature.
- ❖ Chromogen (R9) should be pink. Another color indicates a reagent contamination : in this case,
- ❖ Chromogen has not to be used. Prepare only the amount of the reagent to be used within 6 hours, ensuring that the volume of diluted reagent will be adequate for the entire run. Use the following table as a guide:

Preparation of Working diluted substrate solution by strip

Number of Strips to be used	1	2	3	4	5	6	7	8	9	10	11	12*	24**
Amount of Chromogen R9 (µl)	100	200	300	400	500	600	700	800	900	1000	1100	1200	2400
Amount of Substrate Buffer R8 (ml)	1	2	3	4	5	6	7	8	9	10	11	12	24

** 1 Complete Plate ** 2 Complete Plates*

STORAGE AND VALIDITY

The kit should be stored at +2-8°C. When stored at this temperature, each reagent contained in the kit can be used until the expiry date mentioned on the package (except for specific instructions).

- ❖ **R1:** After the vacuum-sealed bag has been opened, the microwell strips stored at +2-8°C in the carefully resealed bag can be used for 1 month.
- ❖ **R2:** The diluted washing solution can be stored at +2-30°C during 2 weeks. The concentrated washing solution (R2) can be stored at +2- 30°C.

- **R7a + R7b: Working Conjugate Solution should be protected from light, both at room temperature and at +2-8°C.** After the reconstitution, working conjugate solution can be used for 8 hours at room temperature (+18-30°C) and for 24 hours if stored at +2-8°C.
- **R8 + R9:** After the reconstitution, the reagent stored in the dark can be used for 6 hours at room temperature (18-30°C)
- After opening all the other reagents are stable until the expiration date indicated on the box when stored at +2-8°C.

SAMPLES

Collect a blood sample according to the usual practice.

The test should be performed on serum or plasma. Only the following samples have been tested: serum collected in standard tube or tube containing separative gel, plasma collected with EDTA or heparin. In case of use of plasma collected with citrate or ACD, results are lower than those obtained with serum for 20%.

Samples containing aggregates must be clarified by centrifugation prior to testing. Suspended fibrin aggregates or particles may produce falsely positive results. The samples should be stored at +2-8°C if the screening is carried out within 7 days or deep-frozen at -20°C. Avoid repeated freezing/thawing. Samples that have been frozen and defrozen more than 3 times cannot be used. If the samples are to be shipped, pack them in accordance with the regulations regarding the transport of etiologic agents transport them preferably frozen.

***REMARK**: Samples containing up to 90 g/l albumin and 100 mg/l bilirubin, lipemic samples containing up to the equivalent of 36 g/l triglycerides, and hemolyzed samples containing up to 2.55 g/l hemoglobin do not affect the results.*

Do not use samples after treatment at 56°C for 30 minutes.

ASSAY PROCEDURE

Strictly follow the protocol. Use negative and positive control sera for each test, in order to validate the test quality. Apply good laboratory practice.

Methods

i. Carefully define the sample distribution and identification plan.
ii. Bring all of the reagents to room temperature before beginning the assay procedure.

iii. Prepare Conjugate Working Solution (R7a + R7b), Working Diluted Substrate Solution (R8 + R9) and Diluted Washing Solution (diluted R2).

iv. 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.

v. Dilute specimens, calibrators and controls 3:4 in the Specimen Diluent R6, following one of the two procedures here below :

- ❖ 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).
- ❖ Specimens, calibrators and controls may be prediluted 3:4 in the Specimen Diluent R6 prior to addition to the well (for example, dilute 150 µl of specimen in 50 µl of Specimen Diluent R6, mix gently to avoid foaming, and then transfer 100 µl to the well).

NOTE: After adding the sample, the diluent will change from purple to a blue color. It is possible to verify the presence of samples in the wells by spectrophotometric reading at 620 nm (Refer to section 14 : SPECTROPHOTOMETRIC VERIFICATION OF SAMPLE AND REAGENT PIPETTING).

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

- ❖ **Qualitative determination**
  - ➢ Anti-HBs Negative Control (C0) in well A1,
  - ➢ 10 mIU/ml Calibrator (C1) in wells B1, C1 and D1,
  - ➢ 100 mIU/ml Calibrator-Positive Control (C2) in well E1,
  - ➢ Samples in wells F1, G1, etc.
- ❖ **Quantitative determination**
  - ➢ 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.

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

vii. Cover, if it is possible, the wells with adhesive film by pressing over the whole surface to ensure tightness.

viii. Incubate the plate for 60 ± 5 minutes at 37 ± 2°C.

ix. 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).

x. If an automatic washer is used, follow the same procedure.

xi. Add quickly 100 µl of the Conjugate Working Solution (R7a + R7b) 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.

NOTE: The conjugate is colored green. It is possible to verify the presence of conjugate in the wells by spectrophotometric reading at 620 nm

xii. 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).

xiii. If an automatic washer is used, follow the same procedure.

xiv. Add quickly 100 µl of the Working Diluted Substrate Solution (R8 + R9) 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.

xv. Add 100µl Stopping Solution (R10) by using the same sequence and rate of distribution as for the Working Diluted Substrate Solution. Homogenize the reaction mixture.

xvi. 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.

Before recording the results, check the correspondence between the reading and the microplate and sample distribution and identification plan.

VALIDATION OF THE RESULTS FOR QUALITATIVE AND QUANTITATIVE METHOD

The mean absorbance of the 10 mIU/ml Calibrator (C1) is the Cutoff Value for the assay.

- **For Anti-HBs Negative Control (C0)**

The measured absorbance value must be greater than 0.000 and less than or equal to 0.070 ($0.000 < ODC0 \leq 0.070$).

- **For** Positive **Control (C2)**

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

For Negative Control (C0) and Positive Control (C2), 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 (C1)**

The measured absorbance value must be greater than or equal to 0.050 and less than or equal to 0.200 ($0.050 \leq ODC1 \leq 0.200$).

Each measured absorbance values must be greater than or equal to 1.5 the OD of the absorbance value of the Negative Control (C0): $ODC1 \geq (1.5 \times ODC0)$.

- **In case of Qualitative method**

If one of the 10 mIU/ml Cutoff Calibrator value is outside the acceptable range (the measured absorbance value must be greater than or equal to 0.050 and less than or equal to 0.200), the mean absorbance should then be calculated from the two remaining absorbance values. The assay is valid. If several ODC1 measured values are outside the acceptable range, the assay is invalid and must be repeated.

- **In case of Quantitative method**

If one of the two ODC1 measured values is outside the acceptable range (the measured absorbance value must be greater than or equal to 0.050 and less than or equal to 0.200), the assay is invalid and must be repeated.

CALCULATION AND INTERPRETATION OF THE RESULTS

For each sample, the comparison of measured absorbance values to the calculated cut-off value allows the determination of the presence or absence of anti-HBs antibodies.

a) Qualitative method

Calculate the mean of the measured absorbance values for the 10 mIU/ml Calibrator (C1)

Example: 10 mIU/ml Calibrator (C1):

B1 0.078

C1 0.079

D1 0.089

Total = 0.246

Mean ODC1 = 0.246 / 3 = 0.082

If one of the measured absorbance value is outside the acceptable range (the measured absorbance value must be greater than or equal to 0.050 and less than or equal to 0.200), the mean absorbance should then be calculated from the two remaining absorbance values.

Calculation of the cut off value (CO)

The Cutoff Value for the assay is the mean absorbance of the 10 mIU/ml Calibrator (C1):

CO = Mean ODC1

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.

Those with values greater than the upper linearity limits of the reader should be reported as reactive.

REMARK: Due to the diversity of antibodies and antigen used in each assay, results could be different depending on the assay.

Vaccination strategies: different recommendations are proposed depending on regions and countries involved. In case of change of analysis method during vaccination follow-up, anti-HBs antibodies concentration have to be determined with both methods during a transitional period.

b) Quantitative method

To determine the concentration of anti-HBs antibodies in serum and plasma specimens, the following: Anti-HBs Calibrator must be used: C0 (0 mIU/ml), C1 (10 mIU/ml), C2 (100 mIU/ml), C3 (400 mIU/ml) and C4 (1000 mIU/ml). Calibrators are added directly in each well, without prior washing of the plate, and in succession as described in the assay procedure (Refer to section 11: ASSAY PROCEDURE). Read at the optical density at 450/620-700 nm using a plate reader (A450). For more samples with absorbance values (A450) greater than or equal to C3 measured absorbance value: A450 ≥ ODC3), read at the optical density at 405/620-700 nm.

The A450 of four Calibrators C0, C1, C2 an C3 are graphed versus their assigned concentrations, using a polynomial (quadratic) regression. Please note that the A450 of the 1000 mIU/ml Calibrator (C4) cannot be used in this graph, as that absorbance value should be outside the range of the spectrophotometer, hence the necessity of a second graph. Samples with measured absorbance values less than ODC3 are interpreted with the graph obtained with the A450 of the four calibrators. The A405 of C3 (400 mIU/ml) and C4 (1000 mIU/ml) calibrators are graphed versus their assigned concentrations, using point to point. A straight line is drawn through the points. Then the anti-HBs concentration (mIU/ml) for each sample is read at the intersection of the respective absorbance values. The A405 curve is used to determine the concentrations of serum or plasma samples whose concentrations are greater than 400 mIU/ml and less than or equal to 1000 mIU/ml.Samples with anti-HBs concentrations greater than 1000 mIU/ml can be diluted using Diluted Washing Solution (diluted R2) and re-assayed.

VERIFICATION OF SPECIMEN DILUENT (R6) AND SAMPLE PIPETTING

After sample addition, Specimen Diluent R6 changes from purple to a blue colour.

The presence of sample and Specimen Diluent (R6) in the well can be verified by spectrophotometric reading at 620 nm: the OD value of each well containing sample or control diluted in Specimen. Diluent must be greater than or equal to 0,150 (a value lower than this indicates poor dispensing of the sample or control).

VERIFICATION OF THE CONJUGATE WORKING SOLUTION (R7a + R7b) PIPETTING

The Conjugate Working Solution (R7a +R7b) is coloured green. The presence of Conjugate Working Solution in the well can be verified by spectrophotometric reading at 620 nm: the OD value of each well must be greater than or equal to 0.070 (a value lowers than this indicates poor dispensing of the Conjugate Working Solution).

VERIFICATION OF THE WORKING DILUTED SUBSTRATE SOLUTION (R8 + R9) PIPETTING

The Working Diluted Substrate Solution (R8 + R9) is coloured pink. It is possible to verify the presence of pink development solution into the well by automatic reading at 490 nm: a well with development solution must have an optical density greater than 0.100 (a lower OD indicates a poor dispensing of the development solution). There is a significative colour change for the empty wells from uncoloured to pink after addition of Working Diluted Substrate Solution.

LIMITS OF THE TEST

- The procedure and the interpretation of the results must be followed when testing serum or plasma specimens for the presence of antibodies to HBs. The user of this kit is advised to read the package insert carefully prior to conducting the test. In particular, the test procedure must be carefully followed for sample and reagent pipetting, plate washing, and timing of the incubation steps.
- Failure to add specimen or reagent as instructed in the procedure may produce false negative results. It is advice to retest samples where a suspicion of procedural error occurs.
- Factors that can affect the validity of results include failure to add the specimen to the well, inadequate microplate washing, failure to follow stated incubation times and temperatures, addition of wrong reagents to wells, the presence of metals, or splashing of bleach into wells.
- Due to the variability of immunological reaction from a patient to another one, as well after HBV infection as further vaccination or therapeutic immunoglobulin injection, it is advised to carefully interpret results with low value.

ANNEX VI: SPECIMEN HANDLING AND REAGENT PREPARATION

1. Handling of specimens

- Collect a blood sample according to the current practices. The test should be performed on undiluted serum or plasma (collected with EDTA, heparin, citrate, ACD-based anticoagulants).
- Separate the serum or plasma from the clot or red cells as soon as possible to avoid any hemolysis.
- Extensive hemolysis may affect test performance.
- Specimens with observable particulate matter should be clarified by centrifugation prior testing. Suspended fibrin particles or aggregates may yield falsely positive results.
- The specimens can be stored at +2-8°C if screening is performed within 7 days or they may be deep-frozen at -20°C for several months.
- Avoid repeated freeze/thaw cycles. Samples that have been frozen and defrozen more than 3 times cannot be used.
- If the specimens are to be shipped, they must be packaged in accordance with the regulations in force regarding the transport of etiological agents.
- *REMARK: Samples containing up to 90 g/l albumin, 100 mg/l bilirubin, lipemic samples containing up to the equivalent of 36 g/l triglyceride, and hemolyzed samples containing up to 1 g/l hemoglobin do not affect the results.*

2. Preparation of the reagents for HBsAg

NOTE: Before use, allow reagents to reach room temperature (18-30°C).

1) Ready for use reagents

- ✓ **Reagent 1 (R1) : Microplate**

Each frame support containing 12 strips is wrapped in a sealed foil bag. Cut the bag using scissors or a scalpel 0.5 to 1 cm above the sealing. Open the bag and take out the frame. Put the unused strips back into the bag. Close the bag carefully and put it back into storage at +2-8°C.

- ✓ **Reagent 3 (R3) : Negative control**
- ✓ **Reagent 4 (R4) : Positive control**
- ✓ **Reagent 10 (R10) : Stopping solution**

2) Reagents to reconstitute

- ➢ **Concentrated washing solution (20X) : Reagent 2 (R2)**

Dilute 1:20 in distilled water to obtain the ready-for-use washing solution. Prepare 800 ml for one plate of 12 strips.

- ➢ **Conjugate working solution (R6 + R7)**

Gently tap the vial of the lyophilized conjugate (R7) on the work-bench to remove any substance from the rubber cap. Carefully remove the cap and pour the content of a conjugate diluent vial (R6) into the lyophilized conjugate vial (R7). Put the cap on and let stand for 10 minutes while gently shaking and inverting from time to time to ease dissolution.

- ➢ **Enzyme development solution : Reagent 8 (R8) + Reagent 9 (R9)**

Dilute 1:11 the chromogen (R9) in the Substrate Buffer (R8) (ex : 1 ml reagent R9+10 ml reagent R8). Stability is for 6 hours in the dark once prepared.

3. Reconstitution of the reagents for core antibody(anti-HBc)

Before using the reagents of the Monolisa™ Anti-HBc PLUS kit, allow them to stabilize at room temperature (18-30°C) for 30 minutes.

1) Ready-for-use reagents

- **HBc Ag microplate (R1)**

Each frame support containing 12 strips is wrapped in a sealed foil bag. Cut the bag using scissors or a scalpel 0.5 to 1 cm above the sealing. Open the bag and take out the frame. Put the unused strips back into the bag. Close the bag carefully and put it back into storage at +2-8°C.

- **Negative control serum (R3)**
- **Positive control serum (R4)**
- **Sample diluent (R6)**

Invert gently to homogenize before use.

- **Conjugate (R7)**

Invert gently to homogenize before use.

2) Reagents to be reconstituted

- **Washing solution (20X concentrate) : R2**

Dilute 1:20 in distilled water to obtain the ready-for-use washing solution. Prepare 800 ml for one plate of 12 strips.

- **Working diluted substrate solution (R8 + R9)**

Dilute reagent (R9) 1:11 using reagent R8 (example : 1 ml of R9 reagent in 10 ml of R8 reagent). 10 ml are necessary and sufficient for 1 to 12 strips.

4. Preparation of reagents for quantitative anti-HBs

Before using the reagents allow them to stabilize at room temperature for 30 minutes.

3) Ready-for-use reagents

h. Microplate (R1)

Each frame support containing 12 strips is wrapped in a sealed bag. Cut the bag using scissors or a scalpel 0.5 to 1 cm above the sealing. Open the bag and take out the frame. Put the unused strips back into the bag. Close the bag carefully and put it back into storage at +2-8°C.

i. Specimen Diluent (R6)

j. Anti-HBs Negative Control (C0)

k. 10 mIU/ml Calibrator (C1)

l. 100 mIU/ml Calibrator – Positive Control (C2)

m. 400 mIU/ml Calibrator (C3)

n. 1000 mIU/ml Calibrator (C4)

Homogenize reagents before use by vortex or invert gently.

4) Reagents to be reconstituted

e) Concentrated Washing Solution (20X) : R2

Dilute 1:20 in distilled water to obtain the ready-for-use washing solution. Prepare 800 ml for one plate of 12 strips.

f) Conjugate working solution (R7a + R7b)

Bring Conjugate Diluent (R7b) to room temperature.

Invert Conjugate Diluent (R7b, colorless to pale straw) and Conjugate Concentrate (R7a, green) to mix before using. Prepare a 1:11 dilution for each strip to be tested (example : add 100 µl of Conjugate Concentrate (R7a) to each 1 ml of Conjugate Diluent (R7b) in a clean, polypropylene tube). Use the following table as a guide. Mix well but gently to avoid foaming.

g) Working Conjugate Solution should be protected from light, both at room temperature and at +2-8°C.

Working Conjugate Solution should be green. It remains stable 8 hours at room temperature and 24 hours when stored at +2-8°C. Conjugate Solution can be prepared by pipetting the entire contents of the Conjugate Concentrate vial (R7a) into the Conjugate Diluent (R7b). Always mix working solution by inverting just prior to use. Return unused Conjugate Concentrate (R7a) to the refrigerator immediately after use.

ANNEX VII: SAMPLE IDENTIFICATION PLAN

Hepatitis project Jimma Site

Jimma Blood Bank Laboratory

HBsAg ELISA test

Sample No----------Repeated samples----------Date----------Lot No----------

	1	2	3	4	5	6	7	8	9	10	11	12
A	Nc1											
B	Nc2											
C	Nc3											
D	Pc1											
E	Pc2											
F												
G												
H												

Remark ----------

Negative control (NC) should be----------cut off value= ----------

Positive control should be Pc----------Gray zone----------

Reactive sample----------

Repeat reactive samples----------

Hepatitis project Jimma Site

Jimma Blood Bank Laboratory

Anti-HBc ELISA test

Sample No----------Repeated samples----------Date----------Lot No----------

	1	2	3	4	5	6	7	8	9	10	11	12
A	Nc1											
B	Nc2											
C	Pc1											
D	Pc2											
E	Pc3											
F												
G												
H												

Remark ----------

Negative control (NC) should be----------cut off value= ----------

Positive control should be Pc----------Gray zone----------

Reactive sample----------

Repeat reactive samples----------

Hepatitis project Jimma Site

Jimma Blood Bank Laboratory

Anti-HBs ELISA test

Sample No----------Repeated samples----------Date----------Lot No----------

	1	2	3	4	5	6	7	8	9	10	11	12
A	Nc(c0)											
B	Calb(c1)											
C	Calb(c1)											
D	Pc(c2)											
E	Calb(c3)											
F	Calb(c4)											
G												
H												

Remark ----------

Negative control (NC) should be----------cut off value= ----------

Positive control should be Pc----------Gray zone----------

Reactive sample----------

Repeat reactive samples----------

ANNEX VII: DECLARATION SHEET

I, the undersigned, declare that this M.Sc. thesis is my original work, has not been presented for degree in this or any other university and that all sources of materials used for the thesis have been fully acknowledged.

Name: _____ Signature: _____Date: _____

Approval advisors

This thesis has been submitted with my approval as University advisor:

First advisor: Name: _____Signature:_____ Date: _____

Second advisor: Name: _____Signature:_____ Date: _____

APPROVAL OF INTERNAL EXAMINER

Name_____Signiture_____Date_____