



St. Paul's Hospital Millennium Medical College, Department of
Microbiology, Immunology, and Parasitology

Seroprevalence, Viral Load and Associated Risk Factors of Hepatitis B
and C Virus Infections Among Pregnant Women Attending Antenatal
Care in Selected Health Facilities, Addis Ababa, Ethiopia.

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Addis Ababa, Ethiopia

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Approval by the board of examination

As a member of the examining board of the final MSc open defense, we hereby certify that we have read and evaluated the thesis entitled “ Seroprevalence, viral load and associated risk factors of hepatitis B and C virus infections among pregnant women attending antenatal care in selected health facilities, Addis Ababa, Ethiopia,” presented by Fitsum Girma. The thesis has been examined and approved in its present form as fulfilling the requirements for the degree of Master of Science in Medical Microbiology.

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This is to certify that the thesis prepared and presented by Fitsum Girma Teshome, entitled: “Seroprevalence, Viral Load and Associated Risk Factors of Hepatitis B and C virus infections among Pregnant Women Attending Antenatal Care in Selected Health Facilities, Addis Ababa, Ethiopia” and submitted in fulfillment of the requirement for the Degree of Master of Science (MSc) in Medical Microbiology complies with the regulations of the College and meets the accepted standards with respect to originality and quality.

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Declaration

I, the undersigned, declare that the thesis entitled “Seroprevalence, Viral Load and Associated Risk Factors of Hepatitis B and C virus infections among Pregnant Women Attending Antenatal Care in Selected Health Facilities, Addis Ababa, Ethiopia” submitted for the degree of Master of Science (MSc) to St. Paul’s Hospital Millennium Medical College is my own work and has not previously been submitted at another university. The materials obtained from other sources have been duly acknowledged and cited their sources.

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Abstract

Background: Hepatitis B virus (HBV) and Hepatitis C virus (HCV) infections are global public health problems in pregnancy that have a high risk of mother-to-child transmission (MTCT). Perinatal transmission accounts for more than 50% of cases worldwide. Globally, 65 million women of childbearing age are infected with chronic HBV, with a 90% likelihood of MTCT in HBeAg-positive mothers of the total HCV-infected people, 1 to 8% are pregnant women, and MTCT occurs in about 3 to 10% of HCV-infected mothers. However, although Ethiopia is highly endemic for viral hepatitis, screening for these viruses in pregnancy is not well implemented, and data on viral hepatitis during pregnancy are scarce.

Objective: The objective of this study was to investigate the seroprevalence and associated factors of hepatitis B and C virus infections among pregnant women attending antenatal care at selected health facilities, Addis Ababa, Ethiopia.

Methods: A cross-sectional study was conducted between April and September 2024, targeting pregnant women attending ANC. Using a consecutive sampling method, 456 study participants were enrolled after written informed consent. Kobotoolbox was used to collect socio-demographic data, associated factors for the study participants. Ten ml of venous blood was collected, serum specimens were tested to detect hepatitis B surface antigen (HBsAg), and anti-hepatitis C virus antibody (anti-HCV) using enzyme-linked immunosorbent assay (ELISA). For the HBsAg-positive specimens, Viral load was determined using Abbot m2000/RT-PCR, and Hepatitis e antigen (HBeAg) was detected using the Cobas 6000 analyzer. The data were transferred from Kobotoolbox to SPSS version 25 software. Bivariate and multivariable logistic regression were analysed to assess the relationship between factors associated with HBV and HCV infection.

Results

The seroprevalence of HBsAg among pregnant women in the current study was 5.8% (26/450), while the prevalence of the anti-HCV antibody was much lower at 0.4% (2/450). The Bivariate analysis HBsAg positivity showed associations with gestational stage, family history of liver disease, and contact with an HBV-infected person; however, after multivariate adjustment, only

being in the third trimester remained significantly associated with HBsAg positivity. Among the HBsAg-positive cases, 30.8% (8/26) had detectable hepatitis B viral DNA with viral loads ranging from 48 IU/mL to 222 IU/mL. However, none of the HBsAg-positive subjects was positive for hepatitis B e-antigen (HBeAg).

The HBV vaccination coverage among the study participants was low; only 20.9% of participants had taken the vaccine.

Conclusion

This study revealed an intermediate seroprevalence of HBsAg and low anti-HCV among pregnant women attending ANC in selected health centers in Addis Ababa. None of the HBsAg-positive participants were reactive for HBeAg, while 30.8% exhibited detectable HBV DNA levels.

Keywords: Pregnant women, hepatitis B infection, hepatitis C infection, Antenatal care

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List of abbreviations

AACAHB	Addis Ababa City Administration Health Bureau
ANC	Antenatal care
COI	Cut-off index
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
GHSS	Global Health Sector Strategies
HBeAg	Hepatitis B envelope antigen
HBV	Hepatitis B virus
HCV	Hepatitis virus
HEV	Hepatitis Evirus
KAP	Knowledge, attitude, and practice
MTCT	Mother-to-child transmission
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SPHMMC	St. Paul Hospital Millennium Medical College
SPSS	Statistical Package for Social Sciences
USIN	Unique survey identification number
WHO	World Health Organization

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1. Introduction

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are the major causes of infectious disease morbidity and mortality worldwide (1,2). Globally, more than 350 million people are still living with this life-threatening hepatitis disease. Hepatitis B and C together continue to cause 1.1 million deaths per year as a result of chronic liver disease and cancer (3). Worldwide, two billion people have been infected with the hepatitis B virus, 296 million are chronically infected, and 1.5 million people become newly infected, with an estimated 820,000 people dying each year (4). According to the WHO 2022 report, an estimated 50 million people have chronic HCV infection, with an incidence of 1.0 million new infections annually (5).

Viral hepatitis infections are very important during pregnancy, as they have a high risk of mother-to-child transmission (MTCT), particularly in high-endemic regions. The risk of MTCT of HBV varies widely depending on the maternal viral load and hepatitis B e-antigen (HBeAg) status. In the absence of preventive interventions, the MTCT rate ranges from 10% to 30% among hepatitis B surface antigen (HBsAg)-positive but HBeAg-negative mothers and can reach 70% to 90% among HBeAg-positive women with high viremia (6,7). In contrast, HCV is transmitted from mother to child in approximately 5–7% of pregnancies, and the risk increases to 5.8–10.8% among mothers coinfecting with HIV(8). HBV and HCV are the commonest causes of hepatic dysfunction in pregnancy, have a high risk of maternal complications, and have become a leading cause of fatal death. These infections also have serious effects on the neonate, leading to impaired mental and physical health later in life (9). HBV infection is associated with increased maternal and perinatal death during pregnancy. Placenta abruption, preterm birth, gestational hypertension, and fetal growth restriction have been associated with chronic HBV during pregnancy. HCV infection in pregnancy has increased rates of adverse pregnancy outcomes, including fatal growth restriction and low birth weight (10).

Due to their global health problems, the World Health Organization (WHO) has set the goal of eliminating hepatitis as a public health problem by 2030. According to the strategic plan, it aims to reduce HBV new infections from 1.5 million cases to 170000 cases and HCV new infections from 1.575 million cases to 35000 cases annually, and hepatitis-related deaths to 450,000 (11).

Nevertheless, there has been a significant lack of progress in addressing these infections, and nearly 80% of people with hepatitis B or C virus infection remain undiagnosed, and affordable treatments are not being accessed. As a result, the WHO, in its updated 2022–2023 global health sector strategies, recommends screening all pregnant women for chronic hepatitis B and C virus infection in endemic countries for the elimination of vertical (mother-to-child) transmission. Taking prevention measures, including creating awareness toward MTCT of HBV infection, is a cornerstone of strategies for the elimination of viral hepatitis (12).

1.2. Statement of the problem

Globally, chronic HBV infection is the major cause of liver cirrhosis and hepatocellular carcinoma, leading to 50% of cases worldwide and 80% in high-endemic areas (13). The new Global Health Sector Strategies (GHSS) on the human immune virus (HIV), viral hepatitis, and sexually transmitted infections, 2022-2030, provide an opportunity to refocus global efforts and accelerate the response to the elimination of viral hepatitis by 2030 (14). The GHSS put priority measures for countries to advance their response to viral hepatitis, including epidemiological evaluation, the establishment of a national plan with committed finance, and setting intervention coverage and impact targets (15).

A meta-analysis estimated that there are 367,250 cases of perinatal HBV transmission (MTCT) per year in sub-Saharan African countries, which makes it crucial to implement preventive measures by identifying such high-risk women early (16). During higher viral replication time (HBeAg-positive), there is a high risk of vertical transmission, and to reduce this, HB immunoglobulin must be given within 12-24 hours after birth, which is effective up to 90% (17). Vertical transmission of hepatitis viruses from infected mothers to their fetuses or newborns remains the main route of transmission (13). However, the transmission rate depends on the type of virus: HBV (60%) (18), and HCV (5.8%) (8). Vertical transmission includes many modes of transmission routes from an infected mother to her newborn: intrauterine, intrapartum, and postnatal (8). Especially in the case of HBV, which is 50-100 times more contagious than HIV, it has become more prevalent (19). Infants' contact with infected vaginal blood and secretions during delivery is the most common risk for perinatal HBV infection (13).

According to a systematic review and meta-analysis in Ethiopia, the risk of MTCT of HBV in Ethiopia was 25.5% (20), and the overall pooled prevalence of HCV infection among pregnant women in Ethiopia was 1.83% (21).

Another systematic review shows the prevalence of HBV and HCV among pregnant women in Ethiopia is 5% to 7 % and 2% to 2.9%, respectively (22). Also, the prevalence of HBsAg, ranging from 4.7% to 9.2% (6,23–27), and 8% HCV among pregnant women was reported in Ethiopia (28).

1.3 Significance of the study

Knowing the prevalence and impact of the hepatitis virus in pregnant women is a reliable indicator for the prevention of MTCT, which is key in reducing the global burden of chronic viral hepatitis. However, the burden and prevalence of these viruses among pregnant women are not well studied in developing countries, including Ethiopia (19).

Nowadays, routine antenatal HBV and HCV screening of mothers during pregnancy has become an important area of public health concern worldwide to prevent vertical transmission. However, although Ethiopia is highly endemic for viral hepatitis, screening for these viruses in pregnancy is not well implemented, and data on viral hepatitis during pregnancy are scarce. In light of this, studying the burden of viral hepatitis during pregnancy, including seroprevalence, the risk of perinatal transmission, and issues related to the treatment and prevention of infection, in Ethiopia is crucial. Hence, this study aims to investigate the seroprevalence of hepatitis B and C infections and associated risk factors among pregnant women attending antenatal care at St. Paul's Hospital, Selam, and Felege Meles HC. The results of this study will provide essential data to assist policymakers and healthcare professionals in strengthening hepatitis screening, prevention, and control strategies among pregnant women in Ethiopia.

2. Literature Review

2.1. Hepatitis viruses: the etiology

Viral hepatitis is an inflammation of the liver caused by five hepatitis viruses known as types A, B, C, D, and E. HBV is an enveloped virus with a partly double-stranded circular DNA genome of 3.2kb that belongs to the *Hepadnaviridae* family(27). It is classified into ten genotypes, from A to J, which are related to its geographical origin and are further divided into several sub-genotypes (29). In Ethiopia, the dominant genotype is A followed by D (30).

HCV is a member of the *Flaviviridae* family that bears an approximately 9.6 kb long positive-sense single-stranded RNA (ssRNA) genome (31). This virus is classified into seven recognized genotypes (1-7) and many subtypes (32). This virus showed a distinct level of genetic diversity and geographical distribution (33). A recent study in Ethiopia found that the predominant genotype was 4 (77.6%), followed by 2 (12.2%), 1 (8.2%), and 5 (2.0%). Seven subtypes were found, with the most frequent subtypes being 4d (34.7%), 4r (34.7%), and 2c (12.2%) (34).

2.2. Epidemiology of hepatitis viruses

Viral hepatitis is responsible for 1.34 million deaths annually, which is comparable to deaths from tuberculosis but a higher number of deaths than from HIV alone. Globally, HBV and HCV are responsible for around 887,000 and 290,000 annual deaths, respectively (35). About 71.1 million people around the world have reported chronic Hepatitis C in 2015, which accounts for 1% of the entire population (36). As per 2022 WHO report , an estimated of 254 million people were infected with chronic hepatitis B infection, with annually 1.2 million new cases and 1.1 million were dead due to cirrhosis and hepatocellular carcinoma (37). In Africa more than 60 million peoples live with chronic HBV (38). In 2023, figures showed a substantial burden of the viral hepatitis with an estimated of 7.6 million people in Ethiopia were living with chronic hepatitis B infection, while approximately 690,000 individuals were infected with hepatitis C (39).

A recent meta-analysis in Africa revealed a seroprevalence of HBsAg of about 8.83% (40). Investigators have tried to look into the seroprevalence of HBV in Ethiopia since 1968, with studies showing varied results. One study reported, the seroprevalence of chronic HBV based on HBsAg

positivity to be 3.7% (41), while another community-based study conducted among residents of Addis Ababa, the capital city, found a higher rate of 7% (42). Another community-based HBV prevalence study conducted using a rapid test in East and West Gojjam, Amhara region, among adult residents, reported a seroprevalence of 3.1% (43). Yet the overall HBV prevalence in the nationwide community was 7.4% (44). A meta-analysis study conducted in Ethiopia showed that the prevalence of anti-HCV in Ethiopia ranged from 0% to 22% with a pooled prevalence estimated at 2% (45), and among blood donors, its prevalence was 0.819% (46).

2.3. Burden of hepatitis viruses among pregnant women

The prevalence of Hepatitis B virus (HBV) and Hepatitis C virus (HCV) infections among pregnant women shows marked geographical variation, with the highest rates generally reported in sub-Saharan Africa and the Western Pacific region. For example, a nationally representative study from the United States revealed that maternal HBV infection rates increased annually by approximately 5.5% since 1998, reaching a prevalence of 85.8 cases per 100,000 deliveries (0.09%) among live-born singleton births between 1998 and 2011 (47).

Globally, in 2019, the estimated number of women of childbearing age with active HCV infection was 14.8 million, with the Eastern Mediterranean region recording the highest viraemic prevalence (48). In Iran, the seroprevalence of HBV among women was 1.18% (49). Similarly, in Pakistan, HBV and HCV prevalence rates of 3.7% and 2.1%, respectively, have been reported (50). However, another study in the same country a higher prevalence of HCV, which accounted for 10.84% was reported (51). In sub-Saharan Africa, HBV and HCV remain important public-health concerns. In Tanzania, the seroprevalence of HBsAg, HBeAg, and anti-HCV among pregnant women was 6.3%, 24%, and 5%, respectively (52). In Uganda, the prevalence of HBsAg among pregnant women was reported at 8.7%, while in Kenya, prevalence rates ranged from 3.8% to 8.75% (53,54).

In Ethiopia, several studies conducted in different parts of the country showed varying seroprevalence of the hepatitis viruses among pregnant women. Among the studies conducted on the prevalence of HBV and HCV among pregnant women, HBsAg and anti-HCV were positive at 8.1% and 3.2%, respectively (55). In another study, the seroprevalence of hepatitis B and C virus

infections was found to be 4.4 and 0.26%, respectively (56). It was also reported that the prevalence of HBsAg was 2.4% and that of HCV was 8.07% in pregnant women (28). Similar but separate studies in Ethiopia reported the prevalence of HBsAg among pregnant women at 3.5%, in Dawro zone (57), 6.6% in Hawassa (58), 8.5% in Jijiga (59) and 7.3% in Wolayta Sodo (60) and 3.8% in Bahirdar (43). According to a systematic review and meta-analysis in Ethiopia, the risk of MTCT of HBV was 25.5% (20), and the overall pooled prevalence of hepatitis C virus infection among pregnant women in Ethiopia was 1.83% (21). Another systematic review shows that the prevalence of HBV and HCV among pregnant women in Ethiopia is 5% to 7% and 2% to 2.9%, respectively (22).

Viral hepatitis is a leading cause of maternal complications and vertical transmission that causes fetal and neonatal hepatitis, which causes serious effects on the neonate, leading to impaired mental and physical health later in life (18). It is the leading and most familiar cause of jaundice in pregnant women (61). The presence of HBeAg in the serum of pregnant women is one of the markers correlated with high infectivity and a high HBV DNA concentration (62). In such cases, the risk of vertical transmission reaches about 90%, while the transmission rate in HBeAg-negative pregnant women is about 10 to 20% (63). Among the means of HBV transmission, perinatal transmission is one of the most common routes (49). Even if a woman has been tested or vaccinated for HBV before, the CDC advises that in every pregnancy and during an early prenatal visit, she must be screened for HBsAg, and the newborn should get prophylaxis and vaccination (64). HBV is one of a vaccine preventable pathogen in such that it can be reduced by 85% to 95% through a dose of the hepatitis B vaccine in combination with hepatitis B immunoglobulins in the neonate (65). It is documented that if the viral load of the mother is greater than 200,000 IU/ ml, its transmission to the newborn reaches about 30% (66). Hence, the administration of prophylaxis with specific immunoglobulin and vaccination in the third trimester of the mother with a high viral load and HBeAg-positive can play a great role in reducing MTCT, but there is no vaccine in the case of HCV (67).

2.4. Associated risk factors of HBV and HCV infections

Viral hepatitis infections, particularly in low-and middle incomes countries persistence largely influenced by various risk factors including sociodemographic, personal behavioral and medical procedures. The associated risk factors includes blood transfusion, having multiple sexual partnerships, tattooing, traditional practices and dental procedures. Giving health educations for pregnant womens is essential in eliminating and controlling the impact of viral infections (68).

Several studies conducted in Ethiopia showed different risk factors including, tooth extraction at health facilities, tonsillectomy, multiple sexual partners, tattooing and contact with jaundiced patient were identified as associated risk factors (23,69,70). Similarly, a study conducted in public hospitals of West Hararghe of Oromia identified a history of abortion, traditional tonsillectomy, previous hospital admission, having multiple sexual partners, and a family history of liver disease as factors significantly associated risks (71). Moreover, history of surgery, history of abortion and tattooing were considered as a risk factors as reported from Tirunesh Beijing General Hospital (26). Furthermore, studies reported from Gedeo, Gambella, Borena and southern Ethiopia showed history of blood transfusion, history of hospital admission, history of abortion, HIV status, multiple sexual partner and drinking alcohol had a statistically significant association with HBsAg seropositivity (72–75). Additionally, a study from Arba Minch hospital only the risk factors of history of abortion and having multiple sexual partner were reported (76).

Regarding the risk factors of HCV infection, a systematic review study conducted in Sub-Saharan showed as being living with HIV, contact with families infected with HCV, blood transfusion, hospitalization, medical operation, scarification, and injection of drug uses and patients on hemodialysis and organ transplantation, and needle stick injury were mentioned (77–79). Moreover, the predictors of HCV were, circumcision, positive maternal HCV status, multiple sexual activity and dental and surgical procedures (80,81).

3. Objective

3.1. General Objective

This study aimed to investigate the seroprevalence of HBV and HCV infections and associated risk factors among pregnant women attending antenatal care at SPHMMC, Selam, and Felege Meles health centers.

3.2. Specific Objectives

- To determine the seroprevalence of HBV and HCV infections among pregnant women.
- To determine the viral load of HBV and HBeAg in HBsAg-positive pregnant women.
- To identify associated risk factors for HBV infection among pregnant women.

4 . Materials and Methods

4.1. Study area

This study was conducted at the obstetrics unit of SPHMMC, the ANC unit of FelegeMeles Health Center, and Selam Health Center in Addis Ababa, Ethiopia. The selection of the study site is based on its attendance flow per day and annually. Administratively, Addis Ababa is divided into 11 sub-cities, which are in turn divided into 116 Woredas, the smallest government administrative units. Health centers are managed by the Addis Ababa City Administration Health Bureau (AACAHB) and are mainly focused on primary health care services.

SPHMMC is a tertiary teaching public hospital affiliated with the Federal Ministry of Health. More than 2,800 clinical, academic, administrative, and support employees work for the institution and offer medical specialty services to clients who are vertically or horizontally referred. Thirteen health facilities in three different sub-cities in Addis Ababa, including Kolfe Keranio, Guelle, and Addis Ketema, provide a direct catchment for the hospital. Nearly 3,000 mothers attend antenatal care, and more than 10,000 deliveries are attended in the hospital every year. The obstetrics department has a maternal-fetal medicine unit, which is one of the pioneers in the country. The unit deals with managing high-risk pregnant mothers and conducts advanced fetal diagnostic ultrasounds by maternal-fetal medicine specialists and fellows.

4.2 Study design and period

A cross-sectional study was conducted from April to September 2024 among pregnant women attending obstetrics units and ANC at SPHMMC, Selam, and Felege Meles Health Centers.

4.3. Source of population

The source population included all pregnant women attending antenatal care at SPHMMC, Selam, and Felege Melese health centers.

4.4. Study populations

Pregnant women who visited the ANCs and gave their consent were included in the study.

4.5. Study variable

4.5.1. Dependent variable

- Sero-prevalence of HBV and HCV.
- HBV Viral load and HBeAg
- Associated risk factors for HBV.

4.5.2. Independent variable

- Age
- Residency (Rural, Urban and Sub-urban)
- Employment/Occupation status
- Educational status
- Marital status
- Surgical history
- HBV vaccination status
- Family history of hepatitis B and C infection
- History of sexually transmitted infection (STI)
- Tattooing
- History of jaundice
- Female genital mutilation
- History of multiple sexual partners
- Gravidity (Primigravida or Multigravida)
- History of blood transfusion

4.6. Inclusion and Exclusion Criteria

4.6.1. Inclusion criteria

All pregnant women who visited the ANC of SPHMMC, Selam, and Felege Meles health centers and those who volunteered to participate and give informed consent.

4.6.2. Exclusion criteria

Pregnant mothers who attended the clinic for more than one visit during the study period were excluded by putting a unique mark on the enrolled mothers' cards.

4.7. Sampling technique and sample size determination

4.7.1. Sample size determination

The sample size is determined using a single population formula for seroprevalence of HBV 11.6% (82) and HCV 1.83% (83) among pregnant women in Ethiopia. The sample size (n) is calculated using the following steps, based on the formula for a proportion:

$$n = \frac{(z_{\alpha/2})^2 * p(1 - p)}{d^2}$$

Where:

- Z = Z-score for a 95% confidence level, which is 1.96.
- p = The estimated prevalence of the condition (HBV or HCV).
- d = The absolute precision (margin of error), which is 3% or 0.03.
- Non-response = 5%

1) HBV (p = 11.6% = 0.116)

$$n = \frac{(1.96)^2 * 0.116(1-0.116)}{0.03^2} = 438$$

The maximum adjusted sample size = 438/ (1-0.05) = **461**

2) HCV (p = 2.88% = 0.0288)

$$n = \frac{(1.96)^2 * 0.0288(1-0.0288)}{0.03^2} = 119$$

The maximum adjusted sample size = 119/ (1-0.05) = **126**

Since the study aims to estimate the prevalence of both HBV and HCV, the larger of the two, which is **461**, was the final sample size.

Note: The decision to use a relatively tight absolute precision (margin of error) of d = 3% (instead of a more common 5%) was made specifically to increase the precision of the estimate for the condition with low prevalence of HCV.

4.7.2. Sampling technique

A consecutive sampling technique was used for this study.

4.8. Data Collection tool

All study participants were properly informed about the study and were asked for consent. Then, data were collected from the study participants using an interview with a structured questionnaire by using the koobotool box, which is in English and was adapted from previously published studies (84,85) with some modifications to ensure applicability to our current study, credibility, and quality by a trained nurse.

4.8.1 Sample collection techniques and Laboratory procedures

Ten ml of venous whole blood was collected using a serum separator tube from each study participant by a trained phlebotomist at the sites and properly packed, and transported to the EPHI for laboratory investigations within 2-3 hours. The serum was separated by centrifugation and then transferred to sterile serum storage vials, where it was kept at -80 °C until tests were done. All serum samples were screened for HBsAg, and anti-HCV antibodies by using a fourth-generation enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. HBsAg-positive samples were further tested for HBV viral load using Abbot m2000/RT PCR (which targets the surface gene in the HBV genome) with limit of detection 10 IU/mL, low viral load range (< 2,000 IU/mL), medium viral load range (2,000 – 20,000 IU/mL) and high viral load (> 20,000 IU/mL). HBeAg was tested using the Cobas 6000 analyzer, which can detect HBeAg concentrations ≥ 0.3 Paul-Ehrlich-Institute standard unit/mL (PEI U/mL) with 95% confidence. The result is reported as reactive or non-reactive based on the Cut-off index (COI). Result < 1.0 COI showed non-reactive (HBeAg negative), and result ≥ 1.0 COI reported as Reactive (HBeAg positive).

All the workflow beginning from sample collection to result interpretation are in depicted in (table 1).

Table 1: Workflow from Sample Collection, Laboratory Procedures and result interpretations

Steps	Procedure/Activities	Detail of the Activities
1. Blood Collection	Venous blood collection after consent is secured	10 mL whole blood drawn using serum separator tube (SST) by trained phlebotomist
2. Sample Transport	Transport to EPHI	Samples properly packaged and transported within 2–3 hours
3. Serum Separation	Centrifugation	Serum separated from whole blood and transferred into sterile vials
4. Serum Storage	Preservation until testing	Serum stored at -80°C
5. Initial Screening Tests	ELISA testing	All serum samples screened for HBsAg and anti-HCV antibodies using fourth-generation ELISA, following manufacturer instructions
6. Follow-up Testing for HBsAg-Positive Samples	HBV Viral Load (VL) testing	Performed using Abbott m2000 RT-PCR system, targeting surface (S) gene of HBV
	Limit of detection	10 IU/mL
	Viral load classification	Low VL: $< 2,000$ IU/mL Medium VL: $2,000\text{--}20,000$ IU/mL High VL: $> 20,000$ IU/mL
7. HBeAg Testing	Performed using Cobas 6000 Analyzer	Detects ≥ 0.3 PEI U/mL with 95% confidence
	Reporting	- $\text{COI} < 1.0 \rightarrow$ Non-reactive (HBeAg negative) - $\text{COI} \geq 1.0 \rightarrow$ Reactive (HBeAg positive)

Keys: EPHI- Ethiopian public health institute, ELISA-Enzyme-Linked Immunosorbent Assay, RT-PCR-Real-Time Polymerase Chain Reaction, COI-Cut-Off Index, EPI-Paul-Ehrlich-Institute standard unit

4.9. Quality control

Each sample tube was labeled with a unique identification number (USIN), as specified in the questionnaire. The questionnaire was first developed in English, which was then translated into Amharic and Affanoromo before being returned to English to ensure uniformity. All procedures were rigorously followed throughout the sample collection, storage, and testing process. Along with sample testing, known positive and negative sera supplied with the test kits were included in each batch to ensure the test procedure is reliable and accurate.

4.10. Operational definition

Viral hepatitis: inflammation of the liver caused by HBV and HCV.

HBV infection: liver infection caused by the hepatitis B virus (being positive for HBsAg).

HCV infection: liver infection caused by the hepatitis C virus (being positive for ant-HCV).

Sexually transmitted infection: is spread predominantly by unprotected sexual contact. (HIV, Chlamydia, Gonorrhea, Herpes, Human Papillomavirus, Mycoplasma genitalium, syphilis)

Co-morbidities: if the study participant has more than one disease or condition at the same time.

Associated risk factors: factors that are related to viral hepatitis, and that increase the likelihood of developing viral hepatitis.

HBV viral load: refers to the quantitative measurement of hepatitis B virus DNA in a patient's serum or plasma, expressed in International Units per milliliter (IU/mL) using real-time PCR (Abbott m2000rt) and categorized as low (<2,000 IU/mL), moderate (2,000–20,000 IU/mL), or high (>20,000 IU/mL).

HBV Vaccination status: it could be Complete vaccination, Partially vaccinated, or Not vaccinated.

- **Complete vaccination:** means those who have received three or more doses (booster doses) of the Hepatitis B vaccine.
- **Partially vaccinated:** means those who have had at least one or two doses of the Hepatitis B vaccine.
- **Not vaccinated:** means those who have not received any dose of the Hepatitis B vaccine.

Liver disease: any condition that affects the liver.

Viral hepatitis infected person. An infection that causes liver inflammation and damage due to HBV, and HCV.

Viral hepatitis transmission through mother to child: all HBV, and HCV infections contracted before birth, the Health Center, and after birth.

Surgical procedure: A procedure to remove or repair a part of the body or to find out whether a disease is present.

Dental procedure: Anything that involves a dentist or dental practitioner working on your mouth. it includes filling for your tooth, treatment for gum disease, straightening crowded teeth, dental surgery, or having dentures made.

Tattooing: Marking (a part of the body) with an indelible design by inserting pigment into punctures in the skin. It could be traditional or modern tattooing.

4.11. Data analysis

Data analysis was done using the Statistical Package for Social Sciences (SPSS) version 25 software. Descriptive statistics of frequency were used to present the prevalence of hepatitis viruses and their sociodemographic characteristics. For categorical variables, frequencies and percentages were computed, and for continuous variables, the mean and the standard deviation were calculated. For the associated risk factor analysis of independent variables with outcomes, bivariable and multivariable logistic regression models were used. For risk factor analysis, odds ratios (OR) were also calculated. By estimating a 95% confidence interval, the relationship between the dependent and independent variables was assessed using univariate analysis. Those with a p-value ≤ 0.25 were candidates for multivariate analysis. After performing a multivariable logistic regression model, a p-value ≤ 0.05 was considered statistically significant.

4.12. Ethics approval and informed consent

Ethical clearance was obtained from the institutional review board (IRB) of SPHMMC and the Addis Ababa health bureau before the study began. Participants provided informed written consent after reviewing forms detailing the study's purpose, procedures, risks, and benefits. Confidentiality was maintained by anonymizing data, and access was restricted to the principal investigator and

supervisors. Positive results were communicated confidentially with participants and were linked to appropriate clinical care. These measures ensured ethical compliance and participant well-being.

4.13. Dissemination of results

The result of this study will be publicly defended and shared with the SPHMMC library, and the researcher will present the findings to professional associations as needed and submit them for publication in peer-reviewed journals.

5. Results

Socio-demographic characteristics of the study participants

A total of 456 pregnant women participated in the study. The majority of participants (54.8%) were recruited from SPHMMC, and 29.6% were between the ages of 25 and 29 years. The participants' average age was 29.7 years (± 5.1 SD), with a minimum of 17 years and a maximum of 47 years. Among the participants, 271 (59.45%) identified themselves as housewives. The majority of the study participants, 428 (93.9%), came from an urban area. Furthermore, the majority of pregnant women, 388 (85.1%), did not get the HBV vaccine. One hundred seventeen (25.7%) of the participants had a college diploma or above, while 27 (5.9%) were illiterate. The majority of participants, 332 (72.8%), had multiple pregnancies. Considering their marital status, 438 (96.1%) were married, 16 (3.5%) were single, and 2 (0.4%) were divorced, while 209 (45.8%) were in the third trimester, 169 (37.1%) were in the second trimester, and 78 (17.1%) were in the first trimester pregnancy (Table 2).

Table 2: Socio-demographic characteristics of pregnant women attending antenatal care at a selected health facility in Addis Ababa, Ethiopia (N=456)

Variables	Categories	Frequency n (%)
Age (years)	<20	2 (0.4)
	20-24	66 (14.5)
	25-29	163 (35.7)
	30-34	135 (29.6)
	35-39	78 (17.1)
	≥ 40	12 (2.6)
Facility	SPHMMC	250 (54.8)
	Selam Health Center	106 (23.3)
	Felegemeles Health Center	100 (21.9)

Residency	Urban	428 (93.9)
	Rural	17 (3.7)
	Sub-urban	11 (2.4)
Marital status	Married	438 (96.1)
	Single	16 (3.5)
	Divorced	2 (0.4)
Educational status	Illiterate	27 (5.9)
	Able to write and read	35 (7.7)
	Elementary	146 (32.0)
	Secondary	131(28.7)
	College diploma and above	117 (25.7)
Occupation	Housewife	271(59.4)
	Self-employed	58 (12.7)
	Private employee	43 (9.4)
	Governmental employee	51 (11.2)
	Others*	33(7.3)
Gestational stage	1–12 weeks	78 (17.1)
	13–24 weeks	169 (37.1)
	25 weeks to delivery	209 (45.8)
Gravidity	Primigravida	124 (27.2)
	Multigravida	332 (72.8)
HBV vaccination status	Vaccinated	34 (7.5)
	Non- vaccinated	388 (85.1)
	Unknown	34 (7.5)

Key: Others*- student, daily laborers, cleaners, waitresses, drivers, beauty salon workers, and factory workers, etc

Seroprevalence of HBV and Anti-HCV Antibody among pregnant women

Of the 456 participants, 450 (98.7%) had been tested for HBsAg and anti-HCV antibodies. The overall positivity either for HBsAg or HCV-antibodies was 6.2%. Among those tested, 26 (5.8%) were positive for HBsAg, and 2 (0.4%) were seropositive for anti-HCV antibodies. Importantly, none of the study participants tested positive for both HBsAg and anti-HCV antibodies, indicating that no co-infection occurred (Table 3).

Table 3: Seroprevalence of HBV (HBsAg) and anti-HCV antibody among pregnant women attending ANC at selected health facilities in Addis Ababa, Ethiopia. (N=450)

Variables	Test results	Frequency,n (%)
HBsAg	Positive	26 (5.8)
	Negative	424 (94.2)
Anti-HCV antibody	Positive	2 (0.44)
	Negative	448 (99.6)
Coinfection of HBV and HCV	Positive	0 (0)
	Negative	450 (100)

Status of Viral load and HBeAg among HBsAg-positive pregnant women

All 26 HBsAg-positive for HBsAg using ELISA were examined further to evaluate HBV DNA levels (viral load) and HBeAg status. Quantitative HBV DNA testing revealed detectable viral load in 8 out of the 26 samples (30.8%), with viral load ranging from 48 IU/mL to 222 IU/mL, as presented in (Table 4). Interestingly, all HBsAg positives were negative for HBeAg.

Table 4: Quantitative HBV DNA levels of HBsAg-positive samples among pregnant women attending ANC at selected health facilities in Addis Ababa, Ethiopia. (n = 26)

Sample No	HBeAg status	HBV DNA (IU/mL)	Interpretation
1	Negative	48	Low-level viremia
2	Negative	54	Low-level viremia
3	Negative	60	Low-level viremia
4	Negative	76	Low-level viremia

5	Negative	104	Low-level viremia
6	Negative	129	Low-level viremia
7	Negative	187	Low-level viremia
8	Negative	222	Low-level viremia
9-26	Negative	Undetectable	No active replication

Relationship between HBsAg-positive, anti-HCV, and sociodemographic characteristics of the study participants

As illustrated in Table 4, most of the (65.4%) HBsAg-positive pregnant women were from SPHMMC and 69.2% of HBsAg-positive pregnant women belonged to the age group between 25-29 years (30.77%) and 30-34 years (38.46%). Furthermore, 96.2% of HBsAg-positive cases resided in urban areas, and the prevalence of HBsAg was high in multigravida 20 (76.92%) and married women 25 (96.2%). Occupationally, the majority of the HBsAg-positive cases (69.2%) were housewives (Table 5). Among the participants screened for anti-HCV, only two tested positive, one belonging to the age group 25–29 years and another in 30–34 years. One was a housewife and the other was self-employed; both resided in urban areas and were in their third trimester of pregnancy.

Table 5: Relationship between HBsAg status and sociodemographic characteristics among pregnant women attending ANC at selected health facilities in Addis Ababa, Ethiopia. : N=450

Variables		HBsAg Positive (%)	HBsAg negative (%)	Total
Age catagories (years)	< 20	1 (3.85)	1(0.24)	2 (0.44)
	20-24	3 (11.54)	62 (14.62)	66 (14.67)
	25-29	8 (30.77)	150 (35.38)	162 (36.00)
	30-34	10 (38.46)	125 (29.48)	133 (29.56)
	35-39	2 (7.69)	76 (17.92)	75 (16.67)
	>=40	2 (7.69)	10 (2.36)	12 (2.67)
Health Facility	SPHMMC	17 (65.40)	233 (54.95)	250 (55.56)
	Selam HC	3 (11.50)	103 (24.29)	106 (23.56)
	Felege Meles HC	6 (23.10)	88 (20.75)	94 (20.89)
Residency Area	Urban	26 (100)	396 (93.40)	422 (93.78)
	Rural	0	17 (4.01)	17 (3.78)
	Sub-urban	0	11 (2.59)	11 (2.44)

Marital status	Married	1 (3.9)	14 (3.30)	433 (96.22)
	Single	25 (96.1)	408 (96.23)	15 (3.33)
	Divorced	0 (0.00)	2 (0.47)	2 (0.44)
Educational status	Illiterate	2 (7.7)	24 (5.66)	26 (5.78)
	Able to read and write	3 (11.5)	31 (7.31)	34 (7.56)
	Elementary	7 (26.9)	137 (32.31)	144 (32.00)
	Secondary	8 (30.8)	122 (28.77)	132 (29.33)
	College diploma and above	6 (23.1)	110 (25.94)	114 (25.33)
Occupation	Governmental	5 (19.23)	46 (10.85)	51 (11.33)
	Housewife	18 (69.20)	251 (59.20)	269 (59.78)
	Self/private	2 (7.69)	96 (22.64)	98 (21.78)
	Other	1 (3.85)	31 (7.31)	32 (7.11)
Gestational stage	1-12 weeks	3 (11.50)	73 (17.22)	76 (16.89)
	13-24 weeks	13 (50.00)	152 (35.85)	165 (36.67)
	25 weeks to delivery	10 (38.46)	199 (46.93)	209 (46.44)
Gravidity	Primigravida	6 (23.08)	116 (27.36)	122 (27.11)
	Multigravida	20 (76.92)	308 (72.64)	328 (72.89)

Risk factors associated with HBV infection among pregnant women

A total of 26 pregnant women were HBsAg-positive from the total of 450 study participants. The distribution of socio-demographic and clinical characteristics is summarized in (Table 6). In the bivariate analysis, several variables showed notable crude associations with HBsAg positivity, including gestational stage, family history of liver disease, and contact with an HBV-infected person. However, after adjusting for potential confounders in the multivariate logistic regression model, gestational stage (third trimester) remained the only factor significantly associated with maternal HBsAg positivity. Although in the bivariate analysis, family history of liver disease had almost 6.5 times more risks than those don't have the contact and contact with an HBV-infected person showed strong crude associations with 6.2 times higher in the pregnant women. However, the bivariate analysis, these associations did not remain statistically significant in the adjusted model except the gestational stage of trimester.

Table 6: Bivariate and Multivariate analysis of the factors associated with HBV among pregnant women attending ANC at selected health facilities in Addis Ababa, Ethiopia.

Variables	Categories	HBsAg results		COR (95% C.I)	P-value	AOR (95% C.I.)	P-value
		Positive, n (%)	Negative, n (%)				
Age (years)	< 20	1(3.85)	1(0.24)	Ref			
	20-24	3(11.54)	62 (14.62)	5.00(0.212-117.894)	0.318	0.063(0.003-1.398)	0.08
	25-29	8(30.77)	150 (35.38)	0.242(0.036-1.633)	0.145	0.065(0.003-1.297)	0.073
	30-34	10(38.46)	125 (29.48)	0.267(0.050-1.426)	0.122	0.118(0.006-2.309)	0.159
	35-39	2(7.69)	76 (17.92)	0.400(0.077-2.081)	0.276	0.046(0.002-1.164)	0.062
	>=40	2(7.69)	10 (2.36)	0.132(0.017-1.041)	0.055	0.169(0.006-4.745)	0.296
Gestational stage	First trimester	8(30.77)	67 (15.80)	Ref			
	Second trimester	13(50)	153 (36.08)	0.712 (0.282-1.797)	0.472	0.980(0.342-2.809)	0.969
	Third trimester	5(19.23)	204 (48.11)	0.205 (0.065-0.649)	0.007	0.252(0.072-0.889)	0.032
History of abortion	Yes	6 (23.08)	124 (29.25)	0.726 (0.285-1.851)	0.502	NA	NA
	No	20 (76.92)	300 (70.75)	Ref			
History of Multiple sexual partners	Yes	2(7.69)	45 (10.61)	0.702 (0.161-3.069)	0.638	NA	NA
	No	24 (92.31)	379 (89.39)	Ref			
Residence area	Urban	26(100)	396 (93.40)	Ref			
	Rural	0 (0%)	17 (4.01)	<0.001	0.998	NA	NA
	Suburban	0 (0%)	11 (2.59)	<0.001	0.999	NA	NA
Educational status	Illiterate	2(7.7)	24 (5.66)	Ref		NA	NA
	Able to read and write	3(11.5)	31 (7.31)	1.161(0.180-7.511)	0.875	NA	
	Elementary	7(26.9)	137 (32.31)	0.613(0.120-3.130)	0.556	NA	NA
	Secondary	8(30.8)	122 (28.77)	0.787(0.157-3.937)	0.77	NA	NA
	College diploma and above	6(23.1)	110 (25.94)	0.655(0.124-3.443)	0.617	NA	NA
Marital status	Single	1(3.9)	14 (3.30)	Ref			NA
	Married	25(96.1)	408 (96.23)	0.858(0.108-6.788)	0.884	NA	NA
	Divorced	0 (0.00)	2 (0.47)	<0.001	0.999	NA	NA
History of Surgical Procedure	Yes	5(19.2)	116 (27.36)	0.632(0.233-1.716)	0.368	NA	NA
	No	21(80.8)	308 (72.64)	Ref			
	Yes	0 (0%)	34 (8.02)	Ref			

Vaccination status	No	24(92.3)	359 (84.67)	<0.001	0.998	NA	NA
	Can't remember	2(7.7)	32 (7.55)	1.036(0.234-4.591)	0.963	NA	NA
Family history of liver disease	Yes	5(19.2)	15 (3.54)	6.492 (2.154-19.563)	0.001	3.363(0.646-17.512)	0.15
	No	21(80.8)	409 (96.46)	Ref			
History of STI	Yes	0 (0%)	26 (6.13)	<0.001	0.999	NA	NA
	No	26(100)	398 (93.87)	Ref			
Body tattooing	Yes	4(15.4)	45 (10.61)	1.531(0.505-4.643)	0.451	NA	NA
	No	22(84.6)	379 (89.39)	Ref			
History of jaundice	Yes	2(7.7)	15 (3.54)	2.272(0.491-10.512)	0.294	NA	NA
	No	24(92.3)	409 (96.46)	Ref			
Gravidity	Primigravida	6(23.1)	116 (27.36)	Ref			
	Multigravida	20(76.9)	308 (72.64)	1.255(0.492-3.204)	0.634	NA	NA
Contact with an HBV-infected person	Yes	6(23.1)	23 (5.42)	6.174(1.492-26.678)	0.015	3.045(0.489-18.792)	0.233
	No	17(65.4)	330 (77.83)	Ref			
	I don't know	3(11.5)	71 (16.75)	1.219 (0.348-4.272)	0.757	1.350(0.376-4.851)	0.646
Female genital mutilation	Yes	12(46.2)	167 (39.39)	1.319 (0.596-1.689)	0.495	NA	NA
	No	14(53.8)	257 (60.61)	Ref			
Nose or ear piercing	Yes	19(73.1)	274 (64.62)	1.486(0.611-3.651)	0.383	NA	NA
	No	7(26.9)	150 (35.38)	Ref			
History of dental procedure	Yes	7(26.9)	98 (23.11)	1.226(0.501-3.001)	0.656	NA	NA
	No	19(73.1)	326 (76.89)	Ref			

Keys: COR-crueds odds ratio, STI-sexually transmitted infection, AOR -adjusted odds ratio, CI, confidence interval, Ref-reference, NA-not applicable refers to factors with p-value of ≥ 0.25 in the bivariate analysis was not considered in the multivariate analysis

6. Discussion

Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV) infections pose significant public health threats globally, particularly in developing nations like Ethiopia, due to their profound and direct impact on maternal and child health and long-term morbidity (86,87). To address these problems and the need for local data, the current study aimed to determine the seroprevalence of HBV and HCV and identify associated risk factors among pregnant women attending ANC services in Addis Ababa, Ethiopia. The investigation revealed an overall hepatitis prevalence of 6.22%, which was primarily driven by an HBsAg seroprevalence of 5.8% and supplemented by a lower anti-HCV seroprevalence of 0.44%, with the crucial finding of no detected HBV/HCV co-infection.

The HBsAg seroprevalence observed in our study (5.8%) is comparable to several previously reported findings, including those based on the WHO classification of HBsAg prevalence which is classified as intermediate, with the prevalence of 2% to 7% (88). Moreover, a meta-analysis in Ethiopia estimated an overall HBV prevalence of 5.78% (83), while a systematic review of 28 African countries found a pooled prevalence of 5.89% (89). Similarly, a study in Hawassa, southern Ethiopia, reported a 6.6% prevalence (58), and a study from Ghana documented a 6.0% of HBV (90). Another meta-analysis from East Africa estimated an overall pooled prevalence of 6.0% with variations across countries ranging from 1.5% to 22.2% (91). These similarities suggest that our findings align with broader regional and continental trends, reinforcing the persistent burden of HBV among pregnant women in Africa. Additionally, this study was comparable to studies conducted in various areas of Ethiopia, including Nekemte (5.8%) (78), Borena Zone (5.7%) (79), Dilla (5.1%) (92), and West Hararghe (6.1%) (71). These findings are crucial for accurately quantifying the local disease burden, informing targeted maternal and child health interventions, and ensuring alignment with the United Nations' Sustainable Development Goals (SDGs) for the elimination of viral hepatitis by 2030, that is reducing new cases of chronic viral hepatitis B and C infections by 90% and deaths by 65% (93).

In contrast, the present study finding (5.8%) was lower than rates reported in some areas of Ethiopia and other sub-Saharan African countries. Higher HBsAg prevalence rates, ranging from 7.2% to 13%, have been widely reported across various regions of Ethiopia, including Jijiga,

Eastern Ethiopia (8.5%) (59), Wolaita, Southern Ethiopia (7.3%) (60), Gedeo Zone (9.2%) (72), Wolaita Sodo (7.5%) (94), Yirgalem Hospital 7.2% (24), Tigray 11.6% (82), Debre Markos (8.3%) (95), Borumeda (8.1%) (55), Adigrat, Tigray (9.2%) (6), Southeast Ethiopia (7.6%) (96), Southern Ethiopia (10.9%) (97), Gambella (7.9%) (73) and Debre Tabor (13%) (98), indicating significant regional variability and a higher endemic burden in several areas compared to the current study. Moreover, the HBsAg seroprevalence observed in this study is consistently lower than the estimated pooled prevalence for Africa, which ranges from 6.77% to 6.8% according to recent meta-analyses (99). Compare to our study, significantly higher rates have been reported across the continent, including rates in Sudan (7.5%), Ghana (7.7%) (100) (101), Mali (8.1%) (102), South Sudan (11%) (103), Kenya (9.3%) (104), Gambia (9.2%) (105), Somalia (11.2%) (106), Cameroon, ranging 10.2% to 10.78% (107), Uganda (11.8%), with some countries like Tanzania (25.1%) (108) , and Nigeria (14.1%) (109) showing the highest endemicity. These notable variations in HBsAg prevalence across African studies are likely multifactorial and attributable to differences in HBV vaccination coverage, healthcare access, effectiveness of screening program implementation, and local sociocultural practices. Regions with weaker healthcare infrastructure, lower vaccination uptake, and increased exposure to vertical transmission risks tend to have higher HBV prevalence and endemicity levels, and limited surveillance among these regions.

In contrast, the HBsAg prevalence in our study (5.8%) is significantly higher than reports from both within Ethiopia and other East African countries. Specifically, our finding exceeds rates reported in studies from Ambo (4.99%) (110), Bahirdar (3.8%) (43), Amhara region (4.6%) (111), Arba Minch (4.3%) (76), and the Dawuro zone of Southwest Ethiopia (2.3%) (57) as well as the pooled estimates from Ethiopian meta-analysis (ranging from 4.7% to 4.75%) (112). Our prevalence is also notably higher than those reported from Uganda (2.9%) (113), Turkey, and India (2.1%) and (1.6%), respectively (114,115). These lower prevalence rates are likely attributed to site-specific factors, including differences in diagnostic methodologies, the success of public health interventions, better access to antenatal screening, and the rigorous implementation of infection prevention strategies in those particular regions.

It has been well recognized that HBeAg positivity during pregnancy is correlated with a high maternal viral load, a critical factor associated with the vertical transmission of HBV to their newborn (116). In the present study, we assessed both HBV viral load and HBeAg, and interestingly found low viral load ranging from 48 to 222 IU/mL which is lower compared to the previous study conducted at SPHMMC showed 14.3% HBeAg positive and 11.4% women had HBV DNA viral load >200,000 IU/mL (117) and from Amhara region showed 69.4% had detectable viral DNA with average of 3,162 IU/mL (111). This difference might be due infection phase, type of diagnostic assay sensitivity and increased HBV vaccination coverage.

The detection level of HBeAg among HBsAg-positive pregnant women varies widely, influenced by factors such as endemicity, HBV genotype distribution, and maternal immune status. The present study showed, none of the HBsAg-positive pregnant were detected for HBeAg which inline with the study conducted in Tanzania (118). Moreover, a study from Nigeria and Cameroon reported undetected level of HBeAg among the HBsAg positive pregnant women (116,119). Similarly, a study from Egypt HBeAg level was not detected (120).

Although, the present study found no HBeAg-positive cases, this contrasts with reports from other settings. Including, India (15.7%) (121), Nigeria (14.6%) (122), South Africa 9.48% (123) and 17.1% (124) and from Uganda, 14.9% and 62.5% (125,126). Furthermore, In Ethiopia HBeAg positive level among HBsAg-positive pregnant women at at SPHMMC and Yirgalem Hospital reported 8.2% and 38.2% respectively (117,127). Such discrepancies may arise from differences in study design, sample size, and diagnostic assay sensitivity specificity , genotypic variations. Furthermore, the relatively higher positivity rate in that setting might be associated with differences in vaccination coverage, resulting in partial immunization which potentially a shift toward inactive carrier.

In the present study, we have looked into the seroprevalence of HCV, that was found to be 0.44%, a figure that places the finding within the low endemicity range according to the World Health Organization classification. This classification defines low seroprevalence as a rate of less than 1.5%, intermediate endemicity as 1.5% to 3.5%, and high endemicity as greater than 3.5% (128). The low rate in the present study suggests that, at present, HCV poses a significantly lesser public health burden than HBV in this study population.

Yet, the low HCV seroprevalence observed in our study is consistent with findings reported from different areas of the Amhara region, Bahirdar (0.6%) (129), Felege Gion Hospital (0.26%) (56), and Debretabor (0.5%) (98), and some African countries such as Tanzania (0.3%) (108) and Nigeria (0.5%) (130). In contrast to the low HCV seroprevalence observed in our study, the burden is reported to be higher in other Ethiopian regions and in national pooled analyses. Specifically, our result is considerably lower than the estimated national pooled prevalence, which ranges from 1.83% to 2% (21,45). Moreover, separate studies conducted across the country have documented rates up to 8.1%, including reports from Southeastern Ethiopia (2.2%) (96), Western Ethiopia (8.1%) (70), Amhara region (1.6%) (111), Northwestern Ethiopia (2.4%) (131), Borumeda, Northeast Ethiopia (3.2%) (55), and Western Oromia, Ethiopia (2.4%) (28).

Compared to study findings from other African countries, our result was significantly lower. For instance, a meta-analysis conducted in Africa among pregnant women showed a prevalence of 3.4% (132). Country-specific studies have documented varying rates, such as in Nigeria, 1.3% (133) and 1.5% (134), Gabon, 2.1% (135), the Democratic Republic of Congo, which showed a prevalence of HCV ranging from 4.8% to 7.3% (87,136), and Ghana, 7.7% (137). Beyond Africa, the overall mean seroprevalence of HCV among pregnant women in Italy was reported to be 3.9% (138).

The observed differences in HCV prevalence across different regions within Ethiopia and across African countries could be attributed to differences in risk factors, healthcare access, blood screening practices, socio-economic conditions, educational awareness, and exposure to traditional risk factors such as unsafe injections or scarification practices.

Although in the bivariate analysis, family history of liver disease had almost 6.5 times more risks and contact with an HBV-infected person 6.2 times higher risk. However, the bivariate analysis, these associations did not remain statistically significant in the adjusted model except the gestational stage of trimester. In this study, there was no significant association among the risk factors, including body tattooing, history of abortion, blood transfusion, circumcision, and surgery

procedures. However, studies conducted in different areas, including Ethiopia and other countries, were reported as potential risk factors for HBV infection (43,139,140).

The reason could be due to the sexually active age group and the housewives' limited awareness or access to HBV screening and prevention programs. Trimester-time women may be more frequently tested for HBV as part of routine ANC screening, leading to increased detection rates; also, their physiological changes during pregnancy could lead to increased viral replication.

Screening and vaccination rates varied among studies. This study showed that 48.9%, 20.9% were screened and vaccinated for HBsAg respectively, which is a better practice than the study conducted in Addis Ababa, Ethiopia, where 86.2% had never been screened, and 97.6% were unvaccinated (141). The screening level for HBV showed similarity with the study conducted (51.2%) in Nigeria (142), and 41.7% in Ghana (141,143). The vaccination level of this study (20.9%) is lower compared to the study conducted in Nigeria, which was (36%) (142). This may be due to disparities in the integration of HBV vaccination into ANC services or the presence of catch-up vaccination strategies.

7. Conclusions and recommendations

This study revealed an intermediated seroprevalence of hepatitis B (HBsAg) and low hepatitis C (anti-HCV) among pregnant women attending ANC in selected health centers in Addis Ababa. In current study, we observed low HBV viral load with corresponding to HBeAg negativity.

Based on our findings, we recommend expanding HBV vaccination among women of reproductive age, especially through catch-up vaccination for those unvaccinated during pregnancy, regardless of the risk factors to reduce the risks of HBV infections.

Moreover, further research is needed, including multicenter and longitudinal studies, to monitor infection trends and evaluate the outcomes of interventions.

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9. Annexes

Annex I. Participant's information sheet and consent form (English version)

Information sheet

Title of the study: Seroprevalence and associated factors of hepatitis B and C virus infections among pregnant women attending antenatal care in selected health facilities, Addis Ababa, Ethiopia.

Objective of the study: The objective of this study was to investigate the seroprevalence and associated factors of hepatitis B and C virus infections among pregnant women attending antenatal care in selected health facilities, Addis Ababa, Ethiopia.

Study Participants: Pregnant women who visit the ANC of SPHMMC, Selam, and Felege Meles health center will be enrolled in the study.

Samples Required: After you agree to participate in the study, a venous blood sample of 10 ml will be collected from each of you using a sterile syringe. Your sample will be used for this study and retained for a while for a future virus genotyping study.

Duration: The study will be conducted from April to September 2024.

Risk associated with the study

There is no risk associated with this study.

Benefits

For those who have positive result their result will be communicated and they will be linked with clinical care provision.

Confidentiality

All records will be kept strictly confidential. Participants' identifications will not be collected for the purpose of this study; instead, unique identification numbers will be used. Personal identifying information will not be shared outside of the study, and it will not be used in any of the publications.

Withdrawal rights

Your participation in this study is purely voluntary, and you may stop the participation at any time, refuse to answer some of the questions if you feel uncomfortable, or withdraw your consent at any time without giving reasons. This will not affect the service you will get at the hospital HC.

If you have any questions regarding this study, the address of the principal investigator is:

Address of Principal Investigator: Fitsum Girma

Tel: +251-912715959

Email: fitsmetg23@yahoo.com

Consent form

Name of the participant: _____ Age _____

Code_ _____

I confirm that I have been given adequate information about the research: hepatitis B, hepatitis C, and hepatitis E virus infections and associated factors among pregnant women attending antenatal care at St. Paul's Hospital Millennium Medical College, Selam and FelegeMeles health centers. I have been requested to provide 10ml of venous blood and to answer structured questions related to this study. I have been informed that there is no major risk associated with participating in the study or providing the requested samples. I have also understood that the results of clinical and laboratory diagnoses will be used for research purposes only, and information related to myself and my family will be kept strictly confidential. I am well informed that participation in the study is fully voluntary, and I can withdraw at anytime without giving any reason. I confirm that all the information provided to me is very clear and has been conveyed in a language that I fully understand. Finally, I declare that I have obtained adequate information, and by signing this, I confirm that I have given my consent to participate in the study.

Participant signature _____ Date _____

Annex II. Participant's information sheet and consent form (Amharic version)

ስለጥናቱ መረጃ

የጥናቱ ርዕስ፡ የሄፕታይተስ ቪሼሪያ ታይተስ ሲኦቶራይን ቪሼሪያ ሄፕታይተስ ኢንፌክሽን ዲፍሰሽን ምክንያቶች እና የደዋዊ ምክንያቶች በቅዱስ ጳውሎስ ሆስፒታል ሚሊኒየም ሜዲካል ኮሌጅ የቅድመ ወሊድ እንክብካቤ በሚከታተሉ ነፍሰ ሞት ምክንያቶች መካከል።

የጥናቱ ዓላማ፡-

በኤች.ቢ.ቪ፣ HCV እና HEV ኢንፌክሽን ለኤች.ቢ.ቪ፣ HCV እና HEV ኢንፌክሽን እና የእውቀት አመለካከት እና ልምድ ምርመራ በነፍሰ ሞት ምክንያቶች ላይ ያለውን የሴሮ እና ሞለኪውላር ስርጭት ለመመርመር።

የጥናት ተሳታፊዎች፡ የቅድመ ወሊድ እንክብካቤን የሚጎበኙ እርጉዝ ሴቶች በጥናቱ ውስጥ ይመዘገባሉ።

ተፈላጊና ሙያዎች፡ በጥናቱ ለመሳተፍ ከተስማሙ በኋላ ከእያንዳንዱ የጥናት ተሳታፊ 10

ሚሊሊትር የሆነ የደምና ሙሽር የጸዳ መርፌን በመጠቀም ይሰበሰባል።

ና ሙሽር ውለዚህ ጥናት ጥቅም ላይ ይውላል እንዲሁም ለጂኖታይፕ ጥናት ምላሽ ወሰን ሊጠቀም ይችላል።

የሚፈጀው ጊዜ፡ ጥናቱ የሚካሄደው ከየካቲት እስከ መስከረም 2024 ነው።

ከጥናቱ ጋር የተያያዘ ስጋት፡ ከዚህ ጥናት ጋር የተያያዘ ምንም ዓይነት አደጋ የለውም።

ጥቅሞች፡ ሽይረሱ ለተገኘባቸው ታካሚዎች ውጤቱ ይነገራቸዋል። እንዲሁም ህክምና እንዲያገኙ ከህክምና ባለሙያዎች እንዲገናኙ ይደረጋል።

ሚስጥራዊነት፡ ሁሉም መዝገቦች በጥብቅ ሚስጥራዊ ይሆናሉ። የተሳታፊዎች መለያዎች ለጥናት ዓላማ አይሰበሰቡም ይልቁንም ለደቁ ጥሮች ጥቅም ላይ ይውላሉ። የግል መለያ መረጃ ከጥናቱ ውጭ አይጋራም እና በማንኛውም ህትመት ውስጥ ጥቅም ላይ አይውልም።

የማውጣት መብቶች፡ በዚህ ጥናት ውስጥ ያለዎት ተሳትፎ በፈቃደኝነት ብቻ ነው እና በማንኛውም ጊዜ ተሳትፎውን ማቆም ይችላሉ ይህም ለደምዎች ከተሰማዎት በጥናቱ ለመሳተፍ ፈቃደኝነት ሆኖ ይሠራል። ፈቃድዎን በማንኛውም ጊዜ ማንሳት ይችላሉ እንደጥያቄዎችን ለመመለስ ምክንያቶች ሳይሰጡ እምቢ ማለት ይችላሉ። ይህ በሆስፒታሉ ውስጥ በሚያገኙት አገልግሎት ላይ ተጽእኖ አይኖረውም።

ይህንን ጥናት በተመለከተ ማንኛቸውም ጥያቄዎች ካሉዎት፣ የዋናው ተመራማሪ አድራሻ፡-

የዋና ተመራማሪ አድራሻ፡- ፍፁም ግርማ

ስልክ: +251-912715959

ኢሜል: fitsmetg23@yahoo.com

የፍቃድቅፅ:

የተሳታፊውስም:- _____ እድሜ _____

ኮድ _____

ስለጥናቱበቂመረጃእንደተሰጠኝአረጋግጣለሁ;

በቅዱስጳውሎስሆስፒታልሚሊኒየምሜዲካልኮሌጅየቅድመወሊድእንክብካቤበሚከታተሉንፍሰጡርእናቶች መካከልየሄፕታይትስቢ፣ሄፓታይትስቢእናሄፓታይትስቢቫይረስኢንፌክሽኖችእናተያያዥምክንያቶች 10ml ደምእንድሰጥተጠየቅኩ፡፡ተመራማሪዎቼበጥናቱላይከመሳተፍወይምየተጠየቁትንናሙናዎችከማቅረብጋር የተያያዘምንምአይነትትልቅስጋትእንደሌለአስታውቀዋል፡፡በተጨማሪምየክሊኒካዊእናየላብራቶሪምርመራ ውጤቶችለምርመራዓላማዎችእንደሚውሉእናከራሴ/ቤተሰቤጋርየተያያዙመረጃዎችበጥብቅእንደሚጠበቁ ተረድቻለሁ፡፡በጥናቱውስጥመሳተፍሙሉበሙሉበፈቃደኝነትእንደሆነእናምንምምክንያትሳላደርግበማንኛ ውምጊዜማቋረጥእንደምችልበደንብተረድቻለሁ፡፡ለእኔየተሰጡኝመረጃዎችሁሉበጣምግልጽእናሙሉበሙሉ በምረዳውቋንቋየተላለፉመሆናቸውንአረጋግጣለሁ፡፡በመጨረሻምበቂመረጃእንዳገኘሁይህንንበመፈረምበ ጥናቱለመሳተፍፈቃደኛመሆኔንአረጋግጣለሁ፡፡

የተሳታፊፊርማ _____ ቀን

Annex III. Participant's information sheet and consent form (Affan orommo version)

Annex III. Participant's information sheet and consent form (Affan orommo version)

Waraqaa odeeffannoo

Mata duree qorannichaa: Bu'aa Infeekshinii Vaayirasii Tiruu B, Hepatitis C, fi Hepatitis E Dubartoota Ulfaa Keessatti Kunuunsa Da'umsa Duraa Hospitaala Phaawuloos Kolleejjii Meedikaalaa Mileniyeemii irratti hirmaatan.

Kaayyoo qorannichaa: Seero- fi babal'ina infekshinii HBV, HCV, fi HEV, sababoota balaa infekshinii HBV, HCV, fi HEV waliin walqabatani, fi qorannoo beekumsaa fi ilaalcha dubartoota ulfaa kan xiyyeefate dha..

Hirmaattota Qorannichaa: Dubartoota ulfaa ANC daawwatan.

Saamuda Barbaachisu: Qorannoo kana irratti hirmaachuuf erga walii galtanii booda, Siriinjii haaraa fayyadamuun tokkoon tokkoon hirmaattota qorannichaa irraa saamuda dhiiga mililiitira 10 ni sassaabama. Saamuda keessan qorannoo kanaaf kan itti fayyadamu yoo ta'u, qorannoo jeenootaayipiif yeroo muraasaaf ni qabaata.

Yeroo: Qorannoon kun Fulbaanaa hanga Adoolessa, 2024tti kan gaggeeffamu ta'a.

Balaa qorannoo kanaa wajjin wal qabachuu danda'an:

Balaan qorannoo kanaan walqabatu hin jiru.

Faayidaa

Mucaan keessan bu'aa qorannoo laabraatoorii qorannoon kun argamsiisu argachuuf mirga ni qabaata. Dabalataanis qaama keessan keessatti dhukkubni HBV, HCV ykn HEV yoo argame hakiima dhimmi isdaa ilaallatuu wajjin wal isin quunnamsiifna.

Iccitii

Galmeen ykn bu'aan qorannoo kanaa hundi iccitii cimaan ni eegama. Eenyummaan hirmaattota qorannaa kanaa walitti hin qabamu; haa ta'u malee, lakkoofsi eenyummaa addaa addaan ni kennama. Odeeffannoon eenyummaa dhuunfaa qorannichaan alatti bakka kamitti iyyuu hin qoodamu; akkasumas maxxansa kamiyyuu keessatti hin fayyadamamu.

Mirga qorannoo keessaa of baasuu

Qorannoo kana irratti hirmaannaan keessan fedhii qofa waan ta'eef yeroo barbaaddanitti hirmaannaa dhaabuu dandeessu; ykn deebii kennuu diduu dandeessu. Sababa gaaffilee tokko tokkootiif yoo miirri tasgabbiin isinitti hin dhaga'amne, qorannicha irratti hirmaachuu diduu ni dandeessu.. Kunis tajaajila hospitaala keessatti argattan irratti dhiibbaa hin geessisu.

Qorannoo kana ilaalchisee gaaffii yoo qabaattan teessoon qorataa akka armaan gadiitti ta'a.

Teessoo Qorataa: Fitsum Girma

Bilbila: +251-912715959

Email: fitsmetg23@yahoo.com

Unka hayyamaa

Maqaa hirmaataa: _____Umurii _____Saala_____

Koodii_ _____

Waa'ee qorannichaa odeeffannoon gahaan akka naaf kenname nan mirkaneessa. Miidhaa Infeekshinii Vaayirasii Tiruu B, Hepatitis C, fi Hepatitis E fi Qabxiilee dhukkubichaa wajjin walqabatan ilaalchisuudhaan qorannoo, Dubartoota Ulfaa Hospitaala Phaawuloos Kolleejjii Meedikaalaa Mileniyeemii keessatti Kunuunsa Da'umsa Duraa Hirmaatan Keessatti adeemsifamu. Dhiiga mililiitira 10 akkan kennu gaafatameen jira. Qorattoonni qorannoo kanaa akka naaf ibsanitti balaan qorannoo kanaa wajjin wal qabatu hin jiru. Akkasumas bu'aan qorannoo kilinikaa fi laabraatoorii qorannoof akka ooluu fi odeeffannoon ofii koo/maatii koo wajjin walqabatu iccitii cimaan akka naaf eegamu hubadheen jira. Qo'annicha irratti hirmaachuun fedhii koo akka ta'ee fi sababa tokko malee yeroon barbaadetti addaan kutuu akkan danda'u sirriitti naaf ibsameera. Odeeffannoon naaf kenname hundi baay'ee ifa kan ta'ee fi afaan ani guutummaatti hubadheen kan dabarfame ta'uu isaa nan mirkaneessa. Dhumarratti odeeffannoo gahaa akkan argadhe ibsee, mallattoo kootiin qorannicha irratti hirmaachuuf hayyamamaa ta'uu koo nan mirkaneessa.

Mallattoo hirmaataa _____Guyyaa_____

Annex IV. Questionnaire

Table 7: General questionnaires

S. No	List of questions	Possible choices for answering by pregnant women		
1	Age(years)			
2	Residency Area	1.Urban	2. Rural	3. Both Urban & rural
3	Marital status	1. Single	2. Married	3. Divorced 4. Widowed
4	PICT status	1. Reactive	2. Non-reactive	
5	Vaccination status for HBV	1. Yes	2.No	3. I don't know
6	Pregnancy status during the study time	1. First trimester (1–12 weeks)	2. Second trimester (13–24 weeks)	3. Third trimester (25 weeks - delivery)
7	Educational background	1. Illiterate 2. able to read & write	3.Elementary 4. Secondary	5.College/ University
8	Occupation / Employment	1. Student 2. Housewife 3. Farmer	4. Self-employed 5. Government 6. Privately employed	7. Unemployed 8.health care professional 9.Other_____
9	Do you have any comorbidities	1. Yes, If yes, state it		
		2. No		
10	History of surgical procedure	1. Yes	2.No	
11	History of dental procedure	1. Yes	2.No	

12	History of abortion	1. Yes	2.No	
13	History of Cesarean section	1. Yes	1. Yes	
14	History of STI	1. Yes	2.No	
15	Do you have a Tattoo	1. Yes	2.No	3.If yes from 3.1Modern tattoo site 3.2 traditional tattoo site
16	Number of family members			
17	History of jaundice/liver disease	1. Yes	2.No	
18	Family history of liver disease?			
19	History of multiple sexual partners	1. Yes	2.No	
20	Gravidity	1. Primigravida	2.Multigravida	
21	History of blood transfusion	1. Yes	2.No	
22	Do you have contact with hepatitis infected person	1. Yes, if yes, state the virus type	2.No	I don't know
23	Do you have a history of traditional tonsillectomy	1. Yes	2.No	
24	History of circumcision	1. Yes	2.No	
25	History of nose and ear piercing	1. Yes	2.No	
26	Monogamous relationship and polygamous relationship	1. monogamous	2. polygamous	

Annex VI Standard operating procedures for laboratory analysis

Procedure for Wantai AiD™ anti HCV Ab ELISA^{plus} using serum sample

Background

AiD™ anti HCV Ab ELISA^{plus} is based on double antigen ‘sandwich’ principle ELISA. This novel for the testing of HCV antibodies method allows detection of very early antibodies including IgM and IgA in addition to the IgG which is the main target for detection of the previous generation assays. In addition the method minimizes the unspecific reaction showed by the other methods and thus its utilization increases the specificity in detection.

Principle:

This kit is a two step incubation enzyme immune assay, which uses polystyrene micro well strips pre coated with recombinant HCV antigen expressed in E. coli (recombinant core and NS3/4/5). Patient’s serum or plasma specimen is added together with biotin-conjugated HCV antigens. During the first incubation step , the specific HCV antibodies , if present will be captured inside the wells as double antigen ‘ sandwich’ complex comprising of the coated and the biotin-conjugated HCV antigens. The micro wells are then washed to remove unbound serum proteins. During the second incubation step the captured HCV antibodies are detected by adding of HRP – conjugate. The micro wells are then washed to remove unbound conjugate and chromo gens are hydrolyzed by the bound HRP conjugate to a blue colored product. The blue color turns yellow after stopping the reaction with sulfuric acid. The amount of the color intensity can be measured and is proportional to the amount of antibodies captured in the wells, and the specimen respectively. Wells containing specimens negative for anti- HCV remain color less

Test kit contents:

- Negative control:
- Positive control:
- Antigen-coated micro well plate
- HRP- conjugate
- Biotin conjugate
- Wash buffer

- Chromogen solution A
- Chromogen solution B
- Stop solution

Items required

Consumables:

- Distilled or deionized water
- Absorbent tissues

Equipment:

- Precision pipette with disposable tips to deliver ranges of 20 µl to 200 µl and 200 µl to 1000 µl respectively.
- Micro plate shaker
- Incubator at 37°C
- Micro plate washer (alternatively, washing can be performed manually)
- Photometric reading: micro plate reader, equipped with a 450 nm filter and optional 620 nm filter

Procedure:

Reagent preparation

Allow the reagent to reach room temperature (18-30 °c). check the wash buffer concentrate for the presence of salt crystals. If crystals are formed, resolublised by warming at 37°c until crystals dissolve. Dilute the wash buffer (20*). Use distilled water or deionizer water and only clean vessels to dilute the buffer.

- 1. Preparation:** mark 3 wells as negative control two wells as positive control and one blank.
- 2. Adding biotin-conjugate reagent:** add 50 µl of biotin conjugate reagent in to each well except the blank.
- 3. Adding specimen:** add 50 µl of positive control, negative control and specimen in to their respective wells except the blank mix gently. Mix by tapping the plate gently.
- 4. Incubating:** cover the plate with the plate cover and incubate at 37°C for 60 minutes.
- 5. Washing:** at the end of incubation, remove and discard the plate cover. Wash each plate 5 times with diluted washing buffer.

6. **Adding HRP- Conjugate:** add 100 µl HRP- Conjugate into each well except the blank.
7. **Incubating:** cover the plate with the plate cover and incubate at 37°C for 30 minutes.
8. **Washing:** at the end of incubation, remove and discard the plate cover. Wash each well 5 times with diluted washing buffer.
9. **Coloring:** add 50 µl of chromo gene solution A and then 50 µl of chromo gene solution B in to each well including the blank, mix gently. Incubate the plate at 37°C for 30 minutes avoiding light. The enzymatic reaction between the chromo gene solution and HRP-conjugate produces blue color in positive control and HCV antibody positive specimens wells.
10. **Stopping reaction:** using multichannel pipette or manually add 50 µl of stop solution in each well and mix gently. Intensive yellow color develops in positive control and HCV antibody positive specimen wells.
11. **Measuring the absorbance:** calibrate the plate reader with blank well and read the absorbance at 450nm. If a dual filter instrument is used, set the reference wavelength at 600-650nm. Calculate the cut-off value and evaluate the results.

Quality control and calculation of the results

Each micro plate should be considered separately when calculating and interpreting the results of the assay, regardless of the number of plates concurrently processed. The results are calculated by relating each specimen absorbance (A) value to the cut-off value (c. O) of the plate. If the cut-off reading is based on single filter plate reader, the result should be calculated by subtracting the blank well A value from the print report values of specimens and controls. In case the reading is based on a dual filter plate reader. Do not subtract the blank well A value from the print report values of specimens and controls.

Calculation of the cut-off value (C. O) = NC+0.12

Quality control (assay validation): the test results are valid if the quality control criteria are fulfilled. It is recommended that each laboratory establish an appropriate quality control system with quality control material similar to or identical with the patient specimen being analyzed.

Interpretation of the result:

Negative results: a specimen giving absorbance less than the cut-off value is negative for this assay, which indicates that no hepatitis C virus antibodies have been detected.

Positive results: specimens giving an absorbance equal to or greater than the cut-off value are considered reactive.

Borderline: specimens with an absorbance to cut-off ratio between 0.9 and 1.1 are considered borderline, and retesting of these specimens in duplicate is required to confirm the initial results.

Procedure for Wantai AiD™ HBsAg ELISA using serum sample

Background

AiD™ HBsAg ELISA uses antibody 'sandwich' ELISA method in which polystyrene micro well strips are pre coated with monoclonal antibodies specific to HBsAg

Principle:

Petients sample or serum is added to the micro wells. During incubation the specific immune complex formed in case of presence of HBsAg in the specimen, is captured on the solid phase. Then the second antibody conjugated the enzyme horseradish peroxides (the HRP-conjugate) directed against a different epitope of HBsAg complexes previously formed during the first incubation and the unbound HRP- conjugate is then removed by washing. Chromogen solution containing tetramethyl- benzidine (TMB) and urea peroxide are added to the wells. In the presence of antibody-antigen-antibody (HRP) sandwich immune complex the colorless chromogen are hydrolysed by the bound HRP- conjugate to a blue colored product. The blue color turns yellow after stopping the reaction with sulfuric acid. The amount of the color intensity can be measured and it is proportional to the amount of antigen captured in the wells, and to its amount in the specimen respectively. Wells containing specimens negative for HBsAg remain colorless.

Test kit contents:

- Negative control:
- Positive control:
- Micro well plate
- HRP- conjugate
- specimen diluent
- Wash buffer
- Chromogen solution A
- Chromogen solution B
- Stop solution

Items required

Consumables:

- Distilled or deionized water
- Absorbent tissues

Equipment:

- Precision pipette with disposable tips to deliver ranges of 20 µl to 200 µl and 200 µl to 1000 µl respectively.
- Micro plate shaker
- Incubator at 37°C
- Micro plate washer (alternatively, washing can be performed manually)
- Photometric reading: micro plate reader, equipped with a 450 nm filter and optional 620 nm filter

Procedure.

Reagent preparation

Allow the reagent to reach room temperature (18-30 °C). Check the wash buffer concentrate for the presence of salt crystals. If crystals are formed, resolubilised by warming at 37°C until crystals dissolve. Dilute the wash buffer (20*). Use distilled water or deionizer water and only clean vessels to dilute the buffer.

1. **Preparation:** mark 3 wells as negative control two wells as positive control and one blank.
2. **Adding diluents:** add 20µl of specimen diluents in to each well except the blank.
3. **Adding specimen:** add 100 µl of positive control negative control and specimen in to their respective wells except the blank.
4. **Incubating:** cover the plate with plate cover and incubate at 37°C for 60 minutes.
5. **Adding HRP-conjugate:** at the end of incubation , remove and discard the plate cover. Add 50 µl HRP- conjugate in to each well except the blank, and mix by tapping the plate gently.
6. **Incubating:** cover the plate with plate cover and incubate at 37°C for 30 minutes.
7. **Washing:** at the end of incubation, remove and discard the plate cover. Wash each wall 5 times with diluted washing buffer.
8. **Coloring:** add 50 µl of chromo gene solution A and then 50 µl of chromo gene solution B in to each well including the blank, mix gently. Incubate the plate at 37°C for 30 minutes

avoiding light. The enzymatic reaction between the chromo gene solution and HRP-conjugate produces blue color in positive control and HBsAg positive specimens wells.

9. **Stopping reaction:** using multichannel pipette or manually add 50 µl of stop solution in each well and mix gently. Intensive yellow color develops in positive control and HBsAg positive specimens wells.
10. **Measuring the absorbance:** calibrate the plate reader with blank well and read the absorbance at 450nm. If a dual filter instrument is used, set the reference wavelength at 600-650nm. Calculate the cut-off value and evaluate the results.

Quality control and calculation of the results

Each micro plate should be considered separately when calculating and interpreting the results of the assay, regardless of the number of plates concurrently processed. The results are calculated by relating each specimen absorbance (A) value to the cut-off value (c. O) of the plate. If the cut-off reading is based on single filter plate reader, the result should be calculated by subtracting the blank well A value from the print report values of specimens and controls. In case the reading is based on dual filter plate reader. Do not subtract the blank well A value from the print report values of specimens and controls.

Calculation of the cut-off value (C. O) = NC+0.06

Quality control (assay validation): the test results are valid if the quality control criteria are fulfilled. It is recommended that each laboratory must establish appropriate quality control system with quality control material similar to or identical with the patient specimen being analyzed.

Interpretation of the result:

Negative results: specimen giving absorbance less than the cut-off value is negative for this assay, which indicates that no HBsAg have been detected.

Positive results: specimens giving an absorbance equal to or greater than the cut-off value are considered reactive.

Borderline: specimens with absorbance to cut-off ratio between 0.9 and 1.1 are considered borderline and retesting of these specimens in duplicate is required to confirm the initial results.

Abbot m2000/RT HBV viral load testing procedure

BACKGROUND:

Abbott RealTime HBV assay is an in vitro polymerase chain reaction (PCR) assay for use with the Abbott m2000 System DNA reagents and with the Abbott m2000sp and m2000rt instruments for the quantization of Hepatitis B Virus (HBV) DNA in human serum or plasma (EDTA) from chronically HBV-infected individuals. The assay can be used to measure HBV DNA levels. The results from the Abbott RealTime HBV assay must be interpreted within the context of all relevant clinical and laboratory findings. Assay performance for determining the clinical stage of HBV infection has not been established. Clinical performance characteristics have been established for individuals treated with adefovirdipivoxil. This assay is not intended for use as a screening test in blood or blood products for HBV or as a diagnostic test to confirm the presence of HBV infection.

PRINCIPLES OF THE PROCEDURE:

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

Sample Preparation:

The purpose of sample preparation is to extract and concentrate nucleic acid, to make the target accessible for amplification, and to remove potential inhibitors of amplification from the extract. This process is accomplished by the m2000sp, an automated sample preparation system designed to use magnetic microparticle processes for the purification of nucleic acids from samples. The assay is suitable for use with both 0.5 mL and 0.2 mL sample input volumes. The Abbott mSample Preparation System DNA (4 x 24 Preps) reagents lyse the virion, capture the nucleic acids, and wash the particles to remove unbound sample components. Proteinase K is included in the lysis

step to digest proteins associated with the nucleic acids.^{12, 13} The bound nucleic acids are eluted and transferred to a 96-deepwell plate. The nucleic acids are then ready for amplification. The IC is introduced into the sample preparation procedure and is processed along with the calibrators, controls, and specimens.

Reagent Preparation and Reaction Plate Assembly:

The Abbott m2000sp combines the Abbott RealTime HBV amplification reagent components (HBV Oligonucleotide Reagent, DNA Polymerase, and Activation Reagent). The Abbott m2000sp dispenses the resulting master mix to the Abbott 96-Well Optical Reaction Plate along with aliquots of the nucleic acid samples prepared by the Abbott m2000sp. After manual application of the Abbott Optical Adhesive Cover, the plate is ready for transfer to the Abbott m2000rt.

Amplification:

During the amplification/detection reaction on the m2000rt instrument, the target DNA is amplified by the DNA Polymerase in the presence of deoxynucleotide triphosphates (dNTPs) and magnesium. First, the HBV and IC primers anneal to their respective targets and are extended by the polymerase. After a denaturation step in which the temperature of the reaction is raised above the melting point of the double-stranded DNA product, the newly created DNA strand is denatured from the target DNA. During each round of thermal cycling, amplification products dissociate to single strands at high temperature, allowing primer annealing and extension as the temperature is lowered. Exponential amplification of the target is achieved through repeated cycling between high and lower temperatures. Amplification of both targets (HBV and IC) takes place simultaneously in the same reaction. The target sequence for the Abbott RealTime HBV assay is in the Surface gene in the HBV genome. This region is specific to HBV and is highly conserved. The primers are designed to hybridize to this region with the fewest possible mismatches among HBV genotypes A through H. The IC target sequence is derived from the hydroxypyruvate reductase gene from the pumpkin plant *Cucurbita pepo*, and is provided as a DNA plasmid in a buffer solution.

Detection:

The presence of HBV amplification products is detected during the extension/anneal step by measuring the fluorescence of the HBV probe that binds to the target during the extension/anneal step. Similarly, the presence of IC amplification is detected during the extension/anneal step by measuring the fluorescence of the IC probe. The HBV and IC probes are single-stranded DNA

oligonucleotides consisting of a probe sequence with a fluorescent moiety that is covalently linked to the 5' end of the probe and a quenching moiety that is covalently linked to the 3' end of the probe. In the absence of the HBV or IC target sequences, probe fluorescence is quenched.

In the presence of HBV or IC target, the HBV or IC probes specifically bind to their target. During the extension/anneal step, the DNA polymerase cleaves, or nucleolytically digests, the bound probe as it moves along the template strand. This separates the fluorophore from the quencher, allowing fluorescent emission and detection. The HBV and IC probes are each labeled with a different fluorophore, thus allowing for simultaneous detection of both amplified products at each cycle. The amplification cycle at which the fluorescent signal is detected by the Abbott m2000rt is inversely proportional to the log of the HBV DNA concentration present in the original sample.

Abbott RealTime HBV Amplification Reagent Kit:

- Abbott RealTime HBV Internal Control
- Abbott RealTime HBV Amplification Reagent

Abbott RealTime HBV Control Kit:

- Abbott RealTime HBV Negative Control
- Abbott RealTime HBV Low Positive Control
- Abbott RealTime HBV High Positive Control

EQUIPMENT AND MATERIALS REQUIRED:

- Abbott RealTime HBV Amplification Reagent Kit
- Abbott RealTime HBV Control Kit
- Abbott RealTime HBV Calibrator Kit
- Sample Racks
- 5 mL Reaction Vessels
- 200 mL Reagent Vessels
- Master Mix Vial
- Abbott 96-Well Optical Reaction Plate
- Abbott 96-Deep Well Plate
- Abbott Splash-Free Support Base
- Abbott Optical Adhesive Cover
- Abbott Adhesive Cover Applicator

- Round-bottom 12.5 x 75 mm Sample Tubes
- Vortex Mixer
- 50 mL Polypropylene Centrifuge Tubes
- Centrifuge capable of 2,000g
- Calibrated Precision Pipettes capable of delivering 10 µL-1000 µL
- 20 µL-1000 µL Aerosol Barrier Pipette Tips for precision pipettes
- Serological Pipettes
- Graduated Cylinder, 100 mL
- USP Grade 190-200 Proof Ethanol (95-100% Ethanol).

PROCEDURES:

Sample Preparation:

1. Thaw assay controls and Internal Control (IC) at 15 to 30°C or at 2 to 8°C. Thaw calibrators at 15 to 30°C or at 2 to 8°C only if performing a calibration run
2. Thaw amplification reagents at 15 to 30°C or at 2 to 8°C until required for the amplification master mix procedure
3. Open the Abbott Proteinase K reagent pack. Add 17.15 mL of Molecular Biology Grade water to a 50 mL polypropylene centrifuge tube. Pipet 2.45 mL of Proteinase K into the container of water. Mix by gentle inversion 10 to 15 times. Transfer the entire contents to a reagent vessel labeled with the Proteinase K barcode label.
4. Open the Abbott mSample Preparation pack. If crystals are observed in any of the reagent bottles upon opening, allow the reagents to equilibrate at room temperature until the crystals disappear. Do not use the reagents until the crystals have dissolved.
5. Prepare the mWash2DNA by adding 70 mL of USP Grade 190-200 Proof Ethanol (95-100% Ethanol) to the mWash2DNA bottle as described in the Abbott mSample Preparation System DNA product information. Do not use ethanol that contains denaturants.
6. Vortex the IC vial(s) three times for 2 to 3 seconds before use.
7. Using a calibrated precision PIPETTE DEDICATED FOR INTERNAL CONTROL USE ONLY, add 100 µL of IC to a bottle of mLysis Buffer. Mix by gently inverting the container 5 to 10 times to minimize foaming.

8. Gently invert the Abbott mSample Preparation bottles to ensure a homogeneous solution and pour the contents into the appropriate reagent vessels per the Abbott m2000sp Operations Manual, Operating Instructions.
9. Place the low and high positive controls, the negative control, the calibrators (if applicable), and the patient specimens into the m2000sp sample rack.
10. Place the 5 mL Reaction Vessels into the m2000sp 1 mL subsystem carrier.
11. Load the carrier racks containing the Abbott mSample Preparation reagents and Proteinase K, and the Abbott 96-Deep Well Plate, on the Abbott m2000sp worktable as described in the Abbott m2000sp Operations Manual, Operating Instructions.
12. From the Run Sample Extraction screen, select the appropriate application file corresponding to the sample volume being tested. Initiate the sample extraction protocol as described in the m2000sp Operations Manual, Operating Instructions.
13. Load the amplification reagents, the master mix vial, and the 96-well Optical Reaction Plate on the m2000sp worktable after sample preparation is completed.
 - Each Amplification Reagent Pack supports up to 24 reactions.
 - Ensure the amplification reagents are thoroughly thawed before use.
 - Prior to opening the amplification reagents, ensure that the contents are at the bottom of the vials by tapping the vials in an upright position on the bench.
 - Remove and discard the amplification vial caps.
 - A second Amplification Reagent Pack is required if performing 25 to 48 reactions.
14. Select the appropriate deep well plate from the Run Master Mix Addition screen that matches the corresponding sample preparation extraction. Initiate the Abbott m2000sp Master Mix Addition protocol. Follow the instructions as described in the Abbott m2000sp Operations Manual, Operating Instructions section.

Amplification procedure:

1. Switch on and initialize the Abbott m2000rt in the Amplification Area.
 - The Abbott m2000rt requires 15 minutes to warm up.
2. Seal the Abbott 96-Well Optical Reaction Plate after the Abbott m2000sp instrument has completed addition of samples and master mix according to the Abbott m2000sp Operations Manual, Operating Instructions section.

3. Place the 96-Well Optical Reaction Plate into the Splash-Free Support Base for transfer to the Abbott m2000rt instrument.
4. Export the completed 96-Well Optical Reaction Plate results to a CD or Network Drive.
5. Place the Abbott 96-Well Optical Reaction Plate in the Abbott m2000rt instrument. Import the m2000sp test order via CD or Network Drive per the Import Order instructions in the Abbott m2000rt Operations Manual, Operating Instructions section.

Post Processing Procedures:

1. Remove the Abbott 96-Deep Well Plate from the worktable and dispose according to the Abbott *m2000sp* Operations Manual.
2. Place the Abbott 96-Well Optical Reaction Plate in a sealable plastic bag and dispose according to the Abbott *m2000rt* Operations Manual, along with the gloves used to handle the plate.
3. Clean the Splash Free Support Base before next use, according to the Abbott *m2000rt* Operations Manual.

RESULTS:

The concentration of HBV DNA in a sample or control is calculated from either a stored calibration curve, or a calibration curve created by calibrators within a calibration or sample run. The Abbott m2000rt instrument automatically reports the results on the m2000rt workstation. Assay results are reported in IU/mL or log IU/mL. Results can also be reported in copies/mL or log copies/mL using a conversion factor of 3.41 (1 IU = 3.41 copies).

Note: If negative or positive controls are out of range, all of the specimens and controls from that run must be reprocessed, beginning with sample preparation.

Standard procedure for analysing HBeAg using Cobas 6000 analyser

Introduction

The hepatitis B e antigen (HBeAg) is a product of the pre-C/C gene that has been found in hepatocytes during proliferation of the hepatitis B virus (HBV) and is an important diagnostic tool to determine the status of ongoing HBV infections. The detection of HBeAg is generally associated with the presence of large quantities of virus as it is a surrogate of viral replication. HBeAg can be detected in serum shortly after HBsAg during acute HBV infections and usually disappears before

HBeAg, when alanine aminotransferase (ALT) levels peak, followed by the presence of the corresponding antibody (anti-HBe). HBeAg can usually be detected when viral replication is high, both in self-limited infections and in chronic hepatitis B; its presence for more than 10 weeks is indicative of a transition to persistent infection. HBeAg seroconversion to anti-HBe suggests the end of active viral replication and is therefore associated with clinical resolution (self-limited) or remission (chronic disease), marking a transition from the immune-active phase of the disease to the inactive carrier state. HBV infections can occur without detectable HBeAg due to infection with HBV variants containing precore stop codon mutants; while the virus can no longer produce HBeAg, disease activity is ongoing and anti-HBe may be present. The HBeAg test is, therefore, meaningful in association with the anti-HBe test for monitoring the course of HBV infection and the effect of treatment for chronic hepatitis B. The Elecsys HBeAg assay uses monoclonal anti-HBe antibodies (mouse) for detection of HBeAg.

Principle

Sandwich principle

1st incubation: HBe antigen from 35 µL sample, a biotinylated monoclonal HBeAg-specific antibody, and a monoclonal HBeAg-specific antibody labeled with a ruthenium complex^a) form a sandwich complex.

2nd incubation: After addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin.

The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

Results are determined automatically by the software by comparing the electrochemiluminescence signal obtained from the reaction product of the sample with the signal of the cutoff value previously obtained by calibration.

Reagent preparation

the reagent rackpack (M, R1, R2) is labeled as HBEAG.

M- Streptavidin-coated microparticles (transparent cap), 1 bottle, 6.5 mL: Streptavidin-coated microparticles 0.72 mg/mL; preservative.

R1-Anti-HBeAg-Ab~biotin (gray cap), 1 bottle, 12 mL: Biotinylated monoclonal anti-HBeAg antibody (mouse) > 0.8 mg/L; TRIS buffer 50 mmol/L, pH 7.4; preservative.

R2- Anti-HBeAg-Ab~Ru(bpy) (black cap), 1 bottle, 12 mL: Monoclonal anti-HBeAg antibody (mouse) labeled with ruthenium complex > 0.3 mg/L; TRIS buffer 50 mmol/L, pH 7.4; preservative.

HBEAG Cal1- Negative calibrator 1 (white cap), 2 bottles of 1.0 mL each: Human serum; preservative.

HBEAG Cal2- Positive calibrator 2 (black cap), 2 bottles of 1.0 mL each: HBeAg (E. coli, rDNA) ≥ 3.5 IU/mL in HEPESb) buffer, pH 7.4; preservative.

Specimen needed

- Serum collected using standard sampling tubes or tubes containing separating gel.
- Li-heparin, Na-heparin, K2-EDTA, K3-EDTA, ACD, CPD, CP2D, CPDA and Na-citrate plasma.
- Plasma tubes containing separating gel can be used.

Material and equipment needed

- PreciControl HBeAg,
- CalSet Vials, empty snap-cap bottles
- General laboratory equipment
- cobas e analyzer (cobas e 411 analyzer)
- Pro Cell, 6 x 380 mL system buffer
- Clean Cell, 6 x 380 mL measuring cell cleaning solution
- Elecsys Sys Wash, ash water additive
- Adapter for SysClean
- AssayCup, 60 x 60 reaction cups
- AssayTip, 30 x 120 pipette tips
- Clean-Liner

Quality control

For quality control, use Preci Control HBeAg. Controls for the various concentration ranges should be run individually.

Calculation

The analyzer automatically calculates the cutoff based on the measurement of HBEAG Cal1 and HBEAG Cal2. The result of a sample is given either as reactive or non-reactive as well as in the form of a cutoff index.

Interpretation of the results

Samples with a cutoff index < 1.0 are non-reactive in the Elecsys HBeAg assay. These samples are considered negative for HBeAg.

Samples with a cutoff index ≥ 1.0 are reactive in the Elecsys HBeAg assay. These samples are considered positive for HBeAg.