DEBRE MARKOS UNIVERSITY

RESEARCH, COMMUNITY SERVICE AND TECHNOLOGY TRANSFER DIRECTORATE

EVALUATION OF HEPATITIS B VACCINE EFFICACY AND FACTORS AFFECTING VACCINE EFFECTIVENESS AMONG HEPATITIS B VACCINE FULLY VACCINATED HEALTH CARE WORKERS IN ALL HOSPITALS OF EAST GOJJAM ZONE AMHARA REGION, NORTH WEST ETHIOPIA:-

A RESEARCH PAPER SUBMITTED TO COLLEGE OF MEDICINE AND HEALTH SCIENCES, RESEARCH, COMMUNITY SERVICE AND TECHNOLOGY TRANSFER DIRECTORATE, DEBRE MARKOS UNIVERSITY

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APRIL, 2025

DEBRE MARKOS, ETHIOPIA

Acknowledgment

We would like to express our deepest gratitude to Debre Markos University for the financial support, which was instrumental in accomplishing this work. Furthermore, we sincerely acknowledge the College of Medicine and Health Sciences, Research, Community Service, and Technology Transfer Directorate, Debre Markos University for the continued support in achieving this research work.

Additionally, we extend our heartfelt thanks to the administrative offices of each hospital for their cooperation and willingness to assist. Finally, we would like to express our profound appreciation to all hospital staff and the study participants for their invaluable contributions.

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Abbreviation and acronyms

CD4- Cluster of Differentiation

CHB - Chronic hepatitis B

COR - Crude odd ratio

DC- Dendritic cell

DNA - Deoxyribonucleic acid

ELISA- Enzyme-linked immune-sorbent assay

EPI - Expanded program for immunization

GHSS- Global health sector strategy

HBcAb -Hepatitis B core antibody

HBcAg - Hepatitis B core antigen

HBIG- Hepatitis B Immunoglobulin

HBsAg - Hepatitis B surface antigen

HBV- Hepatitis B virus

HCC- Hepatocellular carcinoma

HIV - Human immune virus

HRP - Horse radish peroxide

MAb- Monoclonal antibody

MIU/ ml- mili international per milliliter

PCR - Polymerase chain reaction

RT- Reverse transcriptase

WHO- World health organization

Abstract

Background: Hepatitis B virus (HBV) is highly infectious and poses significant global health challenges. Healthcare workers (HCWs) are particularly at risk of HBV infection due to occupational exposure through contact with infected blood or body fluids. To mitigate this risk, hepatitis B vaccination is recommended for all healthcare workers and is administered in three doses at 0, 1, and 6 months, aiming to reduce exposure and provide immunity against the virus. However, the effectiveness of the HBV vaccine remains uncertain in the study area.

Objective: This study aimed to evaluate hepatitis B vaccine efficacy and factors affecting vaccine effectiveness among healthcare workers in all Hospitals of East Gojjam Zone Amhara Region, North West Ethiopia

Materials and Methods: A hospital-based cross-sectional study was conducted among 422 healthcare workers from March 25, 2024, to November 30, 2024. Simple random sampling technique was used to recruit the study participants. Total sample size was allocated proportionally to each hospital. Socio-demographic and clinical data were collected using a pretested structured questionnaire. Moreover, venous blood samples (4–5 ml) were collected from the study participants, and serum samples were analyzed to measure and detect the concentrations of anti-hepatitis B surface antigen (anti-HBs), hepatitis B surface antigen (HBsAg), and anti-hepatitis B core antibody (anti-HBc) using a sandwich enzyme-linked immunosorbent assay (ELISA). Additionally, hepatitis B virus DNA (HBV DNA) was assessed using real-time polymerase chain reaction (qPCR). Data were analyzed using SPSS software version 25. Logistic regression model with 95% CI was used to show the statistical association. P value < 0.05 was considered as statistically significant.

Results: The overall sero-prevalence of HBsAg among all study participants was 23/422 (5.5%) (95% CI: 3.0-8.0). In multivariable logistic regression, blood transfusion (AOR: 16.5, 95% CI: 1.53, 29.24) (P<0.011) and dental extraction (AOR: 13.5, 95% CI: 3.99, 45.57) (P<0.000) were significantly associated with HBsAg positivity. In addition, the overall sero-reactivity of anti-HBc was 30/422 (7.1%) (95% CI: 5.0-10.0). Injectable medication (AOR: 4.3, 95% CI: 1.73, 10.72) (P<0.002) was significant determinant for anti-HBc sero-positivity. Furthermore, of fully vaccinated healthcare workers, 346/422 (82.0%) (95% CI: 78.0- 86.0 %) of them were sero-protected (had anti-HBs titer ≥ 10 mIU/ml). The paradoxical sero-positivity of HBsAg and anti-HBs among fully vaccinated healthcare workers was 16(4.6%). Moreover, among HBsAg-positive individuals, HBV DNA was detected in 17 (73.9%) of them.

Conclusions and recommendations: The study identified intermediate HBV seroprevalence among vaccinated healthcare workers, indicating suboptimal vaccine efficacy. These findings emphasize the necessity of monitoring post-vaccination anti-HBs antibody levels to address gaps in protection.

Keywords: Hepatitis B vaccine effectiveness, Healthcare workers, Hospitals

1. Introduction

1.1 Background

Infection Due to hepatitis B virus leads to a broad spectrum of clinical presentation from asymptomatic carrier state to chronic hepatitis with hepatocellular carcinoma cirrhosis. Both the viral factors and the host immune response play a great role for the pathogenesis and clinical outcome of HBV infection [1]. It is a critical health problem worldwide causing high mortality as the regular development of progressive chronic hepatitis B (CHB), such as:

- Liver cirrhosis
- Hepatocellular carcinoma.

According to the World Health Organization, one-third of the global population has serological markers of active or occult HBV infection. Moreover, approximately 780000 people die every year due to hepatitis B [2, 3]. World Health Assembly has taken the Global Health Sector Strategy (GHSS) on viral hepatitis in order to minimize HBV by 2030 [4].

Because they frequently come into contact with contaminated bodily fluids and blood, healthcare workers (HCWs) are susceptible to contracting several blood-borne infections. A secure and efficient way to provide long-term protection against HBV infection is through the hepatitis B vaccination. HBV vaccine for HCWs is given at 0, 1, and 6 months [5]. A full-dose (3-dose vaccine series) is required for all healthcare professionals in order to achieve >90% protection against the hepatitis B virus

The target of the (WHO) GHSS is to decrease hepatitis new case from 6-10 million cases to 0.9 million cases and to reduce annual hepatitis deaths from 1.4 million to 0.5 million, by 2030. One of the five core area intervention which is documented by WHO's GHSS to eliminate hepatitis by 2030 is HBV vaccination[6].

The first vaccine used for protection of HBV infection was developed in 1981, which contained purified hepatitis B surface antigen (HBsAg) obtained from the plasma of chronic HBV infection patients. The second generation of recombinant hepatitis B vaccine has been developed and which was first approved in 1986, contained HBsAg generated from yeast or mammalian cells [7].

Nowadays, greater than 200 countries have performed a universal HBV vaccination program for adolescents based on the WHO recommendation. Consequent to this world wide prevention plan,

rapid reduction of HBV infection among healthcare workers has been documented [8, 9]. The purpose of such vaccination is to induce the immune response in the host and resulting in the prevention of HBV replication [10], and helps the body to ready for future infection by generating immunologic memory [11].

The aim of active immunization against HBV is to enhance the immunity in the host and results loss of HBV surface antigen (HBsAg) and control of HBV replication. Vaccination strategies against HBV include the administration of HBsAg vaccine, Human anti-HBV surface antibody (anti-HBs) etc.[12].

The production of anti-HBs antibodies by B lymphocyte is dependent on the stimulation of helper lymphocytes. After vaccination in the presence of alum as an adjuvant, CD4 T-lymphocytes proliferate and secrete Th2 cytokines, which activate B cell differentiation in antibody-producing cells [13].

There are two vaccine protection mechanisms against HBV:

- The immediate neutralization of HBV by anti-HBs antibodies if they are present at a titer of more than10 mUI/ml. If anti-HBs are less than10 mIU/ml, the virus may start to replicate in the liver.
- By the activation of specific CD4 T memory cells that will promote the subsequent activation of memory B cells and the secretion of anti-HBs antibodies [14].

Protective (Neutralizing) antibodies (anti-HBs) induced by vaccination are targeted mainly the amino acid hydrophilic region which is found on HBsAg spanning amino acids from 124 to 149 this provides protection against the genotypes of HBV and is responsible for the broad immunity obtain by hepatitis B vaccination [15].

- After complete vaccination the non-responder, hypo responder, and hyper responder may exist. A non-responder after a complete vaccination protocol is defined by an anti-HBs titer less than 10 mIU/ml. Hypo responder is defined by an anti-HBs titer of 10-100 mIU/ml. Hyper responder is the one whose antibody titters is greater than100 mIU/ml [16-18] .

1.2 Statement of the Problem

Hepatitis B is one of the most critical viral diseases affecting humans all over the world. More than a million deaths each year are resulted from hepatitis B infection. The cause of the disease the HBV-is found more than 350 million chronic carriers, which is greater than 5% of the global population [19].

Based on the recent WHO report, one- third of the world's populations have serologic evidence of present or past HBV infection [20, 21]. It is a leading cause of both chronic and acute liver disease including cirrhosis and liver cancer, which ranks as the 3rd cause of cancer deaths worldwide. WHO estimates that at least 2 billion people have been globally infected with HBV [15, 22], and 1. 2 million die annually from chronic hepatitis, HCC [23].

It causes 60 to 80% of the world's primary liver cancers [24], and plays a significant role for morbidity worldwide. Current estimates suggest that it causes 30% of cirrhosis and approximately 50% of HCC globally [25]. Chronic infection with HBV is a paramount cause of death from cirrhosis and liver cancer, mainly in South-East Asia and sub-Saharan Africa [26, 27]. Africa has the second largest number of chronic HBV carriers after Asia and is considered as a region of highly endemicity [28].

Approximately 60 million people in Africa are chronically infected with HBV, mostly acquired prenatally or in early childhood [29, 30].

It is also an important cause of morbidity and mortality with a very high burden in Africa [31]. The case of chronic infection is lowest after acute infection during adulthood (<10%) [32].

It is an important public health problem in Ethiopia too. The distribution is varying with age, population practice, ways of exposure, and geographical area [33-35]. According to A previous national survey 10.8% of young men had HBsAg , and 73.3% had at least one HBV marker [36].

Even though there is HBV vaccination program for healthcare workers, various literature showed that different factors like s age, gender, obesity , drinking, smoking and the host immune status of been reported to be related with immunization responses [37, 38]. Beside to this, factors like vaccine dose, vaccination schedule, vaccine type, and route of vaccine administration will affect the protective efficacy (PE) of hepatitis B vaccine among fully vaccinated healthcare workers [19].

There are many obstacles which make hepatitis B vaccination service difficult among healthcare workers in Ethiopia. For instance, poor with socioeconomic capital [39], poor access to HBV vaccine, inadequate awareness, fear of vaccine side effects, lack of trust in the vaccine effectiveness, and high dropout rates are possible challenges which cause low HBV vaccine uptake and make healthcare workers at risk for HBV infection [40-42]. Furthermore; ignorance about the vaccination schedule, shortage of vaccine, and perception to accessibility of vaccines were also challenges of HBV vaccination in healthcare workers [43].

Evaluating of HBV vaccine efficacy and identify factors which affect the seroprotection of HBV vaccine provides information about the performance of HBV vaccine in healthcare workers. It is also crucial to accelerate the introduction of new vaccine and stimulate development of better HBV vaccine and also critical to promote immunization, sustain and optimize vaccine uptake.

The outcomes of the findings will be important for policy makers and other concern bodies at the national and local health bureaus by providing information about the protection level of the HBV vaccine among healthcare workers in hospitals of East Gojjam.

In Ethiopia the full dose (3 doses) of hepatitis B vaccination coverage among healthcare workers is 20-80% [44, 45]. However, there is no published data on the protective efficacy of hepatitis B vaccine among healthcare workers in Ethiopia in general and East Gojjam, North West, Ethiopia in particular. Thus; the aim of this study was to assess hepatitis B vaccine efficacy and factors affecting the vaccine responsiveness among HBV fully vaccinated healthcare workers in all hospitals of East Gojjam Zone.

1.3 Rational of the study

Evaluation of hepatitis B vaccine efficacy and factors affecting vaccine responsiveness in post vaccination among health care workers is very crucial to determine the effectiveness of hepatitis B vaccine and to identify factors which affect the hepatitis B vaccine efficacy among health care workers. Because there is a risk of getting infection in vaccinated healthcare workers when their antibody titers <10 mIU/ml and they may also expose to break through infection.

Therefore this study will serve as a starting point for further control of HBV vaccination effectiveness and it will also bring evidence on the level of seroprotection and factors affecting the level of immune response to HBV vaccine. Currently there is no post vaccination evaluation for the effectiveness of hepatitis B vaccine among healthcare workers in all hospitals of East Gojjam Zone.

As we have adequate man power and laboratory to perform various experiments, which have direct link with our research.

1.4 Significance of the study

Generally, the result will be important for:

- Police makers
- Researchers

- Practitioners

For example for policymakers, it will serve as an input to take the right actions in amending of hepatitis B vaccination guidelines. The modification may be in vaccine type, dose, schedule, or route of administration based on the result of this study.

2. Literature review

2.1 Epidemiology of HBV infection

The distribution of chronic HBV infection is different in different part of the world. For instance, it is highly endemic in developing regions with large population such as South East Asia, China, sub-Saharan Africa and the Amazon Basin, and 8% of the populations are HBV chronic carrier. In these regions, 70-95% of the population shows past or present serological evidence of HBV infection. Most infections occur during infancy or childhood [46].

But it is mid endemic in part of Eastern and Southern Europe, the Middle East, Japan, and part of South America and low in developed areas, such as North America, Northern and Western Europe and Australia [47].

Various studies were conducted in different part of the world on the hepatitis B vaccine efficacy and factors affected the protective efficacy of hepatitis B vaccine in fully vaccinated individual. For instance, a cross-sectional study was conducted in Sulaimani City, Kurdistan Region-Iraq, among 384 fully hepatitis B vaccinated individuals, of which 199 (51.8%) were males and 185 48.2%) were females. All of them received the 3 doses of hepatitis B virus vaccine. Blood samples were taken from them, and the sera were tested for serological markers (HBsAg, anti-HBc, and anti-HBs) using ELISA.

Based on this study Hepatitis B surface antigen and total antibody to hepatitis B core antigen were negative in all participants but antibody to hepatitis B surface antigen was positive (antibody to hepatitis B surface antigen ≥ 10 mIU/mL) in 256 (66.7%) and negative (antibody to hepatitis B surface antigen < 10 mIU/mL) in 128 (33.3%) participants and differences in the age groups were statistically significant, p value <0.005 [48].

There was a study conducted at Italy among 522 participants. After immunization the protection provided by the immunization was evaluated. Of the 522 study participants, 17 participants (3.3%) were anti–HB core antigen positive, and 3 also were HBsAg positive, 400 (79.2%) still had protective anti-HBsAg titers ≥10 mIU/mL [49].

In Greenland, Population-based retrospective cohort study was conducted about the effectiveness hepatitis B vaccine among hepatitis B vaccinated participants. HBsAb levels in vaccinated participants during follow-up, were much lower than expected, and 8 (6%) of 140 at-risk for breakthrough infections, with 4 chronically infected (persistently HBsAg positive) [50].

Another study that was done in southern India estimated that participants. Based on this study the vaccine efficacy was 68 % and there was seroconversion 92.4% in participants [51].

A cross-sectional study was conducted in Taiz, Yemen among 198 vaccinated individuals in this study, 143 (72.2%) of them showed anti-HBs protective level of ≥10 IU/L, while 55 (27.8%) showed non protective anti-HBs titer levels of <10 IU/L Protective anti-HBs levels were slightly higher in females (75.7%) than males (68.4%) but this difference was not statistically significant (P = 0.251). Anti-HBs antibody levels were then compared in different age groups. Anti- HBs levels decrease with increasing age, according to this study the protective rate (anti-HBs ≥10 IU/L) [52].

Another study which was conducted in Senegal showed that out of 143 hepatitis B vaccinated participants the protective efficacy rate was 87% [53]. In Nigeria, a cross-sectional study was conducted among 449 vaccinated individuals. Of them, the prevalence of anti-HBc was 10.5, and the rate of HBsAg was 2%. The vaccine effectiveness against anti HBc was 84.6%, and the effectiveness against the infection was 84.7%.Among them 61% had protective antibodies $\geq$ 10 IU/L [54].

Moreover, a cross-sectional study design was also conducted in Burkina Faso among 265 hepatitis B vaccinated individuals so as to measure the level of antibody titers. Among those vaccinated participants 219 (82.6%) were fully vaccinated and 135 (61.6%) of them had an anti HBs $\geq$ 10 milli international per mililiter [55].

In Ethiopia a cross-sectional study was done at jimma town among 900 vaccinated individuals with their ages from 5-9 years old for the evaluation of hepatitis B immunization efficacy. Out of which 58.4% them had a protective level of HB surface antibodies, ≥ 10 mIU/ml [56].

A prospective cross-sectional study design was also conducted In Ethiopia, Addis Abeba among 383 hepatitis B vaccinated participants with their mean age of 7±1 (SD) years. Of them Anti-HBs were detected in 54.3% (208/383) means had a protective level of antibody concentration (anti-HBsAg concentration of > 10mIU/ml), with a slightly higher proportion of protective level in females 98 (54.7%) than males 110 (53.9%). The proportion of participants with a protective level (>10 mIU/ml anti-HBs antibody) decrease as the age increased: 52.6%, 60%, 43.5% and 37.1% at the age of 5, 6, 7 and 8 years, respectively [57].

A Follow-up study was carried out in Ethiopia among 314 individuals for the evaluation of hepatitis B vaccine efficacy five years after vaccination with their age ranging from 2-14 years

old. They were alternatively vaccinated with 10 and 20 micro gram hepatitis B vaccine doses, using the initial, one- and six-month schedule. Five years later, 210 of the vaccines were retested for anti-HBV surface antibody titers and 89 % of them had protective antibody titers (>10mIU/ml) but the rest 11% had antibody titter the protective level (<10%) [58] .

A cross-sectional study was also done in Ethiopia at Gondar among 431 participants with their ages are from 5-9 years old. Of them 49.4% (213/431) were female and the rest were male. Of which, 69.4% (299/431) of children had normal a body mass index (BMI), whereas 20.4% and 10.2% of participants were underweight and over-weight, respectively. Most of the participants (68.8%) were anti-HBs titer < 10 mIU/mL. while, 32.2% had seroprotective titers of anti-HBs (anti-HBs titer >10 mIU/mL). From these, 31 were anti-HBs titer > 100 mIU/mL, while the remaining 99 were anti-HBs titer of 10-100 mIU/Ml [59].

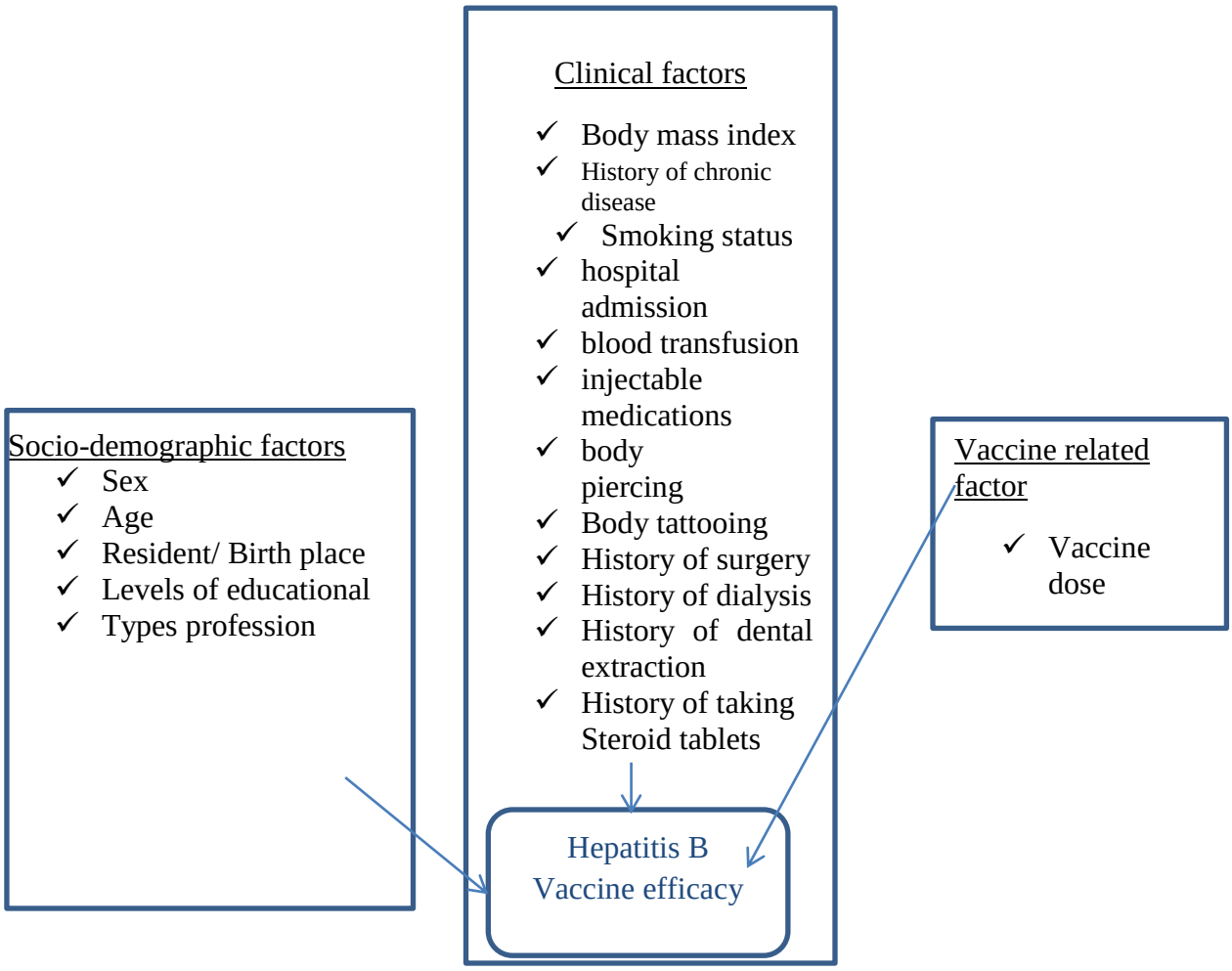

Figure 1: Conceptual framework adopted from the literature

3. Objective

3.1 General objective

To assess hepatitis B vaccine efficacy and factors affecting vaccine effectiveness among hepatitis B fully vaccinated healthcare workers at all hospitals of East Gojjam Zone Amhara Region, North West Ethiopia

3.2 Specific objectives

- To detect hepatitis B surface antigen (HBsAg) among healthcare workers who were fully vaccinated with hepatitis B vaccine
- To determine anti-HBs antibodies (HBsAb) among hepatitis B fully vaccinated healthcare workers
- To detect anti-HBc antibodies (HBcAb) among fully vaccinated healthcare workers
- To determine the paradoxical co-existence of HBsAg and anti-HBs hepatitis B fully vaccinated healthcare workers
- To detect and quantify HBV DNA from HBsAg positive healthcare workers using quantitative real time polymerase chain reaction (qPCR)
- To determine the coverage of hepatitis B vaccination in healthcare workers
- To identify factors associated with hepatitis B vaccine efficacy among healthcare workers

4. Materials and methods

4.1 Study design, period, and area

A hospital based cross-sectional study design was conducted to evaluate the vaccine efficacy and factors associated with hepatitis B vaccine responsiveness among hepatitis B fully vaccinated health care workers at all hospitals in East Gojjam Zone, Amhara regional state, North West Ethiopia, from March 25, 2024, to November 30, 2024. East Gojjam is located from 300Km Northwest of Addis Ababa, the capital of Ethiopia, and 265Km Southeast from Bahir Dar, the Regional capital city of Amhara national regional state. In East Gojjam Zone, there is one Comprehensive Specialized Hospital, one General Hospital, and nine Primary Hospitals.

4.2 Source population

All hepatitis B fully vaccinated healthcare workers who work in all hospitals of East Gojjam Zone was considered as source population.

4.3 Study population

All hepatitis B fully vaccinated healthcare workers who fulfilled eligibility criteria and were available at hospital during the data collection period was taken as study participants.

4. 4. Inclusion and Exclusion criteria

4.4.1 Inclusion criteria

- ✓ All hepatitis B fully vaccinated healthcare workers who were volunteers to participate in the study and gave written informed consent were included in the study.

4.4.2 Exclusion criteria

- ✓ Those study participants who were severely sick during data collection period were excluded in the study.

4.5 Study variables

4.5.1 Dependent variable

- ➢ Hepatitis B vaccine efficacy

4.5.2 Independent variables

- Socio-demographic factors: Age sex, birth place of study participants, participants' profession, and educational status of the study participants.
- Clinical and vaccine related variables: Number of doses of HBV vaccine, BMI status, history of chronic disease, history of smoking cigarette, history of taking steroidal drugs, previous history hospital admission, blood transfusions, injectable medications, participant's body piercing, participant's body tattooing, history of dialysis, history of surgery, and participant's dental extraction

4.6 Operational Definition

Fully vaccinated: Those healthcare workers who took 3 dose of HBV vaccine

Hepatitis B vaccine non-responders: Those are healthcare worker her/ his protective antibody level is less than10 mUI/ml.

Hepatitis B vaccine responders: Those are healthcare workers their protective antibody level greater than or equal to 10 mUI/ml.

4.7. Sample Size Determination and Sampling Techniques

4.7.1 Sample size determination

The required samples size for this study was calculated using 50% via using the following single population proportion formula:

$$= \frac{\left(\frac{Za}{2}\right)^2 pq}{d^2}$$

Where n= sample size

p= proportion

q= 1-p

Z= confidence interval

p =0.89 q= 0.11 with confidence interval of 95% Z= 1.96

$n= \frac{(1.96)^2*0.5*0.5}{(0.05)^2} =384$

The final sample size will be adjusted as follows:

Sample size = n (sample size) + (10% non-respondent). Therefore; final sample size (n) was calculated as n = (384+38) = 422. It was proportionally allocated to each hospital.

4.7.2 Sampling Techniques and Procedures

Simple random sampling technique was used to recruit the study participants until the required sample size was obtained among hepatitis B fully vaccinated healthcare workers in all hospitals of East Gojjam Zone. Total sample size was allocated proportionally to each hospital depending on the total numbers of vaccinated healthcare workers. Then, 5-7 ml of venous blood sample was collected from each study participant.

4. 8. Data Collection Procedure

4.8.1 Sociodemographic data and associated factors

After participants were agreed to take part in the study and signed on informed consent form, Data was collected using a pretested structured questionnaire to collect socio-demographic, clinical, and vaccine-related variables. Information about health care workers' vaccination status was obtained from themselves. Body mass index was measured.

All those data was collected by data collectors who have experience with data collection after giving training about it.

4.8. 2 Laboratory Procedure

Five up to seven (5-7) ml of vinous blood sample was collected by venipuncture from each study participant aseptically and then was packed; labeled in accordance with the existing local and international regulation for clinical specimen transportation. All collected samples from different hospitals of East Gojjam Zone were transported to Molecular lab of Debre Markos comprehensive specialized Hospital (DMCSH) using a cold box with ice packs for storage. Serum was separated from each blood sample by centrifugation, and then stored at -80oC until laboratory analysis was done.

Using the sandwich enzyme-linked immunosorbent assay (ELISA) [BIO-RAD, Monolisa, France], which uses antigens and monoclonal antibodies for capture and detection, HBsAg, anti-HBc, and anti-HBs were detected in Debre Markos Blood Bank Laboratory (DBBL). A qualitative type of sandwich ELISA was used to detect HBsAg and anti-HBc. A quantitative kind was employed to measure the anti-HBs level.

HBsAg was detected by Monolisa™ HBs Ag ULTRA ELISA kit spectrophotometrically at 450/620-700 nm. Moreover, anti-HBcAb was detected by Monolisa™ Anti-HBc PLUS ELISA kit and the absorbance values were read using a spectrophotometer at 450/620-700 nm. In

addition, Monolisa™ Anti-HBs PLUS was used for the qualitative and quantitative detection of anti-HBs and the absorbance values were read at 450/620-700 nm spectrophotometrically. The test kits are certified to have 100% sensitivity and 99.28% specificity.

Every test process was carried out in compliance with the manufacturer's guidelines based on standard operating procedures and the absorbance value was read at 450/620-700 nanometers (nms) spectrophotometrically using ELISA reader [57].

In addition, serum or plasma samples were taken from HBsAg-positive individuals and HBV DNA was isolated using a nucleic acid extraction kit with surfactants (0.4% SDS or NL) at Molecular lab of Armauer Hansen Research Institute (AHRI). A PCR reaction mix was prepared containing a high-performance Taq polymerase (Prime Direct Probe Taq) and HBV-specific primers. Real time PCR was performed to amplify and quantify viral DNA using standardized cycling conditions. Finally, the results were analyzed against established viral load thresholds.

4.9 Data Quality Control

For the generation of quality and reliable data, all quality control was done. All questionnaires were prepared in a clear and precise way. Training was given for data collector how to collect data from study participants. Supervision was conducted on data collector and pre-test was applied to identify the potential problems and to assure the completeness, simplicity, and validity of the questionnaire.

All the laboratory procedures were conducted with experienced professionals and quality control and blank samples were run along with study samples. Pretest was done before data collection to optimize the experimental set up. This was done in samples obtained from 5% of study participants. Standard operating procedures (SOPs) was followed during sample collection, transportation, and processing steps.

4.10 Data Processing and Analysis

After checking the data completeness, data were entered into- Epidata software version 3.1 and exported to SPSS software version 25 for analysis. The results were presented as texts and tables. Binary logistic regression was used to show the association between dependent and independent variables. In bivariate logistic regression, a p-value <0.25 was candidates for multivariate logistic regression. Multivariate logistic regression was used in terms of adjusted odds ratio (AOR) with 95% confidence intervals and a p-value < 0.05 was considered statistically significant.

5. Ethical consideration

Ethical clearance was obtained from Institutional Ethical Review Board of Debre Markos University, College of health sciences. Participants were asked to sign the written informed consent. All the information obtained from the study subjects was coded to keep confidentially.

6. Results

6.1 Socio-demographic characteristics

A total of 422 healthcare workers participated in this study. The median age of the study participants was 36.00 ± 6.64 years. Approximately 78% of participants were over 30 years old. Among the study participants, 55% were male. Additionally, 56.4% held a bachelor's degree **(Table 1)**.

Table 1: Socio-demographic characteristics of the study participants at East Gojjam ZONE Hospitals, North West Ethiopia in 2025

Socio-demographic variables	Age category	Frequency	Percent
Age	≤30	93	22.0
	>30	329	78.0
Sex	Male	232	55.0
	Female	190	45.0
Birth place of study participants	Rural	96	22.7
	Urban	326	77.3
Participants' profession	Nurse	87	20.6
	Midwife	56	13.3
	Doctor	70	16.6
	Laboratory	73	17.3
	Pharmacy	56	13.3
	Radiology	36	8.5
	Anesthesia	27	6.4
	Environmental health	17	4.0
Educational status of the study participants	Diploma	71	16.8
	Bachelor Degree	238	56.4
	Masters	106	25.1
	Specialist	7	1.7
Total		422	100.0

6.2 Sero-prevalence of HBsAg, anti-HBc, and anti-HBs, and their distribution with participants' socio-demographic characteristics

The overall sero-prevalence of HBsAg among all study participants was 23/422 (5.5%) (95% CI: 3.0-8.0). Among HBsAg-positive participants, 19/329 (5.8%) were over 30 years old. Furthermore, 14/232 (6.0%) of HBsAg positive individuals were males. On the other hand, 7/96 (7.3%) HBsAg-positive participants were born in rural areas. In addition, 14/238 (5.9%) of HBsAg-positive participants held a bachelor's degree. The overall sero-reactivity of anti-HBc was 30/422 (7.1%) (95% CI: 5.0-10.0). Moreover, 19(10.0%) of anti-HBc positive study participants were females. Among anti-HBc positive study participants, (7.3%) of them were boron from rural areas. Furthermore, of fully vaccinated healthcare workers, 346/422 (82.0%) (95% CI: 78.0- 86.0 %) of them had anti-HBs titer ≥ 10 mIU/ml. Among fully vaccinated healthcare workers aged ≥30 years, 271 (82.4%) had an anti-HBs titer of 10 mIU/ml or higher **(Table 2)**.

Table 2: Distribution of HBsAg, anti-HBc, and anti-HBs with participants' socio-demographic characteristics

Variables	Category	HBsAg status			Anti-HBc Status			Anti-HBs titers (mIU/ml)		
		Positive	Negative	Total	Positive	Negative	Total	<10	≥10	Total
Age	≤30	4(4.3%)	89(95.7%)	93	5(5.4%)	88(94.6%)	93	18(19.4%)	75(80.6%)	93
	>30	19(5.8%)	310(94.2%)	329	25(7.6%)	304(92.4%)	329	58(17.6%)	271(82.4%)	329
Sex	Male	14(6.0%)	218(94.0%)	232	11(4.7%)	221(95.3%)	232	36(15.5%)	196(84.5%)	232
	Female	9(4.7%)	181(95.3%)	190	19(10.0%)	171(90.0%)	190	40(21.1%)	150(78.9%)	190
Birth place of study participants	Rural	7(7.3%)	89(92.7%)	96	7(7.3%)	89(92.7%)	96	18(18.8%)	78(81.3%)	96
	Urban	16(4.9%)	310(95.1%)	326	23(7.1%)	303(92.9%)	326	58(17.8%)	268(82.2%)	326
Participants' profession	Nurse	6(6.9%)	81(93.1%)	87	8(9.2%)	79(90.8%)	87	17(19.5%)	70(80.5%)	87
	Midwife	1(1.8%)	55(98.2%)	56	3(5.4%)	53(94.6%)	56	11(19.6%)	45(80.4%)	56
	Doctor	3(4.3%)	67(95.7%)	70	3(4.3%)	67(95.7%)	70	20(28.6%)	50(71.4%)	70
	Laboratory	2(2.7%)	71(97.3%)	73	5(6.8%)	68(93.2%)	73	17(23.3%)	56(76.7%)	73
	Pharmacy	8(14.3%)	48(85.7%)	56	6(10.7%)	50(89.3%)	56	3(5.4%)	53(94.6%)	56
	Radiology	1(2.8%)	35(97.2%)	36	4(11.1%)	32(88.9%)	36	2(5.6%)	34(94.4%)	36
	Anesthesia	1(3.7%)	26(96.3%)	27	0(0.0%)	27(100.0%)	27	2(7.4%)	25(92.6%)	27
	Environmental health	1(5.9%)	16(94.1%)	17	1(5.9%)	16(94.1%)	17	4(23.5%)	13(76.5%)	17
Educational status of the study participants	Diploma	2(2.8%)	69(97.2%)	71	6(8.5%)	65(91.5%)	71	12(16.9%)	59(83.1%)	71
	Bachelor Degree	14(5.9%)	224(94.1%)	238	18(7.6%)	220(92.4%)	238	40(16.8%)	198(83.2%)	238
	Masters	6(5.7%)	100(94.3%)	106	6(5.7%)	100(94.3%)	106	23(21.7%)	83(78.3%)	106
	Specialist	1(14.3%)	6(85.7%)	7	0(0.0%0	7(100.0%)	7	1(14.3%)	6(85.7%)	7

6.3 Seroprevalence of HBsAg, anti-HBc, anti-HBs, co-existence of HBsAg/anti-HBs, and their distribution across participants' clinical and vaccine-related characteristics

In addition, the sero-positivity of HBsAg among smokers was 2(9.5%). The sero-positivity of HBsAg among study participants who had history of blood transfusion was 9(37.5%). The seroprevalence of anti-HBc among study participants who had history of dialysis was 0(0.0%). Among study participants with a normal body mass index, 213 (82.6%) had an anti-HBs concentration of 10 mIU/ml or higher. Moreover, concentration of anti-HBs ≥ 10 mIU/ml among non-smokers was 337(84.0%). The co-existence of HBsAg and anti-HBs among fully vaccinated healthcare workers was 16(4.6%) **(Table 3)**. In addition, full-dose coverage of hepatitis B vaccination in the study area was 44.8%.

Table 3: Distribution of HBsAg, anti-HBc, and anti-HBs with participants' clinical and vaccine-related variables

Clinical or vaccine related variables	Category	HBsAg status			Anti-HBc status			Anti-HBs titers (mIU/ml)		
		Positive	Negative	Total	Positive	Negative	Total	<10	≥10	Total
Full dose of HBV vaccine	Yes	23(5.5%)	399(94.5%)	422	30(7.1%)	392(92.9%)	422	76(18.0%)	346(82.0%)	422
	No	0(0%)	0(0%)	0	0(0%)	0(0%)	0	0(0%)	0(0%)	0
BMI status	Uw	0(0.0%)	48(100.0%)	48	5(10.4%)	43(89.6%)	48	6(12.5%)	42(87.5%)	48
	Normal	21(8.1%)	237(91.9%)	258	15(5.8%)	243(94.2%)	258	45(17.4%)	213(82.6%)	258
	Ow	1(1.3%)	74(98.7%)	75	2(2.7%)	73(97.3%)	75	23(30.7%)	52(69.3%)	75
	Obese	1(2.4%)	40(97.6%)	41	8(19.5%)	33(80.5%)	41	2(4.9%)	39(95.1%)	41
History of chronic disease	Yes	5(5.2%)	91(94.8%)	96	9(9.4%)	87(90.6%)	96	18(18.8%)	78(81.3%)	96
	No	18(5.5%)	308(94.5%)	326	21(6.4%)	305(93.6%)	326	58(17.8%)	268(82.2%)	326
History of smoking cigarette	Yes	2(9.5%)	19(90.5%)	21	2(9.5%)	19(90.5%)	21	12(57.1%)	9(42.9%)	21
	No	21(5.2%)	380(94.8%)	401	28(7.0%)	373(93.0%)	401	64(16.0%)	337(84.0%)	401
Medication	Yes	2(3.0%)	64(97.0%)	66	5(7.6%)	61(92.4%)	66	13(19.7%)	53(80.3%)	66
	No	21(5.9%)	335(94.1%)	356	25(7.0%)	331(93.0%)	356	63(17.7%)	293(82.3%)	356
History of taking steroidal drugs	Yes	5(8.6%)	53(91.4%)	58	4(6.9%)	54(93.1%)	58	12(20.7%)	46(79.3%)	58
	No	18(4.9%)	346(95.1%)	364	26(7.1%)	338(92.9%)	364	64(17.6%)	300(82.4%)	364
History hospital admission	Yes	14(15.1%)	79(84.9%)	93	17(18.3%)	76(81.7%)	93	18(19.4%)	75(80.6%)	93
	No	9(2.7%)	320(97.3%)	329	13(4.0%)	316(96.0%)	329	58(17.6%)	271(82.4%)	329
Blood transfusion	Yes	9(37.5%)	15(62.5%)	24	5(20.8%)	19(79.2%)	24	7(29.2%)	17(70.8%)	24
	No	14(3.5%)	384(96.5%)	398	25(6.3%)	373(93.7%)	398	69(17.3%)	329(82.7%)	398
Injectable medications	Yes	11(13.4%)	71(86.6%)	82	18(22.0%)	64(78.0%)	82	20(24.4%)	62(75.6%)	82
	No	12(3.5%)	328(96.5%)	340	12(3.5%)	328(96.5%)	340	56(16.5%)	284(83.5%)	340
Body piercing	Yes	10(13.2%)	66(86.8%)	76	12(15.8%)	64(84.2%)	76	20(26.3%)	56(73.7%)	76
	No	13(3.8%)	333(96.2%)	346	18(5.2%)	328(94.8%)	346	56(16.2%)	290(83.8%)	346
Body tattooing	Yes	10(15.9%)	53(84.1%)	63	12(19.0%)	51(81.0%)	63	19(30.2%)	44(69.8%)	63
	No	13(3.6%)	346(96.4%)	359	18(5.0%)	341(95.0%)	359	57(15.9%)	302(84.1%)	359
History of dialysis	Yes	0(0.0%)	1(100.0%)	1	0(0.0%)	0(0.0%)	0	1(100.0%)	0(0.0%)	1
	No	23(5.5%)	398(94.5%)	421	30(7.1%)	391(92.9%)	422	75(17.8%)	346(82.2%)	421
History of surgery	Yes	10(18.9%)	43(81.1%)	53	10(18.9%)	43(81.1%)	53	19(35.8%)	34(64.2%)	53
	No	13(3.5%)	356(96.5%)	369	20(5.4%)	349(94.6%)	369	57(15.4%)	312(84.6%)	369
Dental extraction	Yes	18(12.2%)	130(87.8%)	148	11(7.4%)	137(92.6%)	148	21(14.2%)	127(85.8%)	148
	No	5(1.8%)	269(98.2%)	274	19(6.9%)	255(93.1%)	274	55(20.1%)	219(79.9%)	274

6.4 HBV DNA detection from HBsAg positive participants

The detection of HBV DNA from HBsAg positive individuals was 17(73.9%) **(Table 4)**.

Table 4:HBV DNA detection from HBsAg positive participants

Groups					
Category	**DNA status**		**HBsAg**		
			Negative	**Positive**	**Total**
HBV DNA in HBsAg positive individuals	Not detected	Count	393	6	399
		within HBV DNA in HBsAg positive individuals	98.5%	1.5%	100.0%
		within hepatitis B surface antigen	98.5%	26.1%	94.5%
		Total	93.1%	1.4%	94.5%
	Detected	Count	6	17	23
		within HBV DNA in HBsAg positive individuals	26.1%	73.9%	100.0%
		within hepatitis B surface antigen	1.5%	73.9%	5.5%
		Total	1.4%	4.0%	5.5%
Total		Count	399	23	422
		within HBV DNA in HBsAg positive individuals	94.5%	5.5%	100.0%
		within hepatitis B surface antigen	100.0%	100.0%	100.0%
		Total	94.5%	5.5%	100.0%

6.5 Factors associated with HBsAg sero-positivity

In multivariable logistic regression, participants with previous history of blood transfusion were 16.5 times (AOR: 16.5, 95% CI: 1.53, 29.24) (P<0.011) more likely to be HBsAg seropositive as compared to those who had no history of blood transfusion. Moreover, participants who had previous history of dental extraction were 13.5 time (AOR: 13.5, 95% CI: 3.99, 45.57) (P<0.000) more likely to be HBsAg sero-reactive as compare to their counterparts **(Table 4)**.

Table 5: Table 5: Bivariate and Multivariate logistic regression of associated factors for HBsAg positivity

Variables	Category	HBsAg status					
		Positive	Negative	COR(95%CI)	P-V	AOR(95%CI)	P-V
History hospital admission	Yes	14(15.1%)	79(84.9%)	6.3(2.63, 15.08)	0.000*	3.3(0.93, 11.55)	0.064
	No	9(2.7%)	320(97.3%)	Ref		Ref	
Blood transfusion	Yes	9(37.5%)	15(62.5%)	16.5(6.16, 44.00)	0.000*	6.7(1.53, 29.24)	0.011**
	No	14(3.5%)	384(96.5%)	Ref		Ref	
Injectable medications	Yes	11(13.4%)	71(86.6%)	4.2(1.80, 9.98)	0.001*	2.4(0.70, 8.23)	0.165
	No	12(3.5%)	328(96.5%)	Ref		Ref	
Body piercing	Yes	10(13.2%)	66(86.8%)	3.9(1.63, 9.22)	0.002*	1.9(0.43, 8.88)	0.385
	No	13(3.8%)	333(96.2%)	Ref		Ref	
Body tattooing	Yes	10(15.9%)	53(84.1%)	5.0(2.10, 12.03)	0.000*	0.7(0.14, 3.01)	0.590
	No	13(3.6%)	346(96.4%)	Ref		Ref	
History of surgery	Yes	10(18.9%)	43(81.1%)	6.4(2.63, 15.40)	0.000*	1.9(0.52, 7.71)	0.315
	No	13(3.5%)	356(96.5%)	Ref		Ref	
Dental extraction	Yes	18(12.2%)	130(87.8%)	7.4(2.71, 20.51)	0.000*	13.5(3.99, 45.57)	0.000**
	No	5(1.8%)	269(98.2%)	Ref		Ref	

NB: *: Candidate variable for multivariate analysis at P < 0.25 and **: significant variable by the multivariate analysis at P < 0.05, COR: crude odds ratio, AOR: adjusted odds ratio, CI: confidence interval, P-V: p-value, Ref: reference

6.6 Factors associated with anti-HBc sero-positivity

Multivariate logistic regression showed that participants who had previous history of injectable medications were 4.3 times (AOR: 4.3, 95% CI: 1.73, 10.72) (P<0.002) more likely to be anti-HBc positive as compared to their counterparts **(Table 5)**.

Table 6: Bivariate and Multivariate logistic regression of associated factors for anti-HBc positivity

Variables	Category	Anti-HBc status					
		Positive	Negative	COR(95%CI)	P-V	AOR(95%CI)	P-V
History of hospital admission	Yes	17(18.3%)	76(81.7%)	5.4(2.53, 11.68)	0.000*	2.5(0.96, 6.61)	0.061
	No	13(4.0%)	316(96.0%)	Ref		Ref	
Blood transfusion	Yes	5(20.8%)	19(79.2%)	3.9(1.35, 11.39)	0.012*	0.8(0.21, 2.79)	0.693
	No	25(6.3%)	373(93.7%)	Ref		Ref	
Injectable medications	Yes	18(22.0%)	64(78.0%)	7.7(3.53, 16.74)	0.000*	4.3(1.73, 10.72)	0.002**
	No	12(3.5%)	328(96.5%)	Ref		Ref	
Body piercing	Yes	12(15.8%)	64(84.2%)	3.4(1.57, 7.44)	0.002*	1.6(0.57, 4.41)	0.373
	No	18(5.2%)	328(94.8%)	Ref		Ref	
Body tattooing	Yes	12(19.0%)	51(81.0%)	4.5(2.03, 9.79)	0.000*	1.1(0.32, 3.43)	0.930
	No	18(5.0%)	341(95.0%)	Ref		Ref	
History of surgery	Yes	10(18.9%)	43(81.1%)	4.1(1.78, 9.24)	0.001*	1.9(0.73, 5.47)	0.179
	No	20(5.4%)	349(94.6%)	Ref		Ref	

NB: *: Candidate variable for multivariate analysis at P < 0.25 and **: significant variable by the multivariate analysis at P < 0.05, COR: crude odds ratio, AOR: adjusted odds ratio, CI: confidence interval, P-V: p-value, Ref: reference

7. **Discussion**

Hepatitis B virus (HBV) poses a significant public health challenge among healthcare workers (HCWs), who face elevated occupational risks due to exposure to blood, bodily fluids, and needlestick injuries [60]. Key drivers include low vaccination uptake, unsafe practices like improper needle handling, and limited adherence to infection control protocols in resource-constrained healthcare systems. Chronic HBV infection among HCWs not only threatens their health but also risks transmission to patients, perpetuating cycles of infection [61]. Addressing this requires enhanced vaccination campaigns, improved occupational safety training, and routine HBV screening to mitigate risks and protect both HCWs and public health. Moreover, hepatitis B vaccine plays a critical role in protecting HCWs in Ethiopia from occupational HBV exposure [62].

Anti-HBs (hepatitis B surface antibody) serve as a protective antibody that indicates immunity from HBV vaccination, with titers ≥10 mIU/mL confirming effective protection due to vaccination [63]. However, anti-HBs levels may decline over time, e.g., 30% of vaccinated healthcare workers develop titers <10 mIU/mL years post-vaccination, particularly those immunized >5 years, enabling rapid antibody resurgence upon HBV exposure [64].

In this study, the overall sero-prevalence of HBsAg among all study participants was 5.5% (95% CI: 3.0-8.0). This prevalence of HBsAg tells us the study area is found under the category of intermediate endemicity for HBV infection. This is comparable with the study done in Hawassa (4.4%) [65], Gondar (4.2%) [59], and green land (3.3%) [50]. On the other hand, this finding is higher than the other studies done in Uzbekistan-Japan (0.8%) [66], Iran (0.6% [67], Italy (0.6%) [49], Egypt (0.6%) [68]. Moreover, the seroprevalence of HBsAg in the current study is lower than the findings reported from china (20.49%) [69]. These variations may be explained by difference in host genetic factors, ethnicity, cultural practice, lifestyles, socioeconomic status, and regional variations. Moreover, this variation also might be because of the difference infection control practice in the hospital setting, immune status, sample size, and sampling technique and type of laboratory method used for HBsAg detection.

Furthermore, in the current study, the seroprevalence of HBsAg among participants aged ≥30 years was higher (5.8% vs. 4.3%). Additionally, HBsAg prevalence was also elevated in male

participants (6.0% vs. 4.7%). Older individuals are more affected by hepatitis B virus (HBV) due to cumulative exposure risks, waning immunity, and biological factors. Moreover, gender-specific immune and hormonal differences, behavioral disparities, and clinical outcomes collectively explain why males are disproportionately affected by HBV infection. Estrogen's protective role mitigates HBV progression by reducing liver inflammation and promoting immune responses, delaying chronic liver disease development and lowering HBV-related cirrhosis and hepatocellular carcinoma (HCC) risk in females. Conversely, testosterone may enhance HBV replication or promote pro-inflammatory pathways, accelerating disease progression in males [70]. Behavioral factors, such as higher alcohol use, and unprotected sex, increase HBV transmission and liver damage risks in males. Genetic mechanisms, including higher HBV X gene prevalence in males and gender-specific cytokine responses, may further influence viral persistence and pathogenesis [71].

In the present study the sero-positivity of anti-HBc in the fully vaccinated healthcare workers was 7.1% (95% CI: 5.0-10.0). This finding is relatively consist with the results reported from Addis Abeba (5.6%) [57] and Gondar (6.3%) [72]. This finding is also comparable with the results reported from Thailand (5.5%) [73]. On the other hand, this is higher than the study done in Brazil (4.4%) [74], Italy (3.3%) [49], Iran (2.1%) [67], and Benisuef-Egypt (2.0%) [75]. However, it is lower than the findings reported from from Hawassa city (19.5%) [76] and other country; Gambian Villages (10.2%) [77]. This difference is might be due to the difference in infection control practice in the hospital setting, immune status of the study participants, sample size, and sampling technique.

In addition, the seroprotection levels of hepatitis B vaccine (anti-HBs titer ≥ 10 mIU/ml) among fully vaccinated healthcare workers in the current study was 82.0% (95% CI: 78.0-86.0%). This is comparable with the study conducted in Italy (79.2%) [78], and higher than the study in India (68%) [79], and Yemen (72.2%) [80], and lower than finding reported from Senegal (87%) [53]. This difference is might be due to difference in the participants' immune status, Difference in vaccine adherence, demographic factors, and methodological differences. In other words, these disparities underscore the need for standardized protocols, booster policies, and post-vaccination monitoring to ensure consistent protection.

In addition, the paradoxical co-existence of anti-HBs and HBsAg in fully vaccinated healthcare workers was 4.6% (95% CI: 2.0-7.0%). Protective healthcare workers (whose anti-HBs titer is ≥

10 mIU/ml) will paradoxically be positive for hepatitis B surface antigen following chronic hepatitis B virus infection and mutations or deletions in the preS/S viral genomic region [81]. A potential explanation for the simultaneous HBsAg and anti-HBs serological profile has also occurred via alteration in the HBV 'a' determinant. The ‘a’ determinant of HBV consists of a serologically defined region that comprises the largest site of recognition of HBsAg by immune response cells and antibodies mapped to this region confer protection against all subtypes of HBV.

Moreover, host immunological state and genetic determinants may also be involved in the HBsAg/anti-HBs coexistence in individuals with chronic HBV infection [82]. Thus, concurrent HBsAg and hepatitis B surface antibodies constitute a peculiar serological pattern that may be observed in the context of persistent HBV infection.

Furthermore, the detection of HBV DNA from HBsAg positive individuals was 73.9% (95% CI: 54.0-93.0%). This finding is higher than the study done in South Africa (40.6%) [83]. The detection rate of HBV DNA in HBsAg-positive individuals varies across countries due to factors such as genotypic variations, methodological differences, and healthcare policies. For instance, genotype A is associated with higher HBV DNA viral loads and high rates of HBeAg negativity, while genotypes like E-J show lower detection rates [84]. Methodological differences, such as the use of extraction-free techniques with surfactants like SDS or NL, can enhance sensitivity but are not uniformly adopted globally. Additionally, national policies and awareness campaigns significantly influence testing adoption, with proactive countries achieving higher detection rates. These factors collectively explain the disparities in HBV DNA detection rates observed in different regions [85].

In this study, associated factors were also assessed. Hence, participants with previous history of blood transfusion were 16.5 times more likely to be HBsAg seropositive as compared to those who had no history of blood transfusion. Furthermore, participants who had previous history of dental extraction were 13.5 time more likely to be HBsAg sero-reactive as compare to their counterparts. This is due to exposure to contaminated blood or bodily fluids, iatrogenic transmission via unsterilized equipment, and viral persistence in occult infections. Dental extraction risks arise from contaminated instruments (e.g., unsterilized drills, syringes) and blood-to-blood contact during procedures, particularly in settings with poor infection control or

shared needles. These factors highlight the need for enhanced screening for blood donors and rigorous sterilization protocols to mitigate HBV transmission in high-risk settings.

Furthermore, participants who had previous history of injectable medications were 4.3 times more likely to be anti-HBc positive as compared to their counterparts. This might be due to bloodborne transmission risks from shared needles/syringes and contaminated equipment. Direct HBV exposure via blood-to-blood contact during drug use drives transmission of HBV infection

8. Limitation of the study

Other HBV infection markers, such as hepatitis B envelope antigen (HBeAg) and anti-HBeAg, were not tested due to limited resources, precluding determination of viral replication rates and infectivity levels. Additionally, participants' pre-vaccination HBV exposure history was not evaluated due to lack of information and resource constraints. Moreover, as this study employed a cross-sectional design, it was difficult to establish causality (i.e., whether the exposure preceded the outcome or vice versa).

9. Conclusions and recommendations

The study revealed intermediate hepatitis B seroprevalence among fully vaccinated healthcare workers in the study area, suggesting suboptimal vaccine effectiveness. The protective efficacy of HBV vaccine in the present study was low. Moreover, the detection rate of HBV DNA among HBsAg positive individuals was high. Blood transfusion and dental extraction were significantly associated with HBsAg sero-positivity. Moreover, injectable medication was significant predicator for anti-HBc positivity. Hence, infection control measures must prioritize closed-system devices for blood transfusions, double-gloving and eye protection during dental procedures, and safety-engineered syringes for injectable medications to mitigate exposure risks. Occupational health interventions should enforce procedure-specific training on PEP protocols and real-time sharps injury reporting with prompt HBV DNA testing for exposed workers. Serological monitoring protocols should target high-risk HCWs (e.g., frequent blood/dental exposure) with biannual HBsAg/anti-HBc screening. Policy revisions must mandate HBV vaccination for high-exposure roles per Occupational Safety and Health Administration (OSHA) requirements. In addition, determination of levels of anti-HBs antibodies should be done, and then booster doses must be given for non-responders to increase the concentration of anti-HBs

levels and prevent waning immunity. In other words, there should be routine anti-HBs titer testing post-vaccination to identify non-responders (anti-HBs <10 mIU/mL).

10. **References**

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11.**Annex**

Annex I: Laboratory testing procedure

Hepatitis B serologic testing involves measurement of several hepatitis B virus (HBV) specific antigens and antibodies. Different serologic "markers" or combinations of markers are used to evaluate hepatitis B vaccine efficacy.

Generally, Serum HBsA, HBsAb, and HBcAb will be measured by commercially available enzyme liked immune sorbent assays according to the manufacturer's instructions.

Note:-Anti-HBS titer: Antibodies will be produced for HBS Ag introduced to the system either by hepatitis infection or vaccination. Since the major immunogenic antigen and the vaccine component is HBS Ag Anti-HBS is the main antibody produced in Hepatitis B vaccinated individuals and it can be measured to follow the effectiveness of HBV vaccine.

General test principles of ELISA

For the detection of qualitative/quantitative Anti-HBs antibody in human serum, the double antigen- non-competitive sandwich ELISA assay will be used. According to the principle of the assay, ELISA microplates will be coated with 250ng commercial HBsAg. After blocking, 100 microliter of human serum will be added to the microwells together with Horseradish Peroxidase (HRP) conjugated commercial HBsAg. After incubate at 60 minutes, the complex of antigen-antibody-antigen (HRP conjugated HBsAg, anti-HBsAg antibody and HBsAg on the wells) will be formed.

Therefor; the amount of HRP-HBsAg bound to the well proportional to the concentration of anti-HBsAg antibody in the samples. The unbound enzyme conjugates will be washed away and then the substrate buffer will added to the wells. A blue color will be developed in proportion to the amount of anti-HBsAg antibody in the human samples. The enzyme-substrate reaction will be stopped by the addition of stop solution. The absorbance of controls and samples will be determined by using EIA reader with wavelength set at 450nm.The ration optical density will be <1, ≥ 1, and 0.9-1.1 for negative, positive and borderline results respectively.

ELISA test system will be applied for HBsAg detection. For detection of HBsAg in human serum, the double antibody-sandwich ELISA will be used. Based on the principle of the assay, anti-HBsAg monoclonal antibody (MAM 2G3) will be bound to the different ELISA microplates

as a capture antibody. Anti-HBsAg linked horseradishe peroxidase (MAM 2G3–HRP) conjugate will be used as detecting antibody.

Unlike the conventional ELISA systems, the same monoclonal antibody (MAM 2G3) against HBsAg will be used as both capturing antibody and detecting antibody. MAb-HRP and MAb-Biotin conjugate will be diluted 1:1000 in PBS, and human serum will be added to the wells simultaneously and incubated for 1 h at 37°C. After repeating the washing step for 5 times with washing buffer, the enzymatic activity will be detected by substrate buffer (3,3′,5,5′-tetramethyl benzidine (TMB) and hydrogen peroxide for HRP conjugate-antibody.

For the detection anti-HBc, there are three steps. A test specimen will be put directly in the test well containing specimen diluent and incubated for a specified length of time in the first step. Antigen-antibody complexes will form on the microwell surface if anti-HBc is present in the specimen. Complexes will not form and the unbound plasma or serum proteins will be avoided in the washing step if anti-HBc is not found.

Antibody conjugate is added to the test well. The antibody conjugate is a mixture of monoclonal antibodies specific for human IgG and IgM In the second stage. In the antibody portion of the antigen-antibody complexes the conjugate will bind specifically. When antigen-antibody complexes are not found, the unbound conjugate will be removed by washing.

An enzyme will be added to the test well. If bound conjugate is present, the enzyme will be oxidized, resulting in a colored end-product in the third stage. Stop solution will be added to stop the reaction. The intensity of color depends on the amount of bound conjugate and therefore is a function of the concentration of anti-HBc present in the specimen. The color intensity is measured with a microwell reader.

Procedure

Reagents and consumables

Kit components

- Coated 96 well strip plate
- Sample diluent
- Assay diluents (A and B)
- Detection reagents (A and B)
- Wash buffer
- TMB Substrate

- Stop solution
- Adhesive plate sealers
- Instruction manual

Materials not provided with the kit

- Micro plate reader with 450 nm wavelength filter
- Distilled water
- Incubator (37^{o}C)
- Pipettes (high precision)
- Multi-channel micropipettes
- Pipette tips (sterile)
- Absorbent paper
- Eppendrof tubes

A. Monolisa™ HBs Ag ULTRA

1 Plate-96 tests 72346

5 plate -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

IVD For *In Vitro* Diagnostic Use

INTENDED USE

Monolisa™ HBs Ag ULTRA assay is a one-step enzyme immunoassay method 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 depend on the principle of the "sandwich" type using monoclonal antibodies and polyclonal anibodies preferred for their ability to bind themselves to the different 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

Lable	NATURE OF THE REAGENTS	PRESENTATION	
		72346	**72348**
R1	**MICROPLATE** : 12 strips of 8 wells each, coated with monoclonal antiHBs antibodies (mouse)	1 Plate	5 Plate
R2	**CONCENTRATED WASHING SOLUTION (20X** Tris NaCl buffer pH 7.4 Preservative : ProClinTM 300 (0.04%)	1 Vial 70 ml	1 Vial 135 ml
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	2 Vial 2x18ml
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 Vial 2x60 ml
R9	**CHROMOGEN PINK COLOURED** Solution containing tetramethyl benzidine (TMB)	1 Vial 5 ml	2 Vial 2x5ml
R10	**STOPPING SOLUTION** 1N sulphuric acid solution	1 Vial 28ml	3 Vial 3x28ml

PRECAUTIONS

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

- The name of the test or 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 INSTRUCTION

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 neutralised 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 PLACE SOLUTIONS CONTAINING SODIUM HYPOCHLORITE IN THE AUTOCLAVE

- 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 sensitization 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)

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 a etiological 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

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.

Follow the following Good Laboratory Practice:

1. Carefully establish the sample distribution and identification plan.
2. Prepare the diluted washing solution.
3. Prepare the conjugate R6+R7 working solution.
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 colouration 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. 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.

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 criteria 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:

Ratio= OD sample /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:

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

Assay Reproducibility

The reproducibility of Monolisa™ HBs Ag ULTRA test has been determined, by the analysis of 4 samples: 1 negative sample, 2 HBs Ag positive samples (samples 2 and 3) and 1 high HBs Ag positive sample.

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.

Depending on the instrument used (washer, reader, automated processor), slight performance variability may be observed.

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 72408)

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. Never observed during evaluation on 939 tested samples.

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 IMMUNOASSA

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:

1. 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.
2. The peroxidase-labelled 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.
3. After removal of the unbound enzymatic conjugate, the antigen-antibody complex is revealed by addition of substrate.

4. 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

Lable		**Description of reagent composition**	**Preparation**	
			72315	**72316**
R1	**Microplate**	12 strips of 8 wells coated with purified recombinant antigen (expressed in E. Coli)	1Microplate	5 Microplates
R2	**Concentrated washing solution (20X)**	Tris NaCl buffer, pH 7.4 Preservative: ProClin™ 300 (0.04%)	1 Vial 70ml	1 Vial 235ml
R3	**Negative control Serum**	Human serum negative for anti-HBc antibodies Preservative: Sodium Azide (0.1%)	1 Vial 1.5ml	1 Vial 3ml
R4	**Positive control Serum**	Human serum containing anti-HBc antibodies. Photochemicaly inactivated. Preservative: Sodium Azide (0.1%)	1 Vial 1.5ml	1 Vial 3ml
R6	**Sample diluent**	Sample diluent PBS buffer with a coloured control for sample deposition (purple) Preservative: ProClin™ (0.1%) and Ciprofloxacin 10 µg/ml.	1 Vial 30ml	2 Vials 2x60ml
R7	**Conjugate**	Peroxidase-labelled goat antibody directed against human IgG and IgM (green) Preservative: ProClin™ (0.1%) and Ciprofloxacin 10 µg/ml	1 Vial 30ml	2 Vials 2x60ml
R8	**Peroxidase Substrate buffer**	Citric acid and Sodium acetate solution pH 4.0 containing H_2O_2 (0.015%) and dimethyl sulfoxide (DMSO) 4%	1 Vial 60ml	2 Vials 2x60ml

R9	**Chromogen: tetramethylbenzidine (TMB) solution (11X)**	Solution containing 3.3' , 5.5' tetramethylbenzidine (TMB)	1 Vial 5ml	2 Vials 2x5ml
R10	**Stopping solution**	Sulphuricacid solution (H2SO4 1N)	1 Vial 28ml	3 Vials 3x28ml

HEALTH AND SAFETY INSTRUCTIONS

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

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-HBc PLUS : Specific ID number = 14.

1. Verify the specific identification number before any use. If the identification number is missing, or different from the stated number above, the strip should not be used.
2. Wear disposable gloves when handling reagents and samples and thoroughly wash your hands after handling them.
3. Do not pipette by mouth.
4. Human origin material used in the preparation of the negative control (R3) has been tested and found non-reactive for hepatitis B surface antigen (HBs Ag), anti-HBc antibodies and antibodies to hepatitis C virus (HCV) and human immunodefiency virus (HIV 1 and HIV 2).
5. Human origin material used in the preparation of the positive control (R4) has been tested and found reactive for hepatitis B surface antigen (HBs Ag) and anti-HBc antibodies and non -reactive for antibodies to hepatitis C virus (HCV) and human immunodefiency virus (HIV 1 and HIV 2). It has been photochemicaly inactivated.
6. No method can absolutely guarantee the absence of the HIV, HBV, HCV viruses or other pathogens. Consider these reagents as well as patient samples, as potentially infectious and handle them with the customary precautions
7. Consider any material directly in contact with samples and reagents of human origin, as well as washing solutions, as infectious materials.
8. Avoid spilling samples or solutions containing samples.
9. Spills must be rinsed with bleach diluted at 10%. If the contaminating fluid is an acid, spills must be initially neutralized with sodium bicarbonate, then cleaned with bleach and

dried with absorbent paper. The material used for cleaning must be discarded in a contaminated waste container.

10. Samples, reagents of human origin, as well as, contaminated material and products should be discarded after decontamination either by immersion in bleach at the 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.

CAUTION: do not introduce solutions containing sodium hypochlorite into the autoclave.

11. Avoid any contact of the substrate buffer, the chromogen or the stopping solution with the skin or mucosa.
12. Do not forget to neutralize and/or autoclave the solutions or washing wastes or any fluid containing biological samples before discarding them into the sink.

PRECAUTIONS

The quality of results is dependent upon following good laboratory practice:

a. Do not use expired reagents.

b. Do not mix reagents from different lots within a given test run.

REMARK: For washing solution (R2, label identification: 20X coloured green), peroxidase substratebuffer (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. All reagents must be brought to room temperature before proceeding (30 minutes).

c. Carefully reconstitute the reagents avoiding any contamination.

d. 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.

e. Use glassware thoroughly washed and rinsed with deionized water or preferably, disposable material.

f. Do not allow the microplate to dry between the end of the washing operation and the reagent distribution.

g. The enzymatic reaction is very sensitive to any metals or metal ions. Consequently, no metal

lement must be allowed to come into contact with the various solutions that contain conjugate or substrate solution.

h. 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.

i. Use a new pipette tip for each serum.

j. Well washing is a critical step in this procedure: follow the recommended number of wash cycles and make sure that all wells are completely filled and then completely emptied. Incorrect washing may lead to inaccurate results.

k. Never use the same container to distribute the conjugate and the development solution.

l. Check the pipettes and other equipment for accuracy and correct operation.

m. Do not change the assay procedure.

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.

A. Ready- for-use reagents

1. HBc Ag microplate (R1)

Each frame support containing 12 strips is wrapped in a sealed foil bag. Cut the bag using scissorsor 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.

2. Negative control serum (R3)

3. Positive control serum (R4)

4. Sample diluent (R6)

Invert gently to homogenize before use.

5. Conjugate (R7)

Invert gently to homogenize before use.

B. Reagents to be reconstituted

1. **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.

2. **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.

C. 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).

D. METHOD

1. Strictly follow the protocol.
2. Use negative and positive control sera for each test, in order to validate the test quality.
3. 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)	

1. Carefully define the sample distribution and identification plan.
2. Prepare the wash solution to working strength.
3. Remove the microplate frame and ready to use strips (R1) from their protective bag.
4. Add quickly, directly and in succession :

200 µl of diluent (R6) into each well

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

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

20 µl of the first sample in F1 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.

5. Cover the wells with adhesive film by pressing over the whole surface to ensure tightness
6. Incubate the microplate in a thermostat-controlled water-bath or in a dry microplate incubator for :

   Method 1: 30 min ± 5 min at 37°C ± 1°C

   Method 2: 30 min ± 5 min at 40°C ± 1°C
7. 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.
8. 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.

9. 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
10. 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).
11. Prepare the substrate solution (reagent R8 + R9).
12. 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.

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.

Note: 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).

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.
15. 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.

A. 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**

Mean of OD R4 = Total optical density/3 =5.450/3 = 1.817

B. Calculate the cut-off value (Vs)

Vs =Mean of OD R4/5= 1.817/5 = 0.363

Validation criteria

1. For the negative control: each individual measured absorbance value must be <0.100.
2. For the positive control: each absorbance value must be ≥1.000 and ≤ 2.900.

NB. If one of the positive control value is out of these norms or differs by more than 30% from the mean value, perform 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 < the cut-off value are considered to be negative with the Monolisa™ Anti-HBc PLUS test.

Samples with an optical density ≥ 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.

SPECTROPHOTOMETRIC VERIFICATION OF SAMPLE AND REAGENT PIPETING (OPTIONAL)

VERIFICATION OF THE SAMPLE PIPETING

After the sample distribution, the purple diluent turns blue.

Method 1: without the use of a control reagent

The presence of sample into the well can be verified by automatic reading at 620 nm: compare the measured optical densities for each well, after the distribution of sample diluent (R6) and after the sample pipeting :

1. The OD values of the wells containing sample diluent (R6) only must be over 0.500.
2. Each well containing sample must have an OD variation greater than 0.200 (an OD variation strictly lower than 0.200 indicates a poor dispensing of the sample).

Method 2: using control reagent

The presence of sample into the wells can be verify by automatic reading at 620 nm with the use of a control well containing the sample diluent (R6) only: read the plate and compare the OD obtained for each sample with the OD obtained for the control well:

1. The OD value of the control well containing sample diluent (R6) only must be over 0.500.
2. The OD variation between well with sample and the control well must be greater than 0.200 (an OD variation strictly lower than 0.200 indicates a poor dispensing of the sample).

VERIFICATION OF CONJUGATE PRESENCE

The conjugate (R7) is coloured green.

The presence of conjugate (R7) into the wells can be controled by automatic reading at 450 nm: the OD value of each well must be greater than 0.300 (a value lower this norm indicates a poor dispensing of the conjugate).

DEVELOPMENT SOLUTION PIPETTING VERIFICATION

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).

NOTE: the acuracy of Monolisa™ Anti-HBc PLUS test has been determined by the analysis of 4 samples: 1negative sample (sample 1), 2 low anti-HBc positive samples (samples 2 and 3) and 1 high anti-HBc positive sample (sample 4). The intra assay reproducibility has been evaluated by testing these 4 samples 30 times in the same run, the inter assay reproducibility has been evaluated by testing these 4 samples 3 times on 2 microplates performed on 2 independant runs each day during 5 days.

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 phenomena has not been observed during evaluation on 939 tested samples.

C. MONOLISA™ ANTI-HBS PLUS

192 tests **72566**

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

INTENDED USE

Monolisa™ 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.

TEST PRINCIPLE

In the assay procedure, patient specimens and controls are incubated with the antigen-coated microwells. If antibodies to HBs are present in a specimen or control, they bind to the antigen. Excess sample is removed by a wash step. The conjugate is then added to the microwells. The conjugate binds 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 tetramethylbenzidine (TMB) in the chromogen solution wich 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 the 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 value readings for patient specimens are compared to a cutoff value determined by the 10 mIU/mL calibrator.

COMPONENTS OF THE KIT

LABLE	NATURE OF THE REAGENTS	PRESENTATION
R1	**MICROPLATE :** 12 strips of 8 wells sensitized by a mixture 2 microplates of Hepatitis B surface antigen, subtype ad and ay (human origin)	2 Microplate
R2	**CONCENTRATED WASHING SOLUTION (20X)** Tris NaCl pH 7.4.Preservative agent: ProClinTM 300 (0, 04%)	1 Vial 235ml
CO	**ANTI-HBS NEGATIVE CONTROL** Preservative agent: ProClinTM 300 (0,04%) Preservative agent: ProClin™ 300 (0.5%)	1 Vial 2.2ml

C1	**10 mIU/ml CALIBRATOR** Buffer with Anti-HBs antibodies of human origin, fetal calf serum, protein stabilizers and sample indicator dye.Preservative agent: ProClin™ 300 (0.5%)	1 Vial 3ml
C2	**100 mIU/ml CALIBRATOR – POSITIVE CONTROL** Buffer with Anti-HBs antibodies of human origin, fetal calf serum, protein stabilizers and sample indicator dye. Preservative agent: ProClin™ 300 (0.5%)	1 Vial 2.2ml
C3	**400 mIU/ml CALIBRATOR** Buffer with Anti-HBs antibodies of human origin, fetal calf serum, protein stabilizers and sample indicator dye. Preservative agent: ProClin™ 300 (0.5%)	1 Vial 2.2ml
C4	**1000 mIU/ml CALIBRATOR** Buffer with Anti-HBs antibodies of human origin, fetal calf serum, protein stabilizers and sample indicator dye/.Preservative agent: ProClin™ 300 (0.5%)	1 Vial 2.2ml
R6	**SPECIMEN DILUENT** Buffer with fetal calf serum, protein stabilizers and sample indicator dye. Preservative agent: ProClin™ 300 (0.1%)	1 Vial 27ml
R7a	**CONCENTRATED CONJUGATE (11X)** Buffer with HBsAg (human ad and ay subtypes) coated with peroxydase and protein stabilizers Preservative agent: ProClin™ 300 (0.5%)	1 Vial 2.5ml
R7b	**CONJUGATE DILUENT** vial Buffer with calf serum and protein stabilizers Preservative agent: ProClin™ 300 (0.1%)	1 Vial 25ml
R8	**SUBSTRATE BUFFER** Citric acid and Sodium acetate solution pH 4.0 Containing H2O2 (0.015 %) and DMSO (4%)	1 Vial 60ml
R9	**CHROMOGEN (**TMB)	1 Vial 5ml
R10	**STOPING SOLUTION (HSO4 S/ON)**	1 Vial 28ml

Store the kit at 2-8°C. Bring all reagents except Conjugate Concentrate to room temperature (18-30°C) before use. Return reagents to 2-8°C immediately after use. Store all unused strips/plates in pouch and reseal. Do not remove desiccant. Store strip plates at 2-8°C.

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.

a. Do not use expired reagents.
b. Do not mix reagents from different lots within a given test run.
c. Before use, wait for 30 minutes for the reagents to stabilize at room temperature.
d. Carefully reconstitute the reagents avoiding any contamination.
e. Do not perform the test in the presence of reactive vapours (acid, alkaline, aldehyde vapours) or dust that could alter the enzymatic activity of the conjugate.
f. Use glassware thoroughly washed and rinsed with deionized water or preferably, disposable material.
g. Do not allow the microplate to dry between the end of the washing operation and the reagent distribution.
h. 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.
i. 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.
j. 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.
k. Use a new distribution tip for each serum.
l. 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.
m. Never use the same container to distribute the conjugate and the development solution.

HEALTH AND SAFETY INSTRUCTIONS

n. R43: May cause sensitization by skin contact.
o. S28-37: After contact with skin, wash immediately with plenty of soap and water.
p. Wear suitable gloves.
q. Monolisa™ Anti-HBs PLUS contains human blood components that have been tested and found non-reactive for Hepatitis B surface antigen (HBsAg), antibodies to Hepatitis C virus (HCV), and antibodies to Human immunodeficiency viruses (HIV-1 and HIV-2).

r. Consider any material directly in contact with samples and reagents of human origin, as well as washing solutions, as infectious materials.

s. Avoid spilling samples or solutions containing samples.

t. Wear disposable gloves when handling reagents and samples and thoroughly wash your hands after having handled them.

u. Do not pipette by mouth.

v. Samples, reagents of human origin, as well as contaminated material and products should be discarded after decontamination either by immersion in bleach at the 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.

w. Do not forget to neutralize and/or autoclave the solutions or washing wastes or any fluid containing biological samples before discarding them into the sink.

x. The Security Data Sheet is available on request.

REAGENT PREPARATION

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

A. Ready-for-use reagents

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.

Specimen Diluent (R6)

Anti-HBs Negative Control (C0)

10 mIU/ml Calibrator (C1)

100 mIU/ml Calibrator – Positive Control (C2)

400 mIU/ml Calibrator (C3)

1000 mIU/ml Calibrator (C4)

Homogenize reagents before use by vortex or invert gently.

B. Reagents to be reconstituted

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**.**

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.

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.

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.

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 separate 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.

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

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

3. Prepare Conjugate Working Solution (R7a + R7b), Working Diluted Substrate Solution (R8 + R9) and Diluted Washing Solution (diluted R2).
4. 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.
5. Dilute specimens, calibrators and controls 3:4 in the Specimen Diluent R6, following one of the two procedures here below :
    a. 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).
    b. 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).

NB: 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.

6. 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.m

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.

7. Cover, if it is possible, the wells with adhesive film by pressing over the whole surface to ensure tightness.
8. Incubate the plate for 60 ± 5 minutes at 37 ± 2°C.

9. 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).
10. If an automatic washer is used, follow the same procedure.
11. 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.

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

12. 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).
13. If an automatic washer is used, follow the same procedure.
14. 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.

    NB: The Working Diluted Substrate Solution is colored pink. It is possible to verify the presence of conjugate in the wells by spectrophotometric reading at 490 nm.
15. 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.
16. 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 BOTH QUALITATIVE AND QUANTITATIVE METHODS

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 ($0.0 < ODC0 \leq 0.070$).

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 of ODC1=0.246/3= 0.082

Note: 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.

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

PERFORMANCES

1Intra-assay reproducibility: five positive samples and one negative sample have been tested 10 times in triplicate inside the same series.

Inter-assay reproducibility: 3 positive samples and one negative sample have been tested 2 times per day in duplicate during 20 days following the EP5 NCCLS procedure (National Commitee for Clinical Laboratory Standards).

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.

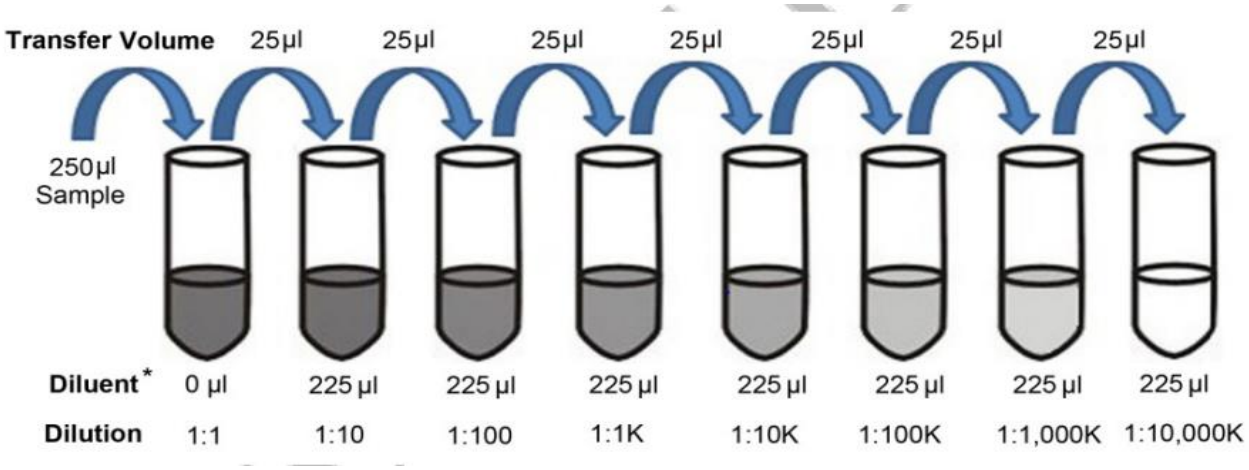
Failure to add specimen or reagent as instructed in the procedure may produce false negative results. It is advice to retest samples where a suspicion of procedural error occurs.

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.

Standard preparation

- Standard dilution series will be used to generate standard curve then standard curve used to determine concentration of target Ag in unknown samples.
- Reconstitute one tube of lyophilized standard with 1 ml of sample diluents.
- Incubate at RT for 10 mins with gentle agitation.
    - D1(500pg/ml) pipette 500 µl of standard into 0 µl of sample diluents
    - D2(250 pg/ml) Pipette 250 µl of D1 in to 250 µl of sample diluent
    - D3 (125 pg/ml) pipette 250 µl of D2 in to 250 µl of sample diluent
    - D4 (62.5 pg/ml) pipette 250 µl of D3 in to 250 µl of sample diluent
    - D5 (31.25 pg/ml) pipette 250 µl of D4 in to 250 µl of sample diluent
    - D6 (15.63 pg/ml) pipette 250 µl of D5 in to 250 µl of sample diluent
    - D7 (7.815 pg/ml) pipette 250 µl of D6 in to 250 µl of sample diluent
    - Zero standard (0 pg/ml) use 250 µl sample diluent alone



Reagent preparation

Note: Bring all reagents to RT (18-25^{0}c) before use

- Dilute detection reagent A and B to ratio of 1:100 using assay diluent A and B respectively for the number of wells you are planning to run. Prepare 10.000 µl of detection reagents assuming for 100 wells. To prepare this you need to add 100µl of

detection reagent A and 9.9 ml of diluent A in a reservoir labeled Detection reagent A. Detection reagent B will also be diluted in similar way and put in separate reservoir.

- Prepare 20 ml of working wash buffer by diluting the supplied 800 µl of 25x wash buffer with 19.2 ml of distilled water. Put this on a reservoir labeled wash buffer. With similar 1:25 dilution you can prepare another working wash buffer when you run out of it.

Assay procedure

1. Prepare the layout of all standards, samples and blank beforehand.
2. Bring all reagents and samples to room temperature without additional heating and mix thoroughly by gently swirling before pipetting (avoid foaming).
3. Prepare all reagents, working standards, and samples as manufacturer directions.
4. Add 100µl of **Standard, Blank, or Sample** per well, cover with a plate sealer, and incubate for 2 hours at 37 ^{o}C.
5. Aspirate the liquid of each well, don t wash
6. Add 100µl of **Detection Reagent A** working solution to each well, cover with a plate sealer, and gently agitate to ensure thorough mixing
7. Incubate for 1 hour at 37 ^{o}C
8. Aspirate the liquid from each well and wash 3 times
9. Wash by adding approximately 350 µl of 1x **Wash Buffer** using a squirt bottle, multi-channel pipette. Allow each wash to sit for 1-2 minutes before completely aspirating. After the last wash, aspirate to remove any remaining Wash Buffer then invert the plate and tap against clean absorbent paper
10. Add 100µl of **Detection Reagent B** working solution to each well, cover with a new plate sealer, and incubate for 60 minutes at 37 ^{o}C
11. Aspirate the liquid from each well and wash 4 times as outlined in step 8.
12. Add 90µl of **TMB Substrate** solution to each well, cover with a new plate sealer, and incubate for minutes at 37 ^{0}C. Protect from light and monitor periodically until optimal color development has been achieved.
13. . Add 50µl of **Stop Solution** to each well. The blue color will change to yellow immediately. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. The Stop Solution should be added to wells in the same order and timing as the TMB Substrate solution.

14. Determine the optical density (OD value) of each well immediately using a microplate reader set to **450 nm.**

Test Quality control (Assay validation)

The resulting Optical Density (OD) values sample must fall within the OD values of the standard curve in order for the calculated antigen concentration to be accurate. The procedure on the SOP should be strictly followed according to manufacturer instructions for utilizing the reagents including appropriate storage to get best result in this procedure. Plus blank should be done with the samples treated similarly in all procedures.

- The A value of the Blank well, which contains only chromogen Stop solution is <0.08o at 450 nm.
- The A value of the positive control must be ≥ 0.800 at450/600-650nm or at 450nm after blanking.
- The A value of the negative control must be ≤ 0.100 at450/600-650nm or at 450nm after blanking.

❖ If one of the negative values a value does not meet the quality control criteria, it should be discarded and the mean value calculated again using the remaining two values. If more than one negative control A does not meet the quality control range specification, the test is invalid and must be repeated.

Registration of results

The result will be expressed as Concentration in IU/ml so average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. The resulting data should be recorded on pre-prepared for both controls and samples accordingly. Anti-HBs

Interpretation of the result

❖ After measuring the anti HBsAg antibody, the result will be interpreted as:

- No Protective if the HBsAb titer is less than 10mUI/ml.
- Protective if HBsAb titer is greater than or equal to 10mUI/ml.

❖ After detection of hepatitis B surface antigen it will be also interpreted as:

Negative results (A/C.O<1) : Specimens giving absorbance less than the cut-off value are negative for this assay indicates that no hepatitis B surface antigen has been detected by ELISA .

Positive results (A/C.O≥1): Specimens giving absorbance or equal greater than the cut-off value are considered initially reactive, which indicates that hepatitis B surface antigen has probable been detected by ELISA.

Borderline (A/C.O = 0.9-1.1): Specimens with absorbance to cut-off ratio between 0.9 and 1.1 are considered borderline and retesting of this specimen in duplicate is required to confirm the initial result.

- After detection of HBsAb , Anti-HBc-total, and HBsAg, it will be interpreted as:

If HBsAb positive, Anti-HBc-total and HBsAg are negative suggest the child is immune due to HBV vaccine.

If HBsAb negative, Anti-HBc-total and HBsAg are positive implies that the child is not immune.

If Anti-HBc-total and HBsAb are positive but HBsAg negative indicates the child is immune due to prior HBV infection.

General SOP

1. Prepare Coating Solution by diluting the Capture antibody in Coating buffer. Refer to manufacturer for dilution recommendations.
2. Coat plates with 100 µL per well of Coating Solution. Cover plates, and incubate overnight (12–18 hours) at 2–8 °C.
3. Aspirate wells and wash 1 time with >200 µL of Wash buffer per well. Following wash, invert and tap on absorbent paper to remove excess liquid.
4. Block plate with 200 µL per well with Blocking buffer for 1 hour at room temperature.
5. Aspirate, invert, and tap on absorbent paper to remove excess liquid.
6. Prepare standards and sample dilutions in blocking buffer.
7. Pipette 100 µL of standards (in duplicate) and samples into designated wells. Incubate for 1 hour at room temperature with gentle continual shaking (~500 rpm).
8. Aspirate and wash 5 times with >200 µL of Wash buffer per well. Following wash, invert and tap on absorbent paper to remove excess liquid.
9. Prepare detection antibody solution by diluting the Detection antibody in blocking buffer. For recommended antibody dilution, refer to manufacturer's instruction.
10. Add 100 µL of the detection antibody solution into each well. Incubate for 2 hours at room temperature with gentle continual shaking (~500 rpm).

11. Aspirate and wash 5 times with >200 µL of Wash buffer per well. Following wash, invert and tap on absorbent paper to remove excess liquid.

12. Make working solution of Streptavidin-HRP with blocking buffer by diluting 1:5,000. For example, to make enough for 1 plate, add 2 µL of streptavidin-HRP to 9.998 mL of blocking buffer.

13. Add 100 µL of working streptavidin-HRP solution into each well. Incubate for 30 minutes at room temperature with gentle continual shaking (~500 rpm).

14. Aspirate and wash 5 times with >200 µL of Wash buffer per well. Following wash, invert and tap on absorbent paper to remove excess liquid.

15. Add 100 µL of TMB substrate solution to each well. Incubate plate for 30 minutes at room temperature.

16. Add 100 µL of Stop solution to each well.

17. Measure absorbance at 450 nm within 30 minutes of adding Stop solution.

18. Calculate results using a log-log or 4-parameter curve fit

D. REAL-TIME PCR ASSAY (QPCR) FOR THE DETECTION OF HBV DNA:

Amplification is performed in a 50 µL reaction mixture containing: 1X TaqMan Universal PCR Master Mix, 20 µM of each primer and probe, and 5 µL of extracted DNA. All samples are performed in duplicate. As an internal control, we use the TaqMan Exogenous Internal Positive Control Reagents kit with 1X IPC Mix (primers and TaqMan probe labeled with VIC) and 1X IPC DNA. All reagents are obtained from Applied Biosystems (Foster City, CA, USA).

Amplification starts with an incubation at 50 ºC for two minutes to inactivate possible contaminating amplicons with uracil N'-glycosylase, followed by 10 minutes at 95 ºC that activates AmpliTaq Gold Polymerase and inactivates uracil N'-glycosylase. The PCR cycling program consists of 45 two-step cycles of 15 seconds at 95 ºC and 60 seconds at 60 ºC (universal conditions)