PREVALENCE OF ANTI-HBV SEROMARKERS AND GENOTYPIC CHARACTERIZATION OF HEPATITIS B VIRUS AMONG ANTENATAL CARE ATTENDEES IN ERITREA

NAHOM FESSEHAYE SEULU

MASTER OF SCIENCE

(Medical Laboratory Science)

JOMO KENYATTA UNIVERSITY OF

AGRICULTURE AND TECHNOLOGY

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Prevalence of anti-HBV seromarkers and genotypic characterization of hepatitis B virus among antenatal care attendees in Eritrea

Nahom Fessehaye Seulu

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Signnature…….. Date……

Nahom Fessehaye Seulu

This thesis has been submitted for examination with our approval as university supervisors

Signature……Date……

Dr. Eddy Okoth Odari, PhD
JKUAT, Kenya

Signature……Date……

Prof. Joseph Kangangi Gikunju, PhD
JKUAT, Kenya

DEDICATION

I dedicate this work to my beloved parents; my mother Mihret Michael and my late Father Fessehaye Seulu for their unconditional love and support.

To my dear wife Luwam Mebrahtu and our lovely children, Salem, Neby, Abynabi and Bethel for being patient and loving.

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ACRONYMS AND ABBREVIATIONS

ANC	Antenatal care
Anti-HBS	Antibody to HBsAg
Anti-HBc	Antibody to HBcAg
Anti-HBe	Antibody to HBeAg
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immuno Assay
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
HBIG	Hepatitis B immune globulin
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HBx	Hepatitis B x antigen
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDV	Hepatitis Delta virus
HIV	Human Immunodeficiency virus
LHBs	Large HBs protein
MHBs	Middle HBS protein
MoH	Ministry of Health
MTCT	Mother to child transmission
NHL	National Health Laboratory
ORF	Open reading frame
PCR	Polymerase chain reaction
PMTCT	Prevention of mother to child transmission
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Real Time-polymerase chain reaction
SHBs	Major surface antigen protein
WHO	World Health Organization

ABSTRACT

Mother-to-child transmission (MTCT) of hepatitis B virus (HBV) is a major cause of chronic HBV infection. Therefore understanding the epidemiology of HBV infection among pregnant women is critical to prevent MTCT. The aim of this study was to assess the seroprevalence of anti-HBV markers and determine circulating HBV genotypes among women seeking antenatal care within different health facilities in Eritrea.

This cross-sectional study was conducted on archived patient samples and records collected during the 2016 antenatal care HIV sentinel surveillance. A total of 5009 samples were screened for anti-HBc and anti-HBs seromarkers using Enzyme Linked Immunosorbent Assay (ELISA) technique. Viral DNA was then extracted from the HBsAg positive samples using Qiagen® Miniviral DNA isolation kit and the HBV-S gene amplified by nested PCR. The amplified PCR product was sequenced and from the generated sequences genetic diversity determined using MEGA v7. The socio-demographic and serological data were analysed using SPSS version 21. Pearson's Chi-square tests were used to measure the strength of association. The threshold for statistical significance was set at $p < 0.05$.

The mean age of the women was 26.7 ±5.9 years and findings of the socio-demographic data showed that 92.6% of the women were married with 88.4% being housewives. Approximately 70% of the women had attended formal education. Results of the serological markers showed that 25.8% (1241) were positive for anti-HBc and the anti-HBs marker seroprevalence was 14.2% (706). The HBV exposure rate exhibited marked differences among the zobas (regions) ranging from 15.5% to 29.1% and this difference was statistically significant with p-value < 0.001. The phylogenetic analysis revealed presence of HBV variants with genotype A 77.8% (32), D 20% (9) and E 2.2% (1).

Findings of the study revealed a substantial exposure to HBV among pregnant women indicated by 25.8% prevalence of anti-HBc. Further the anti-HBs rate stands at 14.2% with the prevalence of vaccine induced anti-HBs at 5.3%. In light of this immunization against HBV therefore remains key to prevention and mitigation of HBV in the Eritrean population. The study provided the first description of HBV genetic diversity in Eritrea

confirming presence of genotypes A, D and E. This demonstrates that there could be a high genetic diversity of HBV in Eritrea.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

World Health Organization (WHO) estimates the number of people who have had Hepatitis B virus (HBV) infection at 2 billion, with more than 240 million patients developing chronic infection (WHO, 2016). Annually 686,000 patients die as a result of HBV-related liver complications such as cirrhosis and hepatic carcinoma (WHO, 2016). Almost one third of those who develop chronic HBV infections acquires the infection via vertical transmission or horizontally during early childhood (Kew, 1996, 2008). Eritrea is geographically located in sub-Saharan Africa, a region considered endemic to HBV infection with an intermediate to high prevalence (WHO, 2016). The epidemiology of HBV infection in the general public of Eritrea is not well documented and there are only limited studies conducted to explore the extent of the problem. A study conducted among blood donors reported a low intermediate prevalence of HBsAg seromarker 2.6% (Fessehaye, Naik, & Fessehaye, 2011).

The available HBV exposure rate as measured by the prevalence of anti-HBc in the African population is highly varied ranging from 1.8-98% (Kramvis & Kew, 2007). In the Eastern Africa region the reported HBV exposure prevalence rate is 65-85% (Kramvis & Kew, 2007). Moreover, the available HBV prevalence data from the region show a varied prevalence for instance studies conducted among pregnant women in Ethiopia reported an HBsAg prevalence ranging from 3.8-7.8% (Desalegn, Wassie, Beyene, Mihret, & Ebstie, 2016a; Desalegn, Mihret, Beyene, Yilma, Tamiru *et al*., 2016b; Metaferia, Dessie, Ali, & Amsalu, 2016; Seid, Gelaw, & Assefa, 2014; Yohanes, Zerdo, & Chufamo, 2016; Zenebe, Mulu, Yimer, & Abera, 2014). Similarly a study done in Kenya had reported an overall HBsAg prevalence of 9.3% ranging from 3-17.8% for the different regions of the country (Okoth *et al*., 2006). Other neighbouring geographical locations have reported HBsAg prevalence of 5.6%, 11% and 10.8% for Sudan (Elsheik, Daak, Elshiek, Karsany, & Adem, 2007), South Sudan (Kirbak *et al*., 2017) and Yemen (Murad, Babiker, Gasim, Rayis & Adam, 2013). The above prevalence data shows Eritrea

is located in a region where HBV is a public health problem affecting a significant proportion of the population.

Although, HBV is mainly transmitted through sexual route and exposure to blood and body fluids from infected individuals, transmission can occur at all stages of life with a higher chances of developing chronic infection from vertical transmission taking place during pregnancy from infected mother to child especially during delivery (Kew, 2008; Kramvis & Kew, 2007). There is also a significant risk of developing chronic HBV infections with exposure and transmission occurring during early childhood (Kew, 2008; WHO, 2015, 2017). The WHO recommended that countries with a more than 2% prevalence of HBV carriers should add hepatitis B vaccine into their routine infant immunization schedules (WHO, 2015). Consequently the routine screening of pregnant women for hepatitis B surface antigen (HBsAg) is also recommended by the World Health Organisation (WHO, 2017). Even though, most countries incorporated HBV vaccination in their national immunization program the introduction of HBV screening of pregnant women is not yet implemented by most developing countries including Eritrea. HBV vaccine was introduced in Eritrea in 2002 as part of the national immunization program and is given at 6, 10 and 14 weeks of age.

Currently there are ten genotypes of HBV circulating worldwide designated A to J (Zhan, Wu, Chen, Li, & Lu, 2016). Various studies have demonstrated that genotyping of HBV can provide a valuable information to study geographical distribution of the virus, transmission patterns, clinical course and outcome, treatment outcomes, monitor escape mutants and effectiveness of current controlling strategies such as vaccination (Beck & Nassal, 2007; Kao, 2002; Schaefer, 2005; Seeger & Mason, 2015). Five genotypes i.e. A to E are known to circulate in Africa; genotypes A, D, E in Mauritania and Kenya (Mansour, Malick, Sidiya, & Ishagh, 2012; Mwangi, Nganga, Songok, Kinyua, *et al*., 2008), genotype A and D in Ethiopia (Hundie *et al*., 2016a), genotype A in Malawi (Sugauchi, *et al*., 2003), genotype A–D in South Africa (Kramvis, 2008) and genotype E in Nigeria (Odemuyiwa, *et al.*, 2001) were the reported genotypes in these countries. In case of Eritrea there is no comprehensive data available on circulating HBV genotypes.

1.2 Statement of the Problem

Hepatitis B virus infection in pregnancy is an important global health problem with an increased risk of perinatal HBV transmission (WHO, 2015). Without appropriate immunoprophylaxis, infants born to HBeAg-positive mothers have a 40–90% risk of vertical transmission (WHO, 2015). Moreover, the risk of progression to chronic HBV infection is inversely proportional to the age at infection (WHO, 2015). Even though, Eritrea is located in a region considered to be endemic with HBV infection there is a limited data on HBV infection in the general population including women seeking antenatal care. Furthermore, the available HBV prevalence data is limited to a single seromarker (HBsAg) which indicates current infections and there is no data on other HBV seromarkers including anti-HBc and anti-HBs that could shed light on HBV exposure rates and immunity against HBV.

The existing HBV genotypes and their distribution is not studied in Eritrea and there is lack of data to this extent within the country. Eritrea located in the horn of Africa makes it an important crossing point between Africa and the Middle East. Moreover the movement of people in the costal low lands to neighboring countries of the Middle East such as Yemen which are hyperendemic to HBV infection and within the country from low prevalence areas to high prevalence areas for trade, tourism and mining could have an impact on the dynamics of HBV infection and circulating HBV genotype/s. This study thus addresses a major gap on the lack of HBV exposure prevalence data among ANC attendee women and further interrogates the genotype distribution of the virus within the different regions of Eritrea.

1.3. Justification

Annually 686,000 patients die as a result of HBV-related liver complications such as cirrhosis and hepatic carcinoma and more than 250,000 of those deaths occur in sub-Saharan Africa countries (Kramvis & Kew, 2007; WHO, 2017) . Almost one third of those who develop chronic HBV infections acquires the infection via vertical transmission or horizontally during early childhood (Kew, 1996, 2008). In light of this developing countries had to mitigate challenges posed by infectious diseases such as HBV by smartly utilizing the limited health resources. This requires informed decision making

on the part of health policy makers and implementers. In this sense understanding the national and local epidemiology of HBV is important.

The prevalence of Hepatitis B Virus infection among antenatal population is used as proxy indicator to determine hepatitis B virus prevalence rate in the general population (Fabiani, *et al*., 2003). Two rounds of Human Immunodeficiency Virus (HIV), Syphilis, Hepatitis B, and Hepatitis C antenatal sentinel surveillance were conducted in Eritrea, in 2013 and 2016 by the Ministry of Health to determine the prevalence of the aforementioned infections. In both surveys the targeted seromarker for HBV was HBsAg which is an indicator of active HBV infection. The fact that only a seromarker of active HBV infection has been used in all the existing studies justifies the need to assess other seromarkers constantly used in other regions, as this will present a more comprehensive situation of HBV infection within Eritrea. The latter will provide important information on the exposure to HBV infection, immune clearance and level of HBV immunity among study subjects.

The fact that geographically Eritrea is located at an important crossing point between Africa and the Middle East, potential circulation of a myriad of HBV genotypes in the country, though not understood, is expected. Recent data indicate that HBV genotypes could influence modes of transmission, clinical course and treatment outcome of HBV (Lin & Kao, 2011). With this expectation and the fact that no previous data on genotype circulation in Eritrea exists, justifies the need to determine the genotype variation and their molecular characteristics. In this regard being the first study of its kind this study will not only determine the prevalent HBV genotypes in the country but their distribution as well.

1.4. Research questions

I. What are the socio-demographic and clinical characteristics of study subjects?
II. What is the prevalence of anti-HBV seromarkers among ANC attendee pregnant women in Eritrea?
III. What are the circulating HBV genotype/s with their distribution among HBsAg positive ANC attendee women in Eritrea?

1.5. Objectives of the Study

1.5.1. General Objective

To determine the prevalence of anti-HBV seromarkers, and genotype distribution of HBV among antenatal care (ANC) attendee women in Eritrea.

1.5.2. Specific Objectives

I. To determine the socio-demographic and clinical characteristics of the ANC attendees

II. To determine the prevalence of anti-HBV seromarkers among ANC attendee pregnant women

III. To determine HBV genotype/s with their distribution among HBsAg positive ANC attendee women in Eritrea

CHAPTER TWO

LITERATURE REVIEW

2.1 Biology of hepatitis B virus

2.1.1 General description of virion

Hepatitis B virus is a double-stranded, DNA virus of the *Hepadnaviridae* family (Seeger & Mason, 2015). Common features of all of these viruses are enveloped virions containing 3 to 3.3 kb of relaxed circular, partially duplex DNA and virion- associated DNA dependent polymerases which have a reverse transcriptase activities (Beck & Nassal, 2007). HBV viral DNA and DNA polymerase are contained in the icosahedral nucelocapside made of the core protein (HBcAg), enclosed by an outer lipid envelope containing the large (LHBs), middle (MHBs) and small (SHBs) viral surface proteins (Beck & Nassal, 2007; Seeger & Mason, 2015). The double shelled particle was discovered by Dane and Colleagues in 1970 and hence often referred as the Dane particle.

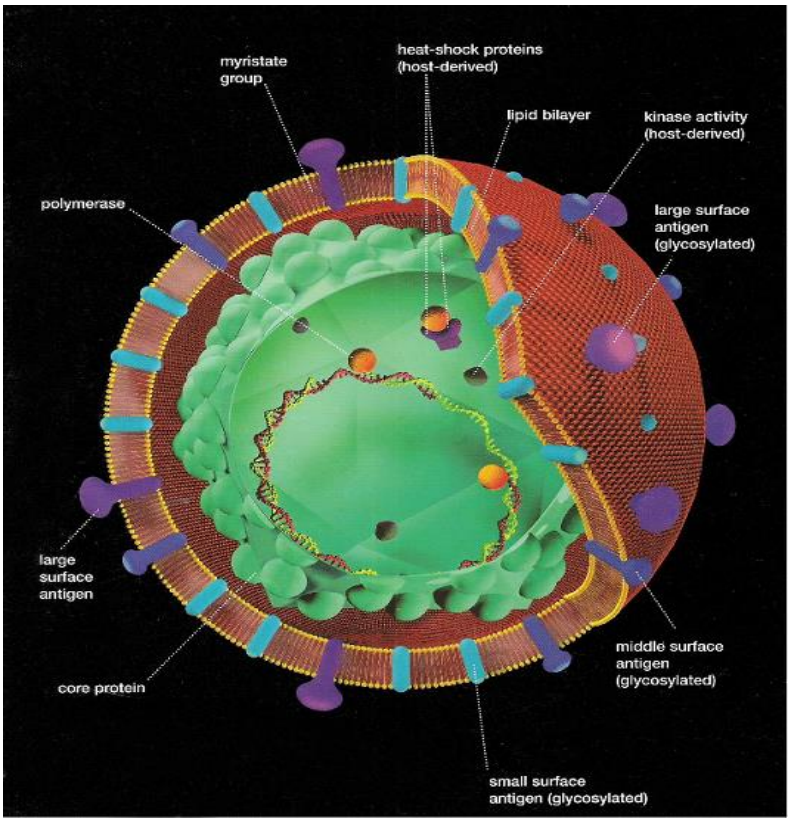

Figure 2 1. Structure of the Hepatitis B Virion
The diagram shows virion surface proteins as well as the lipid bilayer; the core; the polymerase protein; and the partially double stranded DNA genome

2.1.2 Genome organization

Hepatitis B virus genome is uniquely organized in a partly double-stranded, circular pattern and encodes protein from four overlapping genes (Figure 2 2.) (Mahoney, 1999; Schaefer, 2005). Unlike other DNA viruses HBV have an unusual replication strategy involving intermediate RNA synthesis from the DNA by reverse transcription (Seeger & Mason, 2015). This leads to higher error rate due to lack of proof reading of the reverse transcriptase enzyme leading to a higher nucleotide substitution rates of 1.4–3.2 × 10^{-5} /site per year which is almost 10-fold higher than other DNA viruses (Orito, Mizokami, Kameshima, & Yamamoto, 1989).

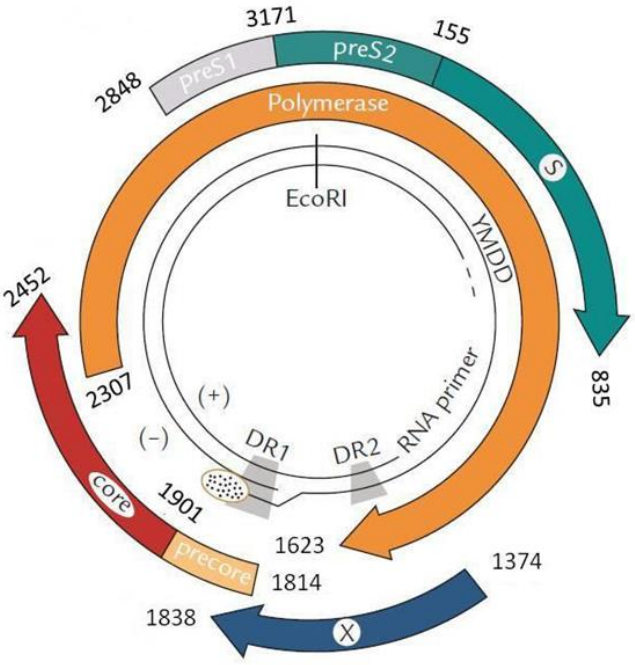

Figure 2 2. Structure and organization of HBV genome
The four protein-coding regions are shown by semi-circular arrows. They include the precore (pre-C) and core gene (C gene); the polymerase gene (P gene); the X gene; and the envelope genes pre-S1, pre-S2, and S (S gene). (Mahoney, 1999)**.**

Four mRNA transcripts of known function have been identified as being involved in HBV transcription and translation (Beck & Nassal, 2007; Seeger & Mason, 2015; Zhang *et al*., 2016). The longest (3.5 kb) is the template for genome replication and the expression of precore/core and polymerase proteins (Seeger & Mason, 2015; Zhang *et al*., 2016). A 2.4 kb transcript encodes pre-S1, pre-S2, and HBsAg (Seeger & Mason, 2015; Zhang *et al.*, 2016). The smallest transcript (0.7 kb) encodes the X protein (Beck & Nassal, 2007). The 42nm virion possess an isometric nucleocapsid or 'core' of 27nm in diameter referred to as hepatitis B core antigen (HBcAg) which are coded for by the C gene (Beck & Nassal, 2007). Surrounding the nucleocapsid core is an approximately 4nm thick outer protein coat termed 'surface antigen' or HBsAg a product of S gene (Beck & Nassal, 2007). The major protein that forms the HBsAg particle is the smallest gene product (SHBs) (Seeger, Zoulim, & Mason, 2007). The middle protein (MHBs), which contains the pre-S2 component, and the large surface protein (LHBs), which contains pre-S1, are also incorporated into HBsAg particles but are found in larger proportions in the intact virus particles (Seeger *et al.*, 2007; Zhang *et al*., 2016).

The other product of C gene is hepatitis B e antigen (HBeAg), a soluble, nonparticulate, nucleocapsid protein that is immunologically distinct from intact HBcAg (Chisari and Ferrari, 1995). The third of the HBV genes is the largest, P gene, which codes for the DNA dependent polymerase (Seeger *et al*., 2007; Zhang *et al*., 2016). DNA polymerase is packed within nucleocapsid core and directs replication and repair of HBV DNA (Schaefer, 2005). The fourth gene X, codes for a small, nonparticulate protein that has been shown to be capable of transactivating the transcription of both viral and cellular genes (Kao, 2002; Schaefer, 2005).

2.1.3 Antigenicity

All three coat proteins of HBV contain the highly immunogenic HBsAg, which elicits the production of anti-HBs antibody (humoral immunity) (Chisari & Ferrari, 1995) A number of different HBsAg sub determinants have been identified but all share the common group-reactive antigen "a" (Seeger & Mason, 2015; Zhang *et al*., 2016). In addition, HBsAg may contain one of several subtype-specific antigens, namely, d or y, w or r. All

combinations of these determinants results in nine subtypes: ayw1, ayw2, ayw3, ayw4, ayr, adw2, adw4, adrq^{+}, adrq^{-} (Seeger *et al*., 2007).

The virion core bears the hepatitis core antigen (HBcAg), and this antigen is not found in the blood as a free form. HBcAg induces the production of anti-HBc antibody.(Baumert *et al.*, 2007; Guidotti & Chisari, 2006). Hepatitis B e antigen is physically and antigenicaly distinct from HBsAg and HBcAg. HBeAg is a soluble protein that can be detected in patients with high virus titers; it indicates an acute active infection and means the patient is infectious (Beasley *et al*., 1977). Ant-HBe is a specific antibody produced against HBeAg (Guidotti & Chisari, 2006). Hepatitis B x antigen is detected in HBeAg positive blood in patients with both acute and chronic hepatitis. HBxAg is a transcriptional activator (Chisari & Ferrari, 1995; Guidotti & Chisari, 2006). It does not bind to DNA. Anti-HBx is a specific antibody to HBxAg and appears when other virological markers are becoming undetectable.(Seeger *et al.*, 2007).

2.1.4. Hepatitis B Virus Replication

Hepatitis B virus has a narrow host range, probably restricted to man and some other primates (Inoue & Tanaka, 2016). Scientific evidences suggest that HBV infects and replicates only in hepatocytes. The HBV virion binds to a recently identified receptor sodium taurocholate cotransporting polypeptide (NTCP) and possible additional hepatocyte-specific factors (Inoue & Tanaka, 2016). The mechanism of HBsAg binding to a specific receptor to enter cells has not been established yet (Beck & Nassal, 2007). Two alternative ways might be used by the virus to enter a cell: either by fusion and penetration at the plasma membrane or by endocytosis of the viral nuclocapsid followed by fusion to the cell membrane (Beck & Nassal, 2007). Once in the cell the viral nucleocapsid is released into the cytoplasm and reach the nucleus, where the viral genome is delivered (Beck & Nassal, 2007).

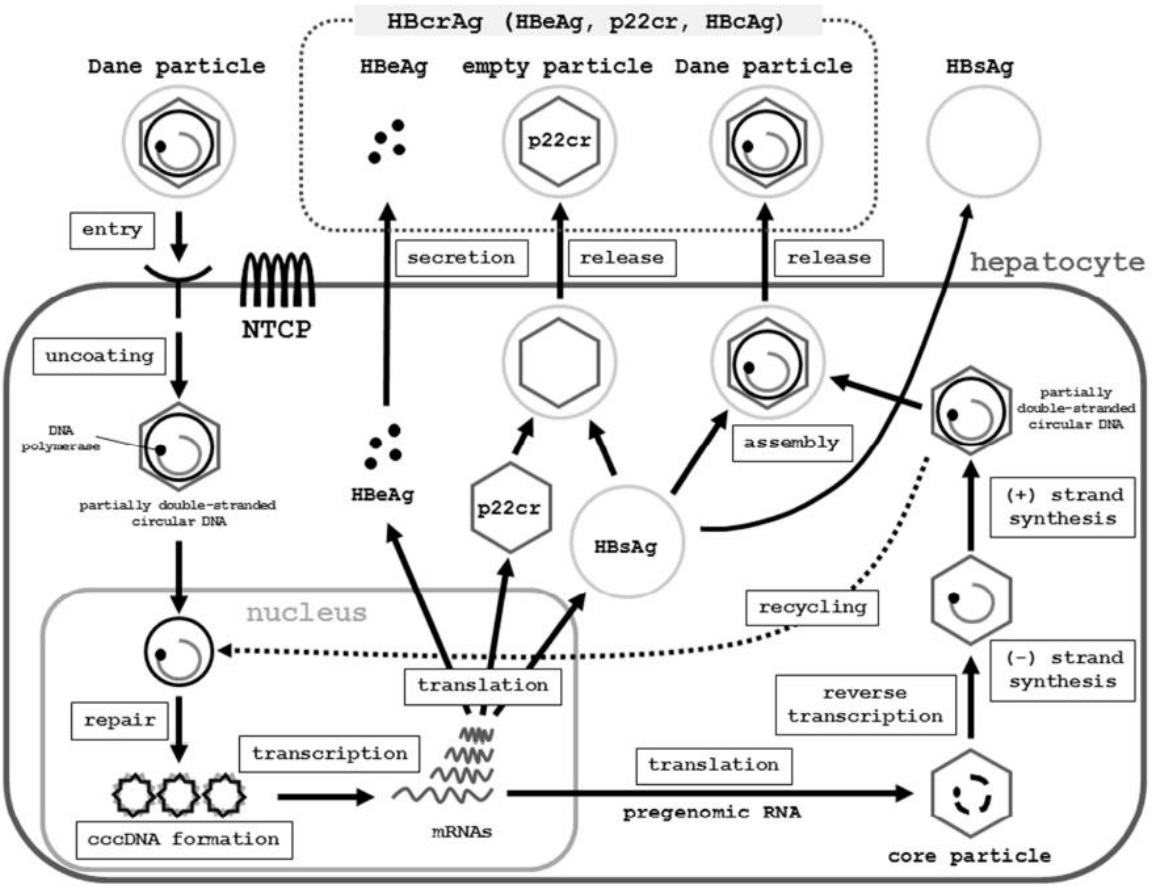

Figure 2 3. Hepatitis B virus replication cycle.
The cycle commences with attachment of the infections virion on to the target cell receptor via the preS1 domain of LHBs protein. Uncoating is followed by relocation of the nucleocapsid into the nucleus where the rcDNA is converted into cccDNA. The cccDNA serves as template for transcription of mRNA and the pgRNA. The mRNAs proceed to the cytoplasm and translated into viral proteins. The pgRNA is reverse transcribed into rcDNA which is either converted into cccDNA or translocated across the endoplasmic reticulum and coated surface proteins and ends with release into the bloodstream (Inoue & Tanaka, 2016)

The virion DNA polymerase synthesizes the missing portion of DNA creating a covalently closed circular supercoiled DNA (Beck & Nassal, 2007). This DNA serves as template for transcription of four viral mRNAs which are transported to the cytoplasm where they are translated into the viral nucleocapsid and precore antigen(C, pre-C), polymerase (P), envelope L (large), M (medium), S (small)), and transcriptional transactivating proteins (Mahoney, 1999; Beck & Nassal, 2007; Seeger & Mason, 2015). After the individual mRNA are made, a full length positive strand transcript is made, which is the strand of the progeny DNA (Beck & Nassal, 2007). The minus strand DNA

then serves as template for positive strand of genome DNA. This RNA dependent DNA synthesis takes place within the newly assembled virion core in the cytoplasm (Beck & Nassal, 2007).

The new, mature, viral nucleocapsids can then follow two different intracellular pathways, one of which leads to the formation and secretion of new virions, whereas the other is recycled back into the nucleus for amplification of cccDNA (Beck & Nassal, 2007; Zhang *et al*., 2016). In the virion assembly pathway, the nucleocapsid reach the endoplasmic reticulum, where they associate with the envelope proteins and bud into the lumen of the endoplasmic reticulum, from which they are secreted via the Golgi apparatus out of the cell (Beck & Nassal, 2007; Seeger & Mason, 2015). In the genome amplification pathway, the nucleocapsids deliver their genome to amplify the intranuclear pool of covalently closed circular DNA (Beck & Nassal, 2007; Seeger *et al*., 2007).

2.2. Epidemiology of Hepatitis B Virus

The prevalence of HBV infection varies greatly around the world with large burden of the diseases being reported in sub-Saharan Africa, East Asia, Oceania and Latin America (Keane *et al*., 2016). In endemic areas of the world the prevalence of hepatitis B surface antigen (HBsAg) ranges from 2-20% Kramvis & Kew, 2007). As measured by the presence of HBsAg, the world can be divided into areas of low (<2%), low intermediate (2-4%), high intermediate (5-7%) and high (>8%) endemicity *(*Keane *et al*., 2016).

In Africa very high endemicity of HBV is seen in developing regions with large population size and in those areas it is estimated that more than 8 % of the population is chronically infected with HBV and 65-98 % of the population bears serological evidence of past or present HBV infection (Kramvis & Kew, 2007). However, the prevalence distribution is not even and some countries including Kenya, Zambia, Cote d'Ivoire, Liberia, Sierra Leone and Senegal, are considered less endemic with an intermediate endemicity (2–8%) (Kramvis & Kew, 2007).

The available HBV prevalence data from Eastern Africa region show a varied prevalence pattern among countries and within each country. Belyhun *et al* in one systematic review and meta-analysis study conducted in Ethiopian reported HBsAg prevalence ranging from

3-27% and HBV exposure rate ranging from 2.5-73.1% among different population categories (Belyhun *et al*., 2016). Similar variation in prevalence is also reported in Ethiopia in various studies conducted among pregnant women with an HBsAg prevalence ranging from 3.8-7.8% (Desalegn *et al*., 2016a; Desalegn *et al*., 2016b; Metaferia *et al*., 2016; Seid *et al*., 2014; Yohanes *et al*., 2016). Likewise a study done in Kenya had reported an overall HBsAg prevalence of 9.3% ranging from 3-17.8% for the different regions of the country (Okoth *et al*., 2006). Other neighbouring geographical locations have reported HBsAg prevalence of 5.6%, 11% and 10.8% for Sudan (Elsheikh, Daak, Elsheikh, Karsany, & Adam, 2007), South Sudan (Kirbak *et al*., 2017) and Yemen (Murad *et al*., 2013). The epidemiology of HBV infection in the general public of Eritrea is not well documented and the available data is very scanty. In one such retrospective study conducted on blood donors data collected from 2006-2009, a low intermediate (2.6%) HBsAg seromarker prevalence was reported (Fessehaye *et al*., 2011).

2.3. Genotypes distribution

Based on divergence either in the entire HBV genomic sequence greater than 8% or more than 4% in the S gene HBV is differentiated into ten genotypes (Beck & Nassal, 2007; Zhang *et al*., 2016). HBV genotypes can be further segregated into subgroups if the genomic sequence divergence is between 4-8% (Kao, 2002; Kao *et al*., 2002; Norder *et al*., 2004; Schaefer, 2005; Zhang *et al*., 2016). Genotypes A-D were the first genotypes to be recognized (Okamoto *et al*., 2018),followed by genotypes E and F (Norder *et al*., 1994), G (Stuyver, *et al.*, 2000), H (Arauz-ruiz *et al*., 2017) and I (Yu *et al.*, 2010) being recognized subsequently. A tenth genotype, J, isolated from a single individual, has been proposed (Tatematsu *et al.*, 2009).

Hepatitis B virus genotypes show a distinct geographic distribution and are believed to play a major role in the clinical outcome of infection (Kao, 2002; Kramvis and Kew, 2007; Schaefer, 2005). Looking into geographic distribution of HBV in Africa; genotype A is predominantly found in Southern, Eastern and Central Africa with subgenotype A1 being dominant subgenotype in those regions (Hundie *et al*., 2016b; Kramvis & Kew, 2007). In Western Africa quasi subgenotype A3 is the main subgenotype reported (Hundie, *et al*., 2016b; Kramvis and Kew, 2007). Even though subgenotype A2 is

predominantly reported in Europe, a few cases of subgenotype A2 have also been reported from South Africa and Kenya (Kramvis & Kew, 2007; Mwangi *et al*., 2008).

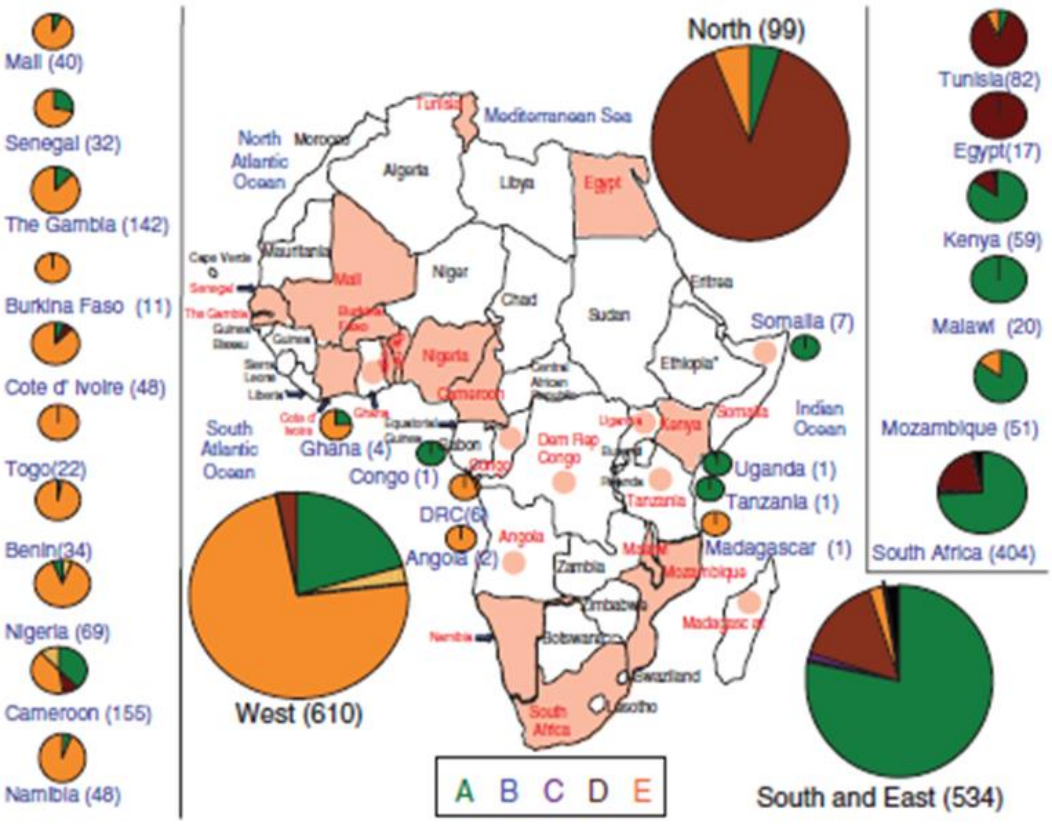

Figure 2 4. Hepatitis B virus (HBV) distribution across the African continent
Distribution of HBV genotypes in Africa. Genotyping studies were performed in countries whose names are shown in red. Countries in which large scale studies were carried out are shaded pink, and smaller scale studies (<10 isolates were genotyped) are marked with a red dot. Small pie charts, genotype distribution within each country; large pie charts, genotype distribution of northern, western, southern/eastern regions; numbers in parentheses, no. of isolates genotyped (Kramvis & Kew, 2007).

Most genotype D cases have been reported in North Africa with D1 being the most dominant subgenotype (Hundie, *et al*., 2016a; Kramvis & Kew, 2007). Other D subgenotypes reported in Africa includes; D2 and D10 in Ethiopia (Hundie, *et al*., 2016a; Hundie, *et al*., 2016b), D3 in South Africa (Kramvis & Kew, 2007), D7 in Tunisia and D8 in Niger (Chekaraou *et al*., 2010). Genotype E is the most prevalent genotype of western and central Africa, it has a low genetic diversity and doesn't have subgenotypes (Mulders *et al*., 2004; Odemuyiwa *et al*., 2001). But isolates from South-West/ Central

Africa cluster separately from those from Western Africa (Kramvis & Kew, 2007; Mulders *et al*., 2004).

In countries where two or more HBV genotypes circulate, co-infection with more than one genotype have been detected leading to intergenotype recombination (Kao, 2002; Kao *et al*., 2002; Kramvis & Kew, 2007; McMahon, 2009; Olinger *et al*., 2008; Schaefer, 2005; Zhang *et al*., 2016). Intergenotype recombination plays a significant role in the evolutionary history of HBV (Beck & Nassal, 2007; Chotiyaputta & Lok, 2009; Schaefer, 2005; Seeger & Mason, 2015; Zhang *et al*., 2016). Recombinants of genotypes D/E, A/E, A/D and even A/D/E were reported in many African countries and countries neighbouring Eritrea (Hundie *et al*., 2016b; Kramvis & Kew, 2007; Ochwoto *et al*., 2016; Yousif *et al*., 2013).

Hepatitis B virus genotypes circulating in Eritrea and their molecular diversity is not documented, the very few data available are from studies conducted among Eritrean immigrants in Italy (Palumbo *et al*., 2007; Scotto *et al*., 2010). Two such studies in Italy reported genotypes A, D and E among Eritrean immigrants (Palumbo *et al*., 2007; Scotto *et al*., 2010). Those three genotypes are known to predominantly circulate in Africa with distinct geographic distribution (Kramvis & Kew, 2007*;* Ochwoto *et al.*, 2016*;* Vray *et al.*, 2006). Therefore these findings should be interpreted with care considering the possibility of those migrants being infected in any of the countries they crossed to reach Europe.

2.4. Clinical relevance of the HBV genotypes

Molecular diversity of HBV genotypes is believed to play a major role in the clinical outcome of infection (Kao, 2002a; Kramvis & Kew, 2007). Different studies have demonstrated that the different genotypes of HBV have a significant effect on severity of liver disease, treatment outcome and immunization (Kramvis & Kew, 2007). Geographical overlapping in co-occurrence of HBV genotypes have made it possible to study in comparison the course of infection and outcome of infections (Kramvis & Kew, 2007).

A number of studies conducted in Asia have demonstrated that genotype C causes more severe liver diseases that progresses more often to HCC when compared to genotype B (Lin & Kao, 2011). In addition patients infected with genotype C has a slow seroconversion rate when compared to patients infected with genotype B. This usually increases the duration of viral replication, which may ultimate result in fibrosis, cirrhosis and HCC (Lin & Kao, 2011). Considering treatment outcome using embolization therapy people with genotype B infection respond favourably with no recurrence of HCC for a prolonged period of time but in case of genotype C patients didn't respond well developing HCC which caused hepatic failure leading to death (Kao, 2002; Kao *et al*., 2002; Tatematsu *et al.*, 2004).

In sub-Saharan Africa, subgenotype A has been associated with high rates of HCC with a higher rate of HBeAg negativity and low HBV DNA level (Kew *et al*., 2005). A similar study reported a 4.5 times higher relative risk of HCC in patients carrier to genotype A compared to patients infected with other genotypes among South African patients (Kew *et al*., 2005). For genotype D different clinical course of hepatic diseases were reported in different researches conducted in different geographical settings. In most patients infected with genotype D generally seroconvert to anti-HBe positivity at a more advanced age; which means they may undergo multiple hepatitis flares, which accelerates the progression of chronic hepatitis to cirrhosis (Lin & Kao, 2011; Mcmahon, 2009). But among Indian patients Thakur *et al* reported a significant correlation with genotype D and severe liver disease with development of HCC in younger patients (Thakur *et al*., 2002). There is limited data on the clinical course and outcome of infection among other genotypes (Kramvis & Kew, 2007).

2.5. Hepatitis B infection in pregnancy

Most studies suggest that HBV is not directly cytopathic to infected hepatocytes and that the cellular response to several viral proteins correlate with the severity of clinical disease and viral clearance (Baumert *et al.*, 2007). The clinical manifestation and outcomes following acute liver injury associated with viral hepatitis are determined by the immunologic responses of the host (Mcmahon, 2009). Replication of virus in hepatocytes

interferes with the function of the liver leading to degenerative changes such as swelling and necrosis (Baumert *et al.,* 2007).

Cell mediated immunity and inflammatory reactions are responsible for causing the symptomatology and effecting resolution of the infection (Baumert *et al.,* 2007; Chisari & Ferrari, 1995). Cell mediated immune responses, particularly those involving cytotoxic T- lymphocytes seems to be very important (Chisari & Ferrari, 1995). The expression of HB viral antigens especially the nucleocapside proteins (HBcAg and possibly HBeAg) and also HBsAg on the surface of hepatocytes invites cytotoxic T cells to destroy the HBV infected hepatocytes (Chisari & Ferrari, 1995). In addition cytotoxic T cells inhibit HBV gene expression through the secretion of antiviral cytokines and that the expression of these cytokines may be the principal mechanism of viral clearance during HBV infection (Baumert *et al*., 2007; Chisari & Ferrari, 1995; Guidotti & Chisari, 2006; Liang, 2010). Moreover, antibody produced in response to viral antigen proteins contributes to clear the virus (Baumert *et al*., 2007; Chisari & Ferrari, 1995; Seeger *et al.,* 2007).

During pregnancy, the maternal immune system becomes tolerant to antigenic insults to accommodate the child and hormonal changes affect immune regulation increasing the likely hood of increased HBV replication (Nguyen *et al.,* 2009). Some studies reported a weaker T-cell response to HBsAg in pregnant women compared to non-pregnant women affecting the course of HBV infection during pregnancy (Nguyen *et al.,* 2009). Moreover it is reported that the ability of HBeAg to cross the placental barrier and accesses foetal circulation could lead to recruitment of T-cells that are tolerant to the HBeAg. This might contribute to the higher potential of chronic HBV infection observed on children infected to HBV during pregnancy (Patton & Tran, 2014).

The clinical presentation of HBV infection show only subtle difference between pregnant women and non-pregnant women. Pregnancy don't alter the clinical course of acute HBV infection; during the prodromal period pregnant women could develop serum sickness followed by constitutional symptoms including anorexia, vague abdominal discomfort, nausea and vomiting, sometimes arthralgias and rash, often progressing to jaundice (Mahoney, 1999). Fever may be absent or mild. The nausea and vomiting of prodromal stage may be confused with the symptoms present in pregnancy. Jaundice is accompanied

by hepatomegaly and splenomegaly and alongside HBV seromarkers possibly indicate acute HBV infections (Jonas, 2009; Han *et al*., 2014).

Possible outcome of acute HBV infection during pregnancy might include increased risk for preterm birth, low birth weight (<2500 g), premature rupture of membranes gestational diabetes and congenital abnormalities (Reddick, *et al*., 2011; Dunkelberg *et al*, 2016; Han *et al*., 2014). Many studies have also indicated that HBsAg clearance and seroconversion are delayed increasing the risk of developing chronic HBV infection (Reddick *et al.*, 2011; Dunkelberg *et al*, 2016; Han *et al*., 2014). Moreover co-infection with HDV viruses during pregnancy increases the risk of fulminant hepatitis with mortality as high as 85% (Reddick *et al*., 2011).

Chronic hepatitis is an important late complication of acute HB occurring in small proportion of acute cases but more common in those who present with chronic infection without having experienced an acute illness (Baumert *et al*., 2007). The clinical features of chronic HBV are highly variable and are not predictive of outcome, but, risk of evolving to cirrhosis is high. Up to 20% of the chronic persistent hepatitis cases progress to cirrhosis (Shi, 2009). HBV associated cirrhosis could lead to HCC, although it has been reported that HCC can occur with or without cirrhosis (Shi, 2009).

The natural history of chronic HBV infection can be divided into five stages: immune tolerant, immune clearance, immune control, immune escape and reactivation phase (Inoue & Tanaka, 2016). Immune tolerant stage is seen in many HBeAg-positive children and young adults, particularly among those infected at birth (Baumert *et al*., 2007; Inoue & Tanaka, 2016). It is characterized by high HBV DNA level (>200 000 IU/mL), normal ALT and lack of liver inflammation (Inoue & Tanaka, 2016). The immune clearance stage, in which there is seroconversion from HBeAg positivity to anti-HBe, followed by inflammation of hepatocytes and increased ALT levels (Kao, 2002). Patients in the immune control (inactive chronic hepatitis) stage are negative for HBeAg having circulating anti-HBe antibodies (Kao, 2002). They have a reduced risk of cirrhosis with a low HBV DNA (2000IU/ml) and a normal ALT level (Kao, 2002). Immune escape (HBeAg- negative chronic hepatitis) stage mostly seen in elderly persons with the possibility of further liver damage, leading to fibrosis and cirrhosis (Inoue & Tanaka,

2016). ALT levels are abnormal with moderate to high levels of HBV replication (> 20,000 IU/ml) (Inoue & Tanaka, 2016). Reactivation or acute-on- chronic hepatitis stage can occur spontaneously or precipitated by antiviral resistance or immunosuppression (Inoue & Tanaka, 2016). There could be seropreversion to HBeAg positivity if the person was HBeAg negative (Inoue & Tanaka, 2016). HBV DNA levels are moderate to high with elevated ALT levels (Inoue & Tanaka, 2016).

Pregnancy is well tolerated by women with chronic hepatitis B infection. Pregnant women will mostly have normal pregnancies unless they develop sever complications including end stage liver diseases or cirrhosis (Piratvisuth, 2013: Dunkelberg *et al,* 2016; Lamberth *et al*., 2015). Many studies have shown that the risk of perinatal complications and poor pregnancy outcome increases in pregnant women with cirrhosis (Piratvisuth, 2013; Dunkelberg *et al,* 2016; Lamberth *et al.,* 2015). These women tend to have an increased risk of spontaneous abortion, gestational hypertension and hepatic decomposition (Piratvisuth, 2013). In case of HCC malignancies the maternal mortality is high with the high level of oestrogen accelerating the HCC evolution leading to death (Piratvisuth, 2013). In chronic HBV infections HBV DNA level and HBeAg levels might increase with delayed seroconversion and this will lead to a higher probability of vertical transmission (Ivan & Borgia, 2014).

2.6. Transmission

Hepatitis B virus is present in many body fluids of infected individuals including blood and semen, compared to other infectious viruses found in body fluids such as HIV and HCV, HBV has a high rate of transmission due to high viral load (Hou *et al*., 2005). The main routes of infection for HBV are perinatal transmission, percutaneous transmission, sexual transmission and blood transfusion (Hou *et al*., 2005; Mahoney, 1999). HBV is spread by either skin puncture or mucous membrane contact with infected blood or other body fluids (Hou *et al*., 2005).

Perinatal transmission occurs primarily in infants born to HBsAg carrier mother or mother with acute hepatitis B during the 3rd trimester of pregnancy or during the early postpartum period (Hou *et al*., 2005). Perinatal transmission usually happens at the time of birth; in utero transmission is relatively rare, accounting for fewer than 2% of perinatal infections

in most studies (Kew, 1996). The risk of perinatal transmission depends on the presence of HBeAg in the blood of mothers infected with HBV. Reports from the African continent have documented that children born to mothers seropositive for both HBsAg and HBeAg are thought to have a pooled risk of 38.3% getting the infection if not given appropriate immunoprophylaxis (Keane *et al*., 2016). This is much higher when compared to the 4.8% reported among infants born to mothers who are HBsAg positive but HBeAg negative (Keane *et al*., 2016).

The perinatal mode of transmission is very common in the Far East countries, including Japan and China, whereas in the African continent horizontal transmission is instrumental in HBV transmission (Kramvis & Kew, 2007). Different studies from the African region had demonstrated that even in setting with high HBV carriage among pregnant women the risk of perinatal transmission is low (Kew, 1996; Kew, 2008; Kramvis and Kew, 2007). A study conducted in Tanzania among expectant mothers reported at 8 months of age HBsAg was detected in 8% and 2% of children born to HBV-infected and noninfected mothers, respectively. Subsequent follow up and testing at 18 months of age the HBsAg prevalence increased to 31% and 21% showing that horizontal transmission was significant among those group of children and similar findings were reported in Tunisia (Mendez *et al.*, 1999; Triki *et al*., 1997)

Infection, following unsafe injection drug use occurs mainly in developed countries. In developing countries, health care professionals and their patients are at risk of getting HBV infection (Coppola *et al*., 2016). Unsatisfactory infection control practices, including the reuse of contaminated medical or dental equipment, failure to use appropriate disinfection and sterilization practices can also result in the transmission of HBV and other blood borne pathogens (Coppola *et al*., 2016; WHO, 2015, 2016). According to the WHO worldwide around 3 million healthcare workers receive an injury with sharp instruments while at work, thus resulting with 2 million subjects contaminated with HBV and 1 million with HCV (Elseviers *et al*., 2014). Cultural customs like tattooing and scarification can also lead to the transmission of HBV. Even though, there is lack of comprehensive data different studies from the African continent had reported association with invasive cultural practices and HBV infections (Bwogi *et al*., 2009; Ducancelle *et al*., 2013; Kirbak *et al*., 2017; Seid *et al*., 2014; Yohanes *et al*., 2016).

HBV is efficiently transmitted by sexual contact, which can account for a high proportion of new hepatitis B infections among adolescents and adults in countries with low and intermediate endemicity of chronic HBV infection (Hou *et al*., 2005). However in countries where HBV infection is highly endemic, sexual transmission does not account for a high percentage of cases because most persons are already infected during childhood (Kew, 1996, 2008; WHO, 2016).

2.7. Laboratory Diagnosis

2.7.1 Sero-diagnosis

Serological tests for HBV antigens and antibodies are typically used for diagnostic screening and can be performed on either serum or plasma. HBsAg can be detected in the serum from several weeks before onset of symptoms to months after onset. HBsAg is present in serum during prodrome and acute disease (Mahoney, 1999; WHO, 2016). It drops to undetectable levels during convalescence in most cases; its prolonged presence (at least 6 months) indicates the carrier state and the risk of chronic hepatitis and hepatic carcinoma (Kew, 2008). The presence of HBsAg indicates that the person is potentially infectious. Anti-HBs replace HBsAg as the acute HBV infection is resolving (Guidotti & Chisari, 2006). Anti-HBs generally persist for a lifetime in over 80% of patients and indicate immunity.

Anti-HBc is the first antibody to appear and demonstration of anti-HBc in serum indicates HBV infection, current or past (Mahoney, 1999). Anti-HBc IgM is present in high titer during acute infection and usually disappears within 6 months, although it can persist in some cases of chronic hepatitis (Mahoney, 1999). This test may therefore reliably diagnose acute HBV infection. Anti-HBc IgG generally remains detectable for a lifetime. HBeAg arises during the incubation period and is present during the prodorme and early acute disease and in certain chronic carriers. Its presence is an important indicator of transmissibility, and, conversely, the presence of anti-HBe indicates low transmissibility (Dunkelberg *et al*., 2016).

Anti-HBe appears after anti-HBc and it replaces HBeAg in the resolution of the disease. Acute hepatitis patients who maintain a constant serum HBsAg concentration, or whose

serum HBeAg persists 8 to 10 weeks after symptoms have resolved, are likely to become carriers and at risk of developing chronic liver disease (Dunkelberg *et al*., 2016; Seeger *et al*., 2007). A complication in the diagnosis of hepatitis B is identification of cases in which viral mutations change the antigen so they are not detectable (Datta *et al*., 2014).

Table 2 1. Interpretation of HBV serological test results

Assay result			Interpretation
HBsAg	**Anti-HBs**	**Anti-HBc**	
-	-	-	Susceptible to HBV
-	+	+	Immune due to natural infection
-	+	-	Immune due to hepatitis B vaccination
+	-	+ IgM	Acutely infected
+	+	+ IgG	Chronically infected
-	-	+	Interpretation unclear: four possibilities: 1. Resolved infection (most common) 2. False positive anti-HBc, thus susceptible 3. "Low level" chronic infection 4. Resolving acute infection

- = Negative, + = Positive. Adopted from CDC (CDC, 2005)

2.7.2. Nucleic acid based detection assays for HBV DNA

Nucleic acid bases assays for detection of HBV are generally categorized into two; the simple but less sensitive direct hybridization tests utilizing probes that directly binds to HBV DNA and the most sensitive but costly indirect tests that involve in vitro amplification of targeted HBV DNA followed by detection (Datta *et al*., 2014). A number of in vitro amplification tests are available in different formats including PCR based assays, nucleic acid sequence based amplification (NASBA), transcription mediated amplification (TMA)**,** rolling circle amplification (RCA) (Datta *et al*., 2014).

To the conventional PCR technique a number of modifications are introduced to increase the sensitivity and specificity of PCR based tests to mention a few multiplex PCR, nested PCR, real time PCR. Some of this modification such as RT-PCR not only enables detection of DNA but it also enables quantitative measurement of DNA (Guirgis *et al.*, 2010). In case of HBV PCR followed by sequencing and phylogenetic analysis is the most frequently used methods to molecularly characterize HBV including genotyping and sub-genotyping (Guirgis *et al.*, 2010).

2.8. Prevention and Control

Treatment of chronic hepatitis B is aimed at eliminating infectivity to prevent transmission and spread of HBV, at halting the progression of liver disease and improving the clinical and histologic picture, and at preventing HCC from developing, by losing markers of HBV replication in serum and liver like HBV DNA, HBeAg and HBcAg (Raimondi, *et al*., 2010; WHO, 2016). Normalization of serum tranaminase activity, resolution of hepatic inflammation and the improvement of patients' symptoms usually accompany these virological changes (Raimondi *et al*., 2010) . There are two main classes of treatment: antivirals aimed at suppressing or destroying HBV by interfering with viral replication and immune modulators aimed at helping the human immune system to mount a defense against the virus (Mahoney, 1999; WHO, 2016).

Currently there are seven drugs used for treatment of HBV infection including interferon (both standard and pegylated), lamivudine, adefovir, entecavir, telbivudine, and tenofovir (Bzowej, 2010; Piratvisuth, 2013). But none are approved to be used during pregnancy therefore before initiation of treatment the risks and benefits for the mother and fetus must be assessed (Bzowej, 2010; Piratvisuth, 2013). The major goal of treatment during pregnancy is to prevent MTCT especially among pregnant women with high viral DNA or are positive to HBeAg (Patton & Tran, 2014; Piratvisuth, 2013).

Hepatitis B virus immunization employs either passive immunoprophylaxis or active immunization (WHO, 2015). Currently the WHO recommends use of the hepatitis B (HepB) vaccine which not only protects children and adults from HBV infection, but clinical trials have established that if given within 24 hours after birth and followed by at least two subsequent doses, the vaccine is approximately 90% effective at preventing

perinatal HBV infection (WHO, 2015). The principal objective of hepatitis B immunization is to prevent chronic HBV infections which tend to occur in people who have acquired HBV during childhood (less than 5years) (WHO, 2015). Failure of HBV vaccine and hepatitis B immunoglobulin (HBIG) have been reported for children born of pregnant women with a high level of HB DNA > 200,000 IU/ml in those condition the use of antiviral drugs such as lamuvidine and telbivudine have shown a promising results (Ivan & Borgia, 2014; Nelson *et al*., 2014)

Other preventive measures includes increasing public awareness on HBV infection especially promoting personal hygiene and introducing environmental control measures to limit transmission (Mahoney, 1999; WHO, 2016). Health care workers should strictly follow universal precautions when handling potentially infectious or contaminated materials. Screening of blood and blood products will prevent transmission of HBV infection via transfusion of those products (Mahoney, 1999; WHO, 2016).

CHAPTER THREE

METHODS AND MATERIALS

3.1 Study design

This cross sectional study was conducted on archived samples and data collected in the 2016 national ANC sentinel surveillance survey of HIV, Syphilis, Hepatitis B, and Hepatitis C. The 2016 ANC sentinel surveillance was conducted in the time frame from January to March, 2016.

3.2 Study Area

Eritrea is located in the horn of Africa between latitudes 12°22′ and 18°02′N, and longitudes 36°26′21″E and 43°13′E. It covers a total area of 122,000 square kilometres and is bordered by the Red sea, Ethiopia, Sudan and Djibouti (Figure 3 1.). The country have three physiographic zones with attitudes ranging from below sea level to 3,000 meters above sea level. These zones include the Western lowlands, the Eastern lowlands (Coastal plains) and the central and northern Highlands. The country is divided into six administrative regions (zobas): Maekel, Debub, Anseba, Gash-Barka, Semenawi Keiyh Bahri, and Debubawi Keiyh Bahri.

The population is estimated at around 5 million with about 53% being above the age of 15 years, the highest within this group, being women of 20-60 years of age (EPHS, 2010). The country's crude birth rate is estimated at 33.9 births/1,000, however with an infant mortality rate of 45/1000 and maternal mortality of 4.8/1000(EPHS, 2010). Major diseases include acute respiratory infection, diarrhoea, anaemia and malnutrition, fever of unknown origin, injuries, heart diseases, diabetes and tuberculosis and recently hepatitis B virus infection being among the 19 key infections identified in the country (Ministry of Health, 2016).

Figure 3 1. Administrative map of Eritrea

The map shows the geographical location of Eritrea and the administrative regions (zobas) of Eritrea. The six regions of Eritrea are represented by different colors. The red circles and green triangles show selected ANC sites from urban and rural areas.

Eritrean is one of the poorest nations in the world with majority of its population dependent on subsistence farming, pastoralism and fishing (African Development Bank, 2016). The main sources of foreign exchange income for Eritrea are worker remittances from abroad and mining (African Development Bank, 2016). However, there is a significant difference in poverty levels among the regions and particularly the coastal and western lowlands had a disproportionate number of poorer households compared to the more urbanized regions located in central and northern highlands (EPHS, 2010). The current study was conducted within 46 health facilities distributed across all the six regions (Table 3.1).

Table 3 1. Distribution of selected ANC facilities per zoba and study site/cluster

Study region (Zoba)	Study site/cluster	No. of Health facilities
Anseba	Joko cluster	5
	Geleb cluster	5
Maekel	Semanawi Asmara Health center	1
	Edaga Hamus Hospital	1
	Akria Health center	1
	Serejeka cluster	7
Debub	Mendefera cluster	2
	Dekemhare MCH	1
	Areza cluster	5
Northern Red Sea	Massawa cluster	3
	Shieb cluster	4
Gash Barka	Barentu Hospital	1
	Agordat Hospital	1
	Tesseny Hospital	1
	Haycota cluster	4
Southern Red Sea	Assab cluster	4

3.3. Study Population

The study population included all the 5,009 pregnant women aged 15-49 years who were part of the 2016 ANC survey. All pregnant women aged 15-49 visiting the ANC clinics for the first time in their pregnancy were selected, with those who were found to have visited any ANC clinic in their current pregnancy excluded from the study. All the samples collected from the 5,009 pregnant women in the 2016 ANC survey were used to screen for anti-HBc and anti-HBs antibody seromarkers. All the 151 HBsAg positive samples were analyzed for HBV DNA detection and genotyping study.

3.4. Sampling methodology

The primary study was conducted following the guidelines published by the world health organization (WHO) for ANC sentinel surveillance (WHO and UNAIDS, 2003). The selection strategies for the 2016 surveillance included maintaining the 2013 sentinel surveillance sites to facilitate prevalence comparisons and generate prevalence that are representative of all zobas (regions) and sub-zobas of the country.

A two-stage sampling design was employed. The first stage involved selecting ANC sentinel sites and in the second selection of ANC attendee women. Accordingly selection of the ANC sites was done using non-probability sampling taking into account representations by zoba and urban-rural; volume of ANC attendance; and availability of facilities for processing and storing blood specimen. A total of 46 health facilities were selected, out of which 20 were from urban areas and the remaining 26 from rural areas (Table 3.1). The selected health facilities were grouped into 16 study sites grouped into urban and rural clusters within their respective zobas. A number of study sites had some satellite sites, which assisted the main site in the effort to reach the target sample size.

A minimum sample size of 300 pregnant women was selected from each sampling site or cluster. Consecutive or convenience sampling method was used to select eligible pregnant women to be included in the study. More specifically, within a given ANC site, the first pregnant woman to meet the eligibility criteria within the designated time frame for data collection was included in the study. Each eligible pregnant woman thereafter was

included until the desired sample size was achieved or until the end of data collection period.

In the current study all the samples collected in the primary survey which fulfilled the sample inclusion criteria were tested for anti-HBV seromarkers. The genotyping targeted all the HBsAg positive samples from the primary survey.

Sample inclusion criteria:

- Sufficient quantity
- Non hemolyzied and non lepeamic
- Not broken or leaking vials
- Clearly labeled

Sample exclusion criteria:

- Not sufficient quantity
- Hemolyzied and lepeamic samples
- Broken or leaking vials
- Samples missing labelling and/or unreadable

3.5. Description of clinical samples

Archived serum samples were used for this study. Each sample was paired to demographic data from the records of ANC sentinel study. The samples had been stored at the National Health Laboratory (NHL) of Eritrea in the immunoserology department and the socio-demographic data were stored at the Communicable Disease Control (CDC) of the Ministry of Health. Samples to be tested were removed from the -80^0C freezer and stored at 4^0C to facilitate thawing for 24hrs. As the samples were stored organized by facility and ANC sites the amount of sample removed from the freezer were limited to the capacity which could be processed within a day. The data set containing information on the socio-demographic characteristics of the study subjects was recovered from the CDC.

3.6. Data on socio-demographic characteristics

In the primary study a structured questionnaire (Appendix III) was developed and used to collect information on important background characteristics of each pregnant woman included in the study. The information collected includes: age, religion, nationality, total number of pregnancies (gravidity), number of previous live births (Parity), marital status, level of education, occupation of the respondents and their partners. Data collection was done by interviewing each eligible pregnant women selected for the study using the structured questionnaire and this was followed by taking anonymous unlinked blood sample from each participant. The pregnant women were selected by a nurse within each selected ANC facilities who was also responsible to fill the study questionnaire through an interview. Thereafter, the pregnant women were referred to the laboratory to give blood samples. Blood samples were collected by a laboratory technician.

3.7. Ethical consideration

The initial study got ethical clearance from the Eritrean Ministry of Health Research and Ethical Committees. All the six zoba Ministry of Health (MoH) branches, and relevant health facilities were informed, through an official letter, on the scope, coverage, and objectives of the study. The coordinators recruited were also oriented about the study. Anonymity was maintained by using questionnaire, which bear no name and was only linked with the sample by study number. Database security measures were added to this protection in such a way that only authorized persons had access to the data and forms.

In the current study ethical clearance was furnished by the UoN/KNH ethical committee, Kenya with study protocol number P965/12/2016 (Appendix I) and the MoH ethical committee, Eritrea (Appendix II). Permissions to use laboratory facility was sought from the National Health Laboratory of Eritrea and HBV laboratory at KEMRI. The individual laboratory test results and laboratory work sheets were kept confidential by the principal investigator. Security measures including password protection were instituted to protect database related with this study.

3.8. Laboratory testing method

3.8.1. Blood sample collection and storage

In the primary study for every participant 5ml of venous blood was collected and labelled in line with the already administered questionnaire. All samples did not contain any personnel identifiers and were labeled alongside the filled questionnaire with a code made up of identifiers for zoba, health facility, and a unique number of the participant. All samples were centrifuged within 1 hour of collection and the serum was divided into two aliquots and immediately placed in a freezer at -20^{0}C. Weekly the collected samples were shipped from the selected health facilities to the NHL for testing and storage at -80^{0}c. All the samples tested in the primary study were archived in the order of zoba of origin and study sites and health facility from which the samples originated from. All the samples were stored at -80^{0}c freezer.

3.8.2. Hepatitis B virus Serology

In the 2016 ANC sentinel surveillance as part of the HBV testing protocol seromarkers for HBsAg, HBeAg and anti-HBe were tested. All serum samples were tested for HBsAg antigen marker using an ELISA - SURASE B-96 (General Biological Corp, Taiwan; Cat number: 4SGE3), sensitivity and specificity of 100% and 99.58% respectively. All HBsAg reactive samples were tested for HBeAg/ anti-HBe seromarkers using EASE BN-96 ELISA test kit (General Biological Corp, Taiwan; Cat number: 4BNE3) with a reported sensitivity and specificity of 98.5% and 99.1% respectively for HBeAg. The sensitivity and specificity for anti-HBe were more than 99%.

In the current study all the serum samples were tested for anti-HBc by ELISA using conjugated anti-HBc in a competition principle manufactured by DIAsource immunoassays, Belgium (Cat number: KAPG4CBE3) (Appendix V). The solid phase of the microtiter plate is made of polystyrene wells coated with HBcAg when a serum or plasma specimen containing Anti-HBc is added to the HBcAg-coated wells together with the human peroxidase conjugated Anti- HBc and incubated, a competition will take place for the binding to the HBcAg on the wells. (HBcAg)-(Anti-HBc peroxidase) complex and/or (HBcAg)-(Anti-HBc) complex will form on the wells. After washing of the

microtiter plate to remove unbound material, a solution of TMB substrate is added to the wells and incubated. Due to the competitive principle a color develops inversely proportional to the amount of Anti-HBc bound to HBcAg deriving from the specimen. The peroxidase-TMB reaction is stopped by addition of sulfuric acid. The optical density of developed color is read with a suitable photometer at 450 nm with a selected reference wavelength within 620 to 690 nm. Cut of values were calculated according to recommendations of the manufacturer and interpreted accordingly. The test have a reported 100 % and 99.8% sensitivity and specificity respectively.

Antibodies against the surface antigen were detected using ANTISURASE B-96 ELISA from the General Biological Corp, Taiwan (Cat number: 4SBE3). The test is designed for qualitative detection for the presence of antibodies to HBsAg in serum or plasma (Appendix VI). The test utilizes a microtiter well coated with HBsAg and a liquid phase of peroxidase conjugated HBsAg. Once a sample containing anti-HBs and the peroxidase conjugate HBsAg provided with the kit are added into the microtiter well coated and incubated, HBsAg-anti-HBs-HBsAg peroxidase complexes will form on the wells. After washing the microtiter plate to remove unbound material, a solution of TMB substrate is added to the wells and incubated. A color develops in proportion to the amount of anti-HBs bound to HBsAg. The peroxidase-TMB reaction is stopped by addition of sulfuric acid. The optical density of developed color is read with a suitable photometer at 450/620nm wavelength. Cut of values were calculated according to recommendations of the manufacturer and interpreted accordingly. The test have a reported sensitivity and specificity of 100% and 99.8% respectively.

3.8.3. HBV DNA extraction and amplification

DNA was extracted from 200 µl serum using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), according to manufacturer's instructions, and was eluted in 60 µl nuclease free water (Appendix VII). The extracted DNA was stored at -80^{0}C. The extracted HBV DNA was amplified in a nested PCR using primers that target the HBsAg (S1) genomic region (nt 155-835) following a slightly modified protocol of Ochwoto *et al* (Ochwoto *et al.*, 2016). Five microliters (5 µl) of the HBV extract was amplified in a nested PCR with two different sets of primer in a total volume of 50 µl per tube. Each tube contained 24 µl of

DNAse free sterile water, 5µl of 10x buffer (without $MgCl_2$), 6 µl of $MgCl_2$ buffer, 5 µl of 1.25 mM dNTPs (Macrogen), 2 µl each of 20 µM forward and reverse primers (Macrogen) and 1 µl of 2.5U Ampli- taq Gold polymerase (KEMTaq).

The reaction conditions for 1st PCR were 94 °C for 7 min, 40 cycles of 95 °C for 45 s, 46 °C for 30 s, extension of 72 °C for 120 s and final extension of 72^0C for 5 minutes. The second amplification was done using nested PCR with two primers taking 5µl of first stage PCR amplicon with a similar master mix preparation. The 2nd stage PCR profile was similar as that of the 1st stage with the only exception being the annealing temperature increased from 46^0C to 50^0C. The final PCR products a 681 bp were viewed following electrophoresis on a 2 % agarose gel.

Table 3 2. Primers for amplification of HBV

Primer name	Primer sequence (5'-3')	Primer position
S1(LLf)	TCCTGCTGGTGGCTCCAG	55-72
S1(LLr)	CGTTGACATACTTTCCAATCAA	995-974
S2n (LLf)	ACCCTGYRCCGAACATGGA	141-159
S2n (LLr)	CAACTCCCAATTACATARCCCA	899-878

3.8.4. Sequencing and phylogenetic analysis

Hepatitis B virus DNA PCR amplified products were shipped and sequenced at Macrogen Inc. Hepatitis B virus genotypes was determined by direct sequencing followed by phylogenetic analyses of, respectively, a 681 bp-long DNA fragment in the HBs region. Directly amplified sequences were assembled using DNA sequence analysis software (GENETYX software v 9.0). HBV sequences together with reference sequences were aligned and edited using ClustalX v2.0.1 software (Thompson, Higgins, and Gibson, 1994). Phylogenetic was constructed using the neighbour-joining method (Saitou and Nei, 1987) using the Kimura 2-parameter + γ substitution model as the most appropriate model by MEGA v 7.0.21. To confirm the reliability of phylogenetic tree topologies,

bootstrap reconstruction was carried out 1,000 times. The phylogenetic tree was edited using INKSCAPE software.

3.9. Data processing and analysis

3.9.1 Data management

All laboratory worksheets were double checked, numbered and kept safely in a cabinet within the Immunoserology laboratory at NHL. Data was coded and entered into SPSS v 21 software. Back up files were stored in flash discs.

3.9.2. Data Analysis

The questionnaires were entered using CSPro version 6.0 (census and survey processing system) software package. All questionnaires were entered twice; that is 100% verification was done to eliminate keying error during entry. Then the data was exported to SPSS (PASW version 21.0) for analysis. All data derived from the laboratory examinations were entered using the statistical package SPSS (PASW version 21.0). Data derived from the questionnaire and laboratory testing were analyzed for statistical significance. As the sample was not allocated in proportion to the distribution of health facilities providing ANC services within each zoba, the sentinel surveillance is not self-weighting. As a consequence a normalized design weights were introduced to compensate for over and under sampling to produce representative results at the zoba and national level. Descriptive statistics such as proportions was used to summarize categorical variables while measures of central tendency such as mean, SD, median and ranges for continuous variables. Pearson's Chi-square tests were used to measure the strength of association. The threshold for statistical significance was set at $p<0.05$.

Out of the two anti-HBV seromarkers used in the current study, the regression analysis used the results for total anti-HBc seromarker as the dependent variable. The main reason behind this decision was, as the total anti-HBc seromarker detects both IgM and IgG antibodies to HBV it captures both current and past infections, which could provide a more comprehensive HBV status of the study subjects.

3.10. Quality Control

In the primary survey a five days intensive training was offered to HIV focal persons from each zoba and the fieldwork staff comprising of one nurse, one lab technician and head of the health facility from each site. Standard operating manual was prepared covering areas concerning how to fill the questionnaires, sample collection, separation and storage, testing and biosafety. Standardized forms and formats were used throughout the survey. Intense supervision visits were carried out during the fieldwork period to enhance the collection of quality and complete information for the study. The zoba HIV focal persons had made two preparatory visits and twice monthly supervisory visits to all sites.

In this study for the laboratory analysis and testing SOPs was prepared and followed by the principal investigator and laboratory assistants. All reagents and test kits used in the study were handled and stored according to the manufacturer's recommendations. Freezers used for storing samples at -80^0c and $2\text{-}8^0$c refrigerators used to thaw samples and store test kits were monitored twice daily and temperatures are logged into a temperature chart. All instruments used in this study as necessary were checked for validation and service checks including preventative maintenance before use. While performing serological tests and PCR appropriate quality control procedure and QC materials were used based on manufacturer's recommendations.

CHAPTER FOUR

RESULT

4.1.Socio-demographic characteristics of study subjects

The zonal distribution of the study subjects revealed Gash Barka and Debub to have the highest number of participants with 36.6% (1831) and 29.1% (1279) women respectively. Zoba Southern Red Sea contributed the least number of study participants with 0.9% (43). The findings on the usual place of residence indicates that 50.4% (2527) of study participants were from rural areas while 49.6% (2482) were from urban areas (Table 4.1).

Table 4 1. Socio-demographic characteristics of ANC attendee and seroprevlance of anti-HBV markers

Socio-demographic Characteristics	Overall	Anti-HBc**		Anti-HBs***	
	N (%)*	Positive N (%)	95% CI	Positive N (%)	95% CI
Regions (Zoba)					
Maekel	824 (16.4)	130 (15.5)	13.3-17.7	96 (11.3)	9.4-13.2
Debub	1279 (25.5)	369 (29.1)	26.0-32.1	206 (16.1)	13.7-18.6
Anseba	367 (7.3)	90 (24.8)	21.3-28.3	51 (14.1)	11.3-16.9
Gash Barka	1831 (36.6)	464 (26.2)	23.1-29.3	245 (13.9)	11.4-16.3
Northern Red Sea	665 (13.3)	177 (27.1)	23.1-31.1	97 (15)	11.8-18.1
Southern Red Sea	43 (0.9)	10 (24.5)	18.7-30.4	11 (25.5)	19.6-31.3
Setting					
Urban	2482 (49.6)	540 (21.2)	19.7-22.7	326 (13)	11.8-14.2
Rural	2527 (50.4)	701 (28.5)	25.8-31.2	380 (15.4)	13.3-17.5
Age Group					
15-19	509 (10.2)	105 (20.4)	15.6-25.2	56 (11.1)	7.9-13.8
20-24	1329 (26.6)	292 (21.7)	18.9-24.4	177 (13.2)	10.9-15.5
25-29	1544 (30.8)	378 (24.3)	21.6-26.9	234 (15.1)	12.9-17.3
≥ 30	1623 (32.4)	466 (28.7)	26.0-31.3	239 (15.6)	12.8-16.9
Marital Status					

Married	4638 (92.6)	1094 (24.1)	22.9-25.4	645 (14.2)	13.2-15.2
Single	255 (5.1)	64 (22)	17.2-26.8	44 (15.2)	11.1-19.4
Living with partner	93 (1.9)	15 (17.6)	9.4-25.9	14 (16.5)	8.4-24.5
Divorced/Widowed	17 (0.3)	7 (46.7)	18.1-75.3	2 (13.3)	6.2-32.8
Occupation					
House wife	4428 (88.4)	1047 (24.3)	23.1-25.6	620 (14.4)	13.3-15.4
Government employed	211 (4.2)	40 (16.4)	11.7-21.1	28 (11.6)	7.5-15.6
Private /self employed	262 (5.2)	75 (26.6)	21.4-31.8	41 (14.6)	10.4-18.7
Unemployed	100 (2)	16 (16.2)	8.8-23.5	16 (16.2)	8.8-23.5
Education					
Illiterate	1204 (24)	345 (29.5)	26.8-32.1	188 (16)	13.9-18.1
Primary	1183 (23.6)	331 (28.4)	25.8-31.0	185 (15.9)	13.8-18.0
Junior	1146 (22.9)	260 (23)	20.6-25.5	162 (14.3)	12.3-16.4
Secondary or higher	1476 (29.5)	244 (16.6)	14.7-18.5	170 (11.6)	9.9-13.2
Ethnicity					
Hidarb	26 (0.5)	4 (16.7)	0.6-32.7	3 (12.5)	0.0-26.8
Blen	156 (3.1)	35 (22.9)	16.1-29.6	13 (8.4)	4.0-12.9
Tigrigna	2880 (57.5)	654 (22.9)	21.3-24.4	409 (14.3)	13.0-15.6
Tigre	1212 (24.2)	264 (22.2)	19.9-24.6	134 (11.3)	9.5-13.1
Nara	187 (3.7)	49 (27.8)	21.2-34.5	22 (12.4)	7.5-17.3
Saho	165 (3.3)	48 (30)	22.8-37.2	33 (13.8)	8.3-19.0
Rashida	65 (1.3)	20 (30.8)	19.2-42.3	20 (30.8)	19.2-42.3
Afar	198 (4)	64 (33.3)	26.6-40.1	53 (27.2)	20.9-33.5
Kunama	104 (2.1)	41 (41)	31.2-50.8	29 (29)	20.0-38.0
Religion					
Christian	2802 (55.9)	647 (23.3)	21.7-24.9	400 (14.4)	13.1-15.7
Muslim	2194 (43.8)	530 (24.8)	22.9-26.6	302 (14.1)	12.6-15.5

*= each socio-demographic characteristics could have missing data, **=86 missing cases, ***= 85 missing cases, 95% CI = 95% confidence interval

The mean age of the study subjects was 26.7 ± 5.88 years with ranges from 15 to 48 years. The highest number of participants 32.4% (1623) and 30.8% (1544) were obtained from the age groups 30 and above and 25-29 years. More than ninety percent

of the respondents 4638 were married with 5.1% (255) being single mothers. Likewise with regards to occupation majority of the women were house wives 88.4% (4428) and the others being government or private employed or unemployed. With regards to educational level almost more than 70% of the women had some level of formal education ranging from primary to tertiary levels. More than half of the research participants were from the Tigrigna ethnic group and Christian by faith, 57.5% (2880%) and 55.9% (2802) respectively as shown in Table 4.1 above.

4.2.Obstetrics history indicators of study subjects

The study also tried to shade some lights on the obstetrics history of the women by looking into gravidity and parity. The mean gravida among the pregnant women was 3.4 ±2.2 with previous pregnancies ranging from 1 to 14. Majority of the women were multigravida 78.5% (3933). The mean parity among the ANC attendee was 2.3 ± 2.1 ranging from first pregnancy to 13 previous full term pregnancies. Accordingly 56.6% (2835) of the ANC attendee were multiparous with the remaining 23.1% (1159) and 20.3% (1015) being nulliparous and primiparous respectively (Table 4.2).

Table 4 2. Obstetric indicators of women and seroprevalence of anti-HBc and anti-HBs markers

Obstetrics indicators		Anti-HBc**		Anti-HBs***	
		Positive		Positive	
	N (%)*	N (%)	95% CI	N (%)	95% CI
Gravidity					
Primigravida	1076 (21.5)	182 (17.1)	14.8-19.4	121 (11.4)	9.5-13.3
Multigravida	3933 (78.5)	999 (25.8)	24.4-27.2	584 (15.1)	14.0-16.2
Parity					
Nulliparous	1159 (23.1)	192 (16.7)	14.6-18.9	131 (11.4)	9.6-13.3
Primiparous	1015 (20.3)	224 (22.4)	19.8-25.0	142 (15.5)	12.0-16.3
Multiparous	2835 (56.6)	765 (27.5)	25.7-29.1	432 (15.5)	14.2-16.8

*= each indicator could have missing data, **=86 missing cases, ***= 85 missing cases, 95% CI = 95% confidence interval

4.3.Seroprevalence of anti-HBc and anti-HBs markers

A total of 4923 samples were tested for anti-HBc total and anti-HBs antibody markers with 1.7% (86) samples being not tested for either of the seromarkers. Out of the 86 samples which were not tested 20 were lost samples, 54 were quantity not sufficient samples and 12 were unfit samples for analysis (grossly hemolyzed and lipeamic). The overall prevalence of total anti-hepatitis B core and anti-hepatitis B surface antibodies markers were 25.8% (95% CI: 24.6-27.0) and 14.2% (95% CI: 13.2-15.1) respectively. By collating the findings of the current study on anti-HBV seromarkers with findings from the primary study on HBsAg revealed that approximately 30% of the study participants were positive for one or more of HBV seromarkers. And based on this the women were grouped into five categories; 3.2% (163) HBsAg positive, 13.9% (661) were isolated anti-HBc, 5.3% (273) were isolated anti-HBs, 8.9% (420) both anti-HBc and anti-HBs positive whereas the remaining 69.5% (3495) of the 5009 were negative for any HBV seromarkers (Table 4.3.).

Table 4 3. Prevalence of hepatitis B seromarkers detected in pregnant women in Eritrea.

Serological markers	N*	%	95% CI
HBsAg	163	3.2	2.5-3.8
Anti-HBc	1241	25.8	24.6-27.0
Anti-HBs	706	14.2	13.2-15.1
HBeAg	7	3.9	1.0-6.8
Anti-HBe	35	17.4	11.1-23.6
Isolated anti-HBc[&]	661	13.9	13.0-14.8
Isolated anti-HBs[&]	273	5.3	4.7-5.9
Dual anti-HBc and anti-HBs	420	8.9	8.1-9.6
Overall exposed to HBV	1525	30.5	29.5-32.0

*= each marker could have missing data, 95% CI = 95% confidence interval,
[&] = isolated means either positive for anti-HBc or anti-HBs but negative for all other seromarker

4.3.1. Socio-demographic characteristics in relation to anti-HBc and anti-HBs prevalence

The study also tried to assess the prevalence of anti-HBc and anti-HBs antibody seromarkers by socio-demographic characteristics. Looking into regional distribution based on place of residence by regions, the highest prevalence of total anti-HBc antibody was reported in zoba Debub and Northern Red Sea with a prevalence of 29.1% (369) and 27.1% (177) respectively. With regards to seroprevalence of anti-HBs marker the highest prevalence was reported in zoba Southern Red Sea with a prevalence of 25.5% (11) and the lowest in zoba Maekel with a prevalence of 11.3% (96). The prevalence of both anti-HBc and anti-HBs seromarkers was higher in rural settings with a prevalence of 28.5% (701) and 15.4% (380) respectively.

Age specific anti-HBc and anti-HBs markers seroprevalence vary among the different age groups and it increases with an increase in the age of participants with age group 30 or above having the highest prevalence for both markers 28.7% (466) and 15.6% (239) respectively (Figure 4.1).

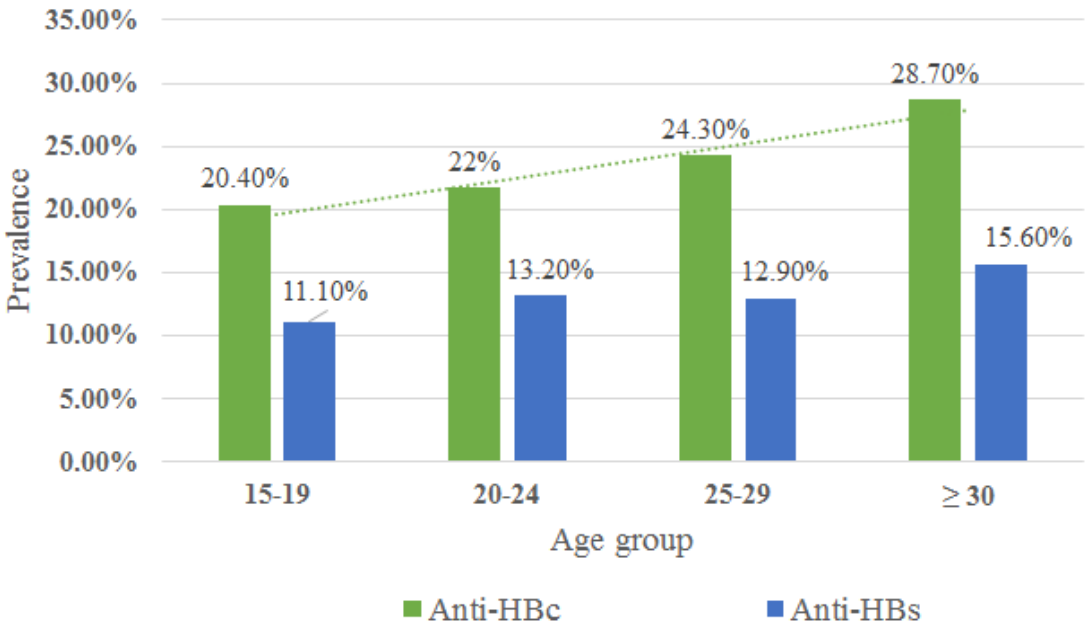

Figure 4 1. Seroprevalence of anti-HBc and anti-HBs by age groups
The prevalence of anti-HBc and anti-HBs seromarker shows an increase with increasing age with age group ≥ 30 years presenting a higher HBV exposure and anti-HBs rate.

Divorced or widowed women were reported to be more exposed to HBV infection as evidenced by the higher seroprevalence of anti-HBc markers which was 46.7% (7). Whereas for anti-HBs seromarkers women living with partners and single women had the highest prevalence with 16.5% (14) and 15.2% (44) respectively. The seroprevalence of anti-HBc was high among privately or self-employed women 26.6% (75) whereas seroprevalence of anti-HBs was higher among unemployed women 16.2% (16). The results of the study demonstrated that illiterate women had the highest prevalence for both seromarkers with 29.5% (345) for anti-HBc and 16% (188) for anti-HBs respectively. Findings of the study revealed that educational level has an inverse relation with prevalence of HBV seromarkers indicated by the fact that as the educational level of the study participants increased the prevalence of HBV seromarkers decrease and vise-versa.

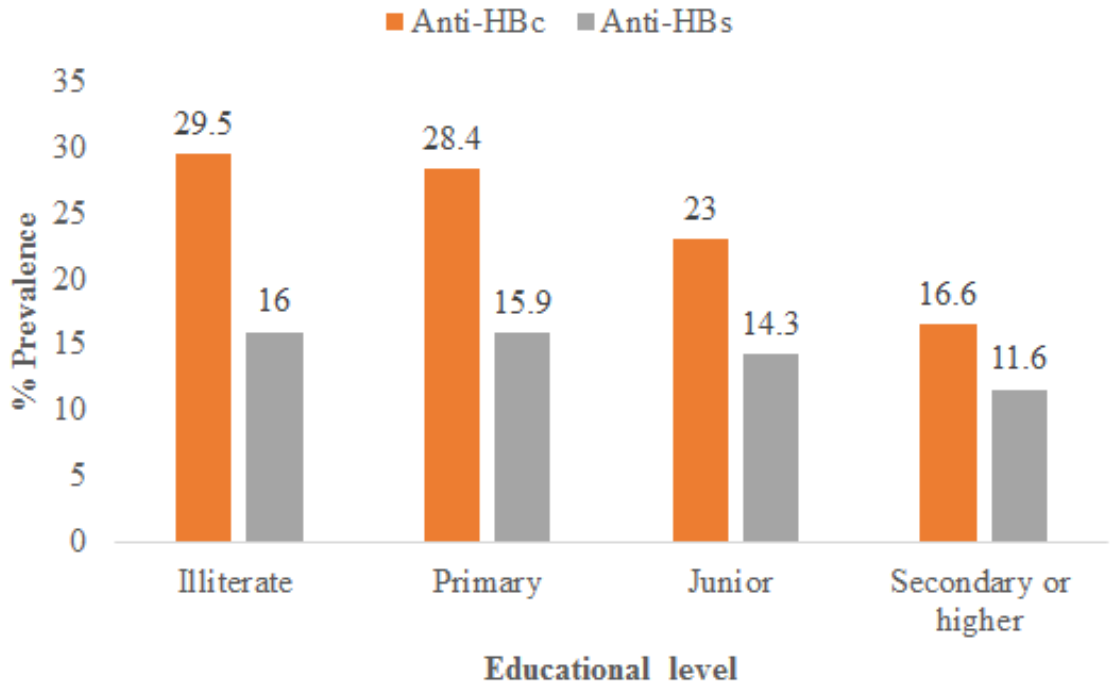

Figure 4 2. Prevalence of anti-HBV markers by educational level

An inverse relation is observed with the illiterate group presenting a higher prevalence of anti-HBV seromarkers compared to the group with secondary education or higher.

The study also assessed the seroprevalence of both markers by ethnic background of the study participants. The two major ethnic groups in Eritrea the Tigrigna and Tigre had a lower prevalence when compared to the other seven ethnic groups grouped under

"others". As presented in Figure 4.3 out of the seven ethnic groups categorized under "others" almost five of them had higher prevalence of anti-HBc and anti-HBs. The highest prevalence for anti-HBc was reported among the Kunama 41% followed by Afar 33.3%; while with regards to anti-HBs the highest prevalence was reported among the Rashida 30.8% and Kunama 29%. The prevalence of anti-HBc was higher among pregnant women reported to be Muslims (24.4%) by faith but the anti-HBs rate was higher among pregnant women who were Christian (14.4%) by faith.

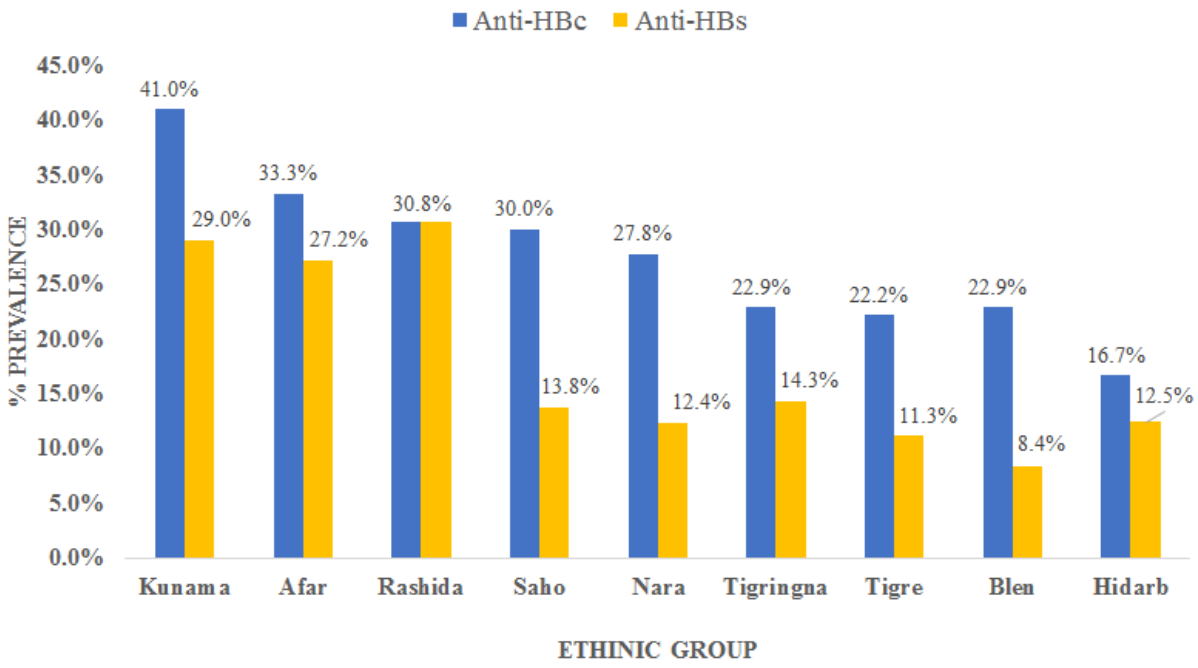

Figure 4 3. Prevalence of anti-HBc and anti-HBs among ethnic groups

The figure illustrates HBV exposure and anti-HBs rates among the nine ethnic groups of Eritrea. The HBV exposure rate particularly was higher among the five ethnic groups from the Eastern and Western low lands of Eritrea.

4.3.2. Obstetrics history indicators in relation to anti-HBc and anti-HBs prevalence

The results of the study showed that women with one or more previous pregnancies (multigravida) had the highest prevalence of anti-HBc and anti-HBs with 25.8% (999) and 15.1% (584) respectively. Likewise the prevalence of both seromarkers was higher among multiparous women with a reported rate of 27.5% (765) and 15.5% (432) for anti-HBc and anti-HBs respectively.

4.4. Association of socio-demographic characteristics and obstetric history indicators in relation to anti-HBc positivity

The study tried to establish association of positivity for anti-HBc antibody seromarker and socio-demographic and obstetric indicators of study participants. Based on regional prevalence of anti-HBc antibody both univariate and multivariate analysis revealed a significant association between anti-HBc positivity and zoba of residence (aOR= 1.84: p < 0.001). Similarly place of residence by urban/rural settings showed a significant association with anti-HBc positivity in the univariate logistic analysis (cOR=1.31: p=< 0.001) but further analysis indicated a non-significant association with a p-value of 0.57. The risk of HBV infection, though not clearly significant in the multivariate analysis was higher within the age groups 30 years or above (aOR=1.38: p=0.055) with a prevalence of 28.7% compared to other groups.

Table 4 4. Binary logistic regression analyses of background characteristics with anti-HBc positivity

Characteristics	cOR (95%CI)	p-value	aOR(95%CI)	p-value
Zoba				
Maekel	reference		reference	
Debub	2.03 (1.64-2.50)	<0.001	1.84 (1.47-2.31)	<0.001
Anseba	1.59 (1.26-2.01)	<0.001	1.46 (1.11-1.94)	0.007
Gash Barka	2.06 (1.70-2.50)	<0.001	1.80 (1.43-2.27)	<0.001
Semenawi Keiyh Bahri	1.79(1.41-2.27)	<0.001	1.67 (1.26-2.22)	<0.001
Debubawi Keiyh Bahri	1.70 (1.20-2.42)	0.003	1.16 (0.99-1.35)	0.071
Setting				
Urban	reference		reference	
Rural	1.31 (1.14-1.50)	<0.001	1.16 (0.99-1.35)	0.057
Age Group				
15-19	reference		reference	
20-24	1.15 (0.874-1.51)	0.316	1.14 (0.849-1.55)	0.367
25-29	1.32 (1.00-1.72)	0.043	1.19 (0.872-1.64)	0.265
≥ 30	1.69 (1.30-2.21)	<0.001	1.38 (0.994-1.93)	0.055
Marital Status				

Living with partner	reference		reference	
Married	1.48 (0.84-2.60)	0.168	0.541 (0.188-1.55)	0.254
Single	1.32 (0.70-2.45)	0.388	0.586 (0.199-1.72)	0.332
Divorced/Widowed	4.08 (1.28-12.99)	0.017	0.401 (0.122-1.32)	0.133
Occupation				
Unemployed	reference		reference	
House wife	1.67 (0.97-2.86)	0.062	1.24 (0.856-1.79)	0.254
Government employed	1.01 (0.54-1.91)	0.958	0.903 (0.456-1.78)	0.769
Private /self employed	1.88 (1.03-3.41)	0.038	1.55 (0.989-2.43)	0.056
Education				
Secondary/higher	reference		reference	
Illiterate	2.08 (1.73-2.51)	<0.001	1.50 (1.18-1.92)	0.001
Primary	1.97 (1.64-2.38)	<0.001	1.53 (1.23-1.91)	0.001
Junior	1.49 (1.23-1.81)	<0.001	1.31 (1.06-1.61)	0.009
Ethnicity				
Tigre	reference		reference	
Tigrigna	1.04 (0.88-1.22)	0.638	1.26 (0.892-1.77)	0.191
Others	1.47 (1.20-1.79)	<0.001	1.46 (1.17-1.82)	0.001
Religion				
Christian	reference		reference	
Muslim	1.08 (0.94 -1.23)	0.242	0.90 (0.67-1.20)	0.480
Gravidity				
Primigravida	reference		reference	
Multigravida	1.68 (1.41-2.01)	<0.001	0.809 (0.472-1.38)	0.441
Parity				
Nulliparous	reference		reference	
Primiparous	1.43 (1.15-1.77)	<0.001	1.55 (0.910-2.66)	0.106
Multiparous	1.88 (1.58-2.24)	<0.001	1.72 (1.01-2.92)	0.046

Although in the univariate analysis marital status, occupation and gravidity showed a statistically significant association ($p < 0.05$) with HBV exposure further analysis

using a multivariate analysis revealed a non-significant association ($p > 0.05$). There was however a significant relationship between the rate of anti-HBc positivity and level of education both in the univariate and multivariate analysis. Those who were categorized in the study as “illiterate” were significantly exposed to HBV infection compared to those who had attained secondary or higher education (aOR=1.50: p=0.001). Likewise ethnicity showed a significant association for “other” ethnic groups (aOR= 1.47: p= 0.001), but it worth noting that there was no significant association between the two main ethnic groups of Tigrigna and Tigre. Similar significant association between anti-HBc positivity and the obstetric indicator parity was also observed. The independent variable “religion” showed no significant association with anti-HBc positivity in both univariate and multivariate logistic regression analysis.

4.5. HBV genotyping and phylogenetic analysis

A total of 151 HBsAg samples were targeted for DNA extraction and PCR out of which 120 samples having sufficient volume were further processed. DNA was extracted successfully from the 120 samples and subsequently subjected to nested PCR with primers targeting the surface antigen coding region of the HBV DNA. When amplified with S-2 primers, a 681 bp (nt 155-835) of amplified PCR product was revealed on agarose gel electrophoresis for 68 of the samples and the rest 52 samples could not amplify (Figure 4 4.).

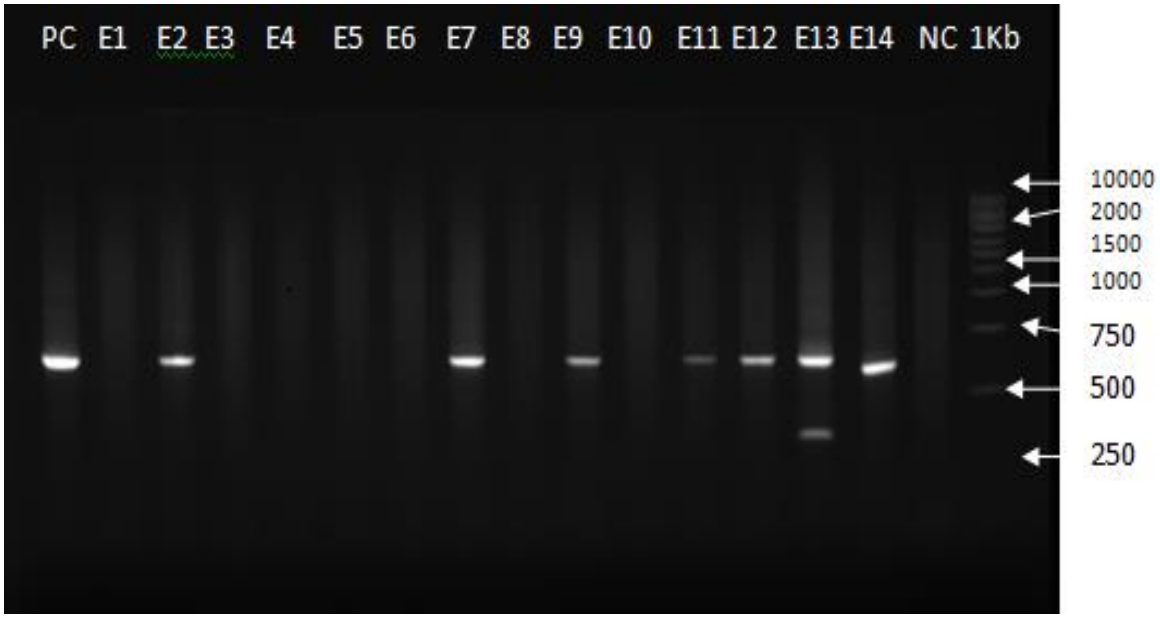

Figure 4 4. A photograph of 2% agarose gel, containing PCR product of HBV S gene

The 681bp amplified PCR product is shown between the 500bp and 750bp. **Abbreviations** NC= negative control, PC= positive control, E= Eritrean samples.

The sixty eight amplified samples were sequenced with 42 samples being successfully sequenced and the remaining 26 samples either no sequence was obtained or contains much interference. The zoba distribution of successfully sequenced samples indicate that 10 samples were from zoba Gash Barka, 8 samples from zoba Northern Red Sea,7 samples from zoba Anseba, 6 samples each from zoba Maekel and Southern Red Seaand the remaining 5 samples from zoba Debub (Table 4.5).

Table 4 5. Zonal distribution of HBV genotypes in Eritrea

Zoba	PCR amplified	Sequenced	Genotypes			
			A1	D		E
				D2	D10	
Maekel	22	10	4	2		
Debub	15	9	4	1		
Anseba	14	10	7			
Gash Barka	41	20	6	3		1
Northern Red Sea	15	10	8			
Southern Red Sea	13	9	3	2	1	

The 42 HBV surface antigen coding genome sequences were phylogenetically analysed with HBV reference sequences of genotypes A-H retrieved from the GenBank database. Phylogenetically the genotype distribution in the 42 sequences was 77.8% (32) genotype A: 20% (9) genotype D and 2.2% (1) genotype E (Figure 4.4). All the 32 genotype A strains were further classified into subgenotype A1, but the strains were distinct and they loosely clustered with the reference sequences separating into two clades. Thirty strains fall in the Asian A1 clade alongside sequences from neighbouring African countries Ethiopia, Sudan, Somalia and also sequences from Asian countries United Arab Emirates, Philippines and Bangladesh. The remaining two strains i.e. ERSK54 and ERAN66 clustered in the African A1 clade with sequences from Congo, Rwanda and Haiti.

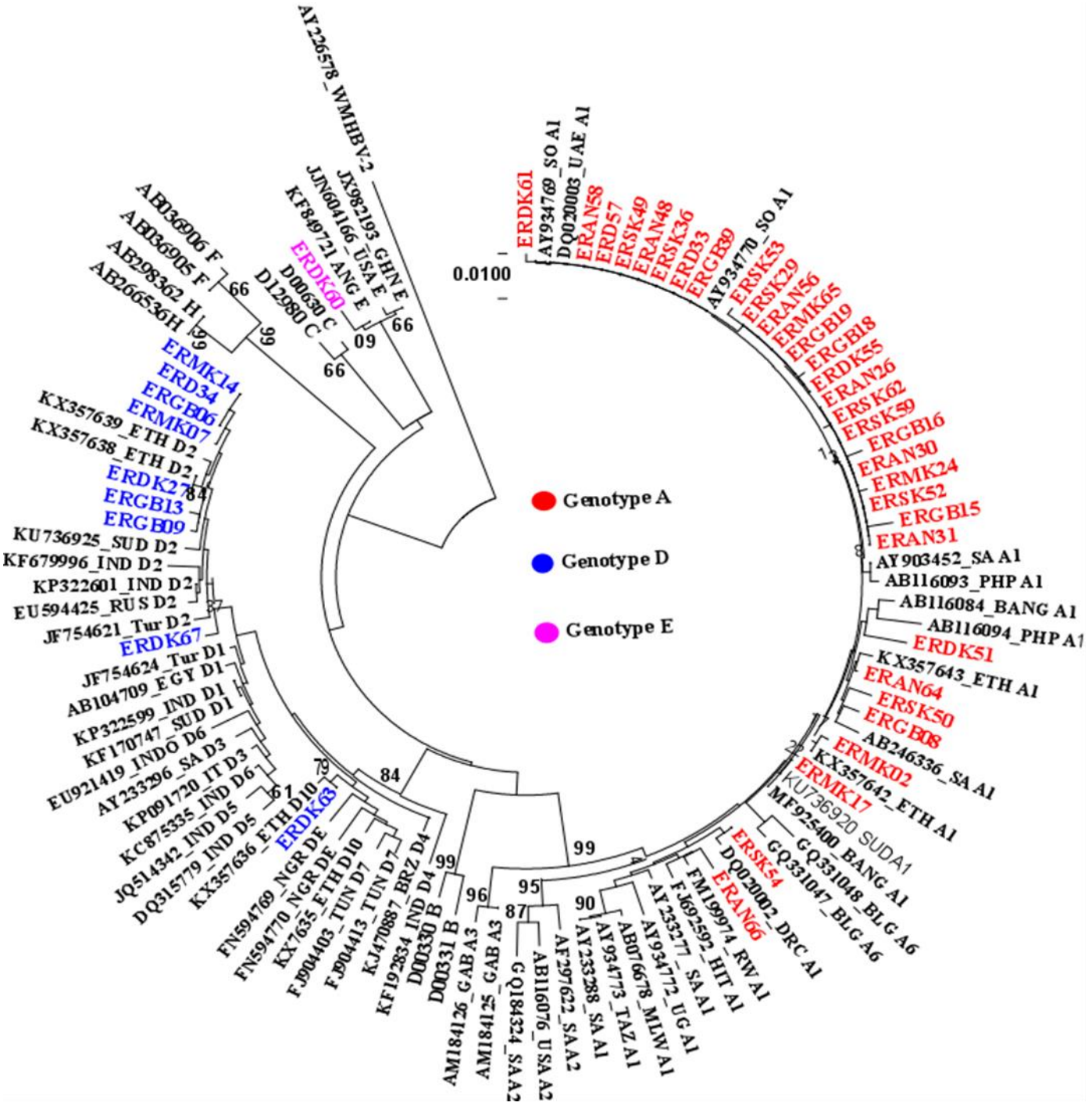

Figure 4 5. Phylogenetic tree constructed by the neighbour-joining method
Based on the surface antigen coding region of HBV genome. Sequences from Eritrea are colour coded based on genotypes. The reference sequences used in this comparison are indicated by their accession numbers, followed by the country of origin and letter of the corresponding genotype/subgenotype. **Abbreviations**: ER= Eritrea, D= Debub, GB= Gash Barka, AN= Anseba, MK= Maekel, SK= Northern Red Sea and DK= Southern Red Sea.

Twenty five of the Eritrean A1 strains of the Asian clade formed two distinct groups; in the first group nine strains clustered with sequences from Somali (accession nos. AY934769 and AY934770) and United Arab Emirates (accession no. DQ020003) (Hannoun *et al*., 2005). In the second group 16 strains clustered apart from the subgenotype A1 reference sequences from the GenBank forming a distinct cluster. A single strain ERDK51 formed a cluster with two reference sequences from Philippines (accession no. AB116094) (Sugiyama *et al*., 2006) and Bangladesh (accession no. AB116084) (Sugiyama *et al*., 2006) and another three strains ERAN64, ERSK50, ERGB08 clustered with a reference subgenotype A1 sequences from Ethiopia (accession no. KX357643) (Hundie *et al*., 2016) and South Africa (accession no. AB246336) (Sugiyama *et al*., 2006). Another sequence ERMK02 formed a sub-cluster with reference sequences from Ethiopia (accession no. KX357642) (Hundie *et al*., 2016) and another sequence ERMK 17 clustered with reference sequences from Sudan (KU736920) (unpublished) and Bangladesh (accession no. MF 925400) (Munshi *et al*., 2017).

The phylogenetical analysis indicated that most of the D strains from Eritrea belonged to D2 (89%, 8/9). Out of the eight D2 strains isolated in this study four strains ERMK14, ERD34, ERGB06 and ERMK07 formed a distinct sub-cluster apart from the D2 reference sequences form the GenBank. Three D2 strains from Eritrea clustered with D2 sequences from Ethiopia with a single strain ERDK27 clustering with a reference sequence from Ethiopia (accession no. KX357638) (Hundie *et al.*, 2016) supported with a boot strap value of 84% but the remaining two strains ERGB13 and ERGB09 clustered more distantly away from the reference sequences. A single D2 strain ERDK 67 clustered with a reference sequences from Europe particularly Turkey (accession no. JF754621) (Cox, Arslan, and Allain, 2011) with a high boot strap value of 87%. A single strain of D (ERDK 63) clustered with D10 from Ethiopia (accession no. KX357636) with a boot strap value of 84% (Hundie *et al*., 2016). The only genotype E sequence identified in the phylogenetic analysis clustered with genotype E sequences from Angola (accession no. KF849721) (Lago *et al*., 2014), Ghana (accession no. JX982193) (Freimanis, Owusu-Ofori, and Allain, 2012) and USA (accession no. JN 604166) (Delwart *et al*., 2012) and it specifically formed a sub-cluster with the strain from Angola with boot strap value of 60%.

The zoba distribution of the genotypes revealed that genotype A subgenotype A1 circulates in all the six regions with 94% (30) of the strains belonging to the Asian A1 clade (Table 4.5). The remaining 6% (2) of subgenotype A1 strains from zoba Anseba and Northern Red Seabelong to the African A1 clade. The geographical distribution of genotype D revealed that it circulates in four regions namely; Maekel, Gash Barka, Debub and Debubawi Keiyh Bahri. Majority of the circulating D genotypes identified from the four regions belong to D2 (8/9) and a single isolate from zoba Southern Red Seabelonged to the novel subgenotype D10. The only genotype E strain isolated was reported from zoba Gash Barka.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

This is the first major study of its kind in determining the extent of HBV exposure among antenatal care attendee in Eritrea. In as much as HBsAg is the commonly targeted seromarker in prevalence studies in Eritrea and other neighbouring countries there is a concrete lack of data on other seromarkers including anti-HBc and anti-HBs which are important in appreciating the extent of HBV exposure and level of immunity against HBV. This is particularly important among women of reproductive age as it could have an implication in understanding underlying risks that could possibly lead to mother to child transmission (MTCT).

The overall prevalence of total anti-HBc and anti-HBs seromarkers among the study subjects was 25.8% (1241) and 14.2% (706) respectively. Collating the result generated by this research to the 3.2% HBsAg prevalence reported in the primary study the ANC attendee were classified into five different categories. The ANC attendee with an active HBV infection whether acute or chronic represent 3.2% (163) of the 5009 study participants (CDC, 2005). Four hundred twenty (8.9%) of the women were positive for both anti-HBc and anti-HBs antibodies indicating immunity due to natural infection (CDC, 2005). Anti-HBs antibody isolated detection possibly due to HBV vaccination was reported among 5.3% (273) of the ANC attendee (CDC, 2005). Isolated anti-HBc was reported among the 13.9% (661) of the pregnant women which most commonly implicate a resolved infection with undetectable anti-HBs antibody titter. However, the isolated anti-HBc could also mean among others chronic infections or resolving acute infections. Majority of the women 69.5% (3495) were negative for any of the three seromarkers indicating that they are susceptible to HBV infection.

In the current study almost one third of the study participants (30.5%) were positive for one or more HBV seromarkers indicating a substantial exposure to HBV infection in Eritrea. But this figure is very low to what is reported in most sub-Saharan African countries for instance Angounda *et al* in a study conducted in Congo, Brazzaville reported 78.3% of pregnant women had at least one HBV seromarker (Angounda *et*

al., 2016). Reports from sub-Saharan countries indicate that HBV infection is mainly transmitted horizontally during early childhood, but contrary to this, the findings of the current study demonstrates that a large pool of women are not exposed to HBV infection. The latter assertion makes these group of women vulnerable to HBV infection through other routes including sexual route. Taking this into consideration the low overall HBV exposure reported might be due to, among others the successes of the ongoing campaigns undertaken to prevent HIV infection (with a prevalence of ≤ 1%) (EPHS, 2010) which shares common risk factors including use of contaminated sharps, blood transfusion and unsafe sex. Therefore in the Eritrean context the introduction and expansion of PMTCT, improved knowledge and change of sexual behaviors associated with the decreased HIV trend might have contributed to lower the HBV exposure rate among the ANC attendee (EPHS, 2010; Teclebirhan *et al*., 2009). Furthermore, reports from the African region have shown that low prevalence rates are to some extent associated with population density (Pirillo *et al*., 2007). Eritrea with a population of 5 million is sparsely populated compared to its highly populated neighboring countries. This could also contribute to the low HBV prevalence reported in this study.

Exposure to HBV as evidenced by the seroprevalence of anti-HBc seromarker among the study participants was 25.8%. This finding is much lower when compared to what is usually reported in most sub-Saharan African countries; Congo 65.7% (Angounda *et al*., 2016), 81.6% in Cote d'Ivoire (Combe *et al*., 2001), 61% in Zimbabwe (Madzime *et al*., 1999) and 41% in Cameroon (Fomulu *et al*., 2013). Even though the anti-HBc rate in this study is low, the regional exposure rates differed with some sites reporting rates as low as 15.5% and some regions recording rates as high as 29.1%. Similar pattern in HBV exposure and carriage rates are reported in many African countries with underlying causes associated with among other things differences in behavioral, socio-economic, socio-cultural practices and viral characteristics (Bwogi *et al*., 2009; Kew *et al*., 2005; Kew., 1996). In developed countries much lower results were reported with 5% in Spain (Salleras *et al*., 2009), 7.1% in Switzerland (Bart *et al*., 1996), and 13.4% in France (Descos *et al*., 1987).

In the current study the anti-HBs antibody prevalence was 14.2% but higher results were reported in Congo 22% (Angounda *et al*., 2016) and Kenya 30.2% (Okoth *et al.*, 2006). In this study 5.3% of the women have ever been vaccinated as indicated by the isolated anti-HBs marker prevalence, this seems a large proportion given HBV childhood vaccination was introduced in the country in 2002. However, as an emerging new nation the Eritrean population had a significant number returning refugees from neighbouring and other countries (EPHS, 2010) including countries with established HBV vaccination programs for children and adults which could have contributed to this effect. Nonetheless, considering the lack of provisions of HBV vaccine for adult in the country including pregnant women and the low vaccine induced anti-HBs prevalence coupled with the reported 69.5% of the women being susceptibility to HBV clearly demonstrates the risk of HBV infection in Eritrea.

In this study total anti-HBc antibody was measured making it difficult to differentiate past and current HBV infections among the pregnant women. However, one important observation among women positive for HBsAg was only 5% of the women seroconvert nonetheless 96% were positive for anti-HBc only which might indicate active virus replication. This might indicate presence of an increased chance of mother to child transmission among those group of pregnant women.

The regional prevalence of both anti-HBc and anti-HBs seromarkers showed variations among the six regions. The highest prevalence of anti-HBc seromarker was reported in Debub 29.1% and Northern Red Sea27.1% and the lowest prevalence was reported in zoba Maekel 15.5%. Regarding anti-HBs seromarker the highest prevalence was reported in zoba Southern Red Sea25.5% and the lowest prevalence once again was reported for zoba Maekel 11.3%. Even though in the African region there are limited studies conducted using these two seromarkers and scarcity of comparable data many researches done targeting the HBsAg seromarker had shown similar variations in HBV prevalence. For instance a study conducted by Okoth *et al* reported a varied HBV prevalence ranging from 4.3-13.4% for the different regions of Kenya (Okoth *et al.*, 2006). In addition a number of reports by different authors in various regions of Ethiopia reported a varied HBV prevalence with lowest (3.8%)

being reported in the central Amhara region (Zenebe *et al*., 2014) and the highest (7.8%) in Southern region (Metaferia *et al*., 2016).

The regional variations in HBV exposure rate was statistically significant with results of the logistic regression analysis indicating that women from zoba Debub or Gash Barka were almost two times more likely to have previous exposer to HBV infection (aOR = 2.06: $p < 0.001$). The differences observed in the regions could be attributed to the socio-economic disparities between those regions. Zoba Maekel which has the lowest HBV exposure rate is located in the central highland of Eritrea. This area has a better infrastructure and socio-economic status as it is much more urbanized with Asmara the nation's capital located at its centre (EPHS, 2010). In contrast the regions with a higher HBV exposure rates are located in the western and eastern lowlands an area characterized by relatively underdeveloped infrastructure and low socio-economic status.

In line with this observation the ethnic groups with a higher HBV exposure rates including Kunama (41%), Afar (33.3%), Rashida (30.8%), Saho (30%), and Nara (27.3%) are almost exclusively found in those regions with higher prevalence. This variation in previous exposure to HBV among the different ethnic group's calls for further investigations taking into account behavioural and cultural practices that could lead to HBV infection. Moreover, the very fact that this ethnic groups are found in areas where there is a relatively dynamic movement of people within Eritrea and outside to neighbouring countries for trade, mining and fishing are important factors to consider.

In the current study the evidence of exposure to HBV and anti-HBs rates were higher among pregnant women from rural areas than those of urban areas. The reported seroprevalence for anti-HBc and anti-HBs markers for women from rural areas was 28.5% and 15.4% respectively which was higher than the 21.2% and 13% reported among pregnant women from urban areas. More frequently many reports from Africa had indicated in rural settings risk of HBV infection is higher as it is influenced among other things by poverty, access to health, poor hygiene and lack of education (Kew., 1996; Ofori-asenso & Agyeman, 2016).

In Eritrea the socio-economic disparities between rural and urban areas is well documented. The 2010 Eritrean population and health survey and the 2014 millennium development goal report indicate that poverty levels are particularly higher in rural areas 72% as compared to urban areas 36% (EPHS, 2010; State of Eritrea, 2014). However, it is unclear to what extent socio-economic conditions could affect the regional variations observed across the country. Similar findings were reported in Uganda, South Africa, Ghana and Yemen in studies conducted among the general public and different population groups (Bwogi *et al*., 2009; Kew., 1996; Ofori-asenso & Agyeman, 2016).

Age specific anti-HBc and anti-HBs seroprevalence showed a steady increase with an increase in age with the highest prevalence being reported among the age groups 30 or above. The evidence of previous exposure to HBV as evidenced by prevalence of anti-HBc seromarker increased from 20.4% in women of age group 15-19 to 28.7% in women of age group 30 years or above. Likewise the prevalence of anti-HBs seromarker frequency also increased from 11.1% in women of age group 15-19 to 15.6% in women of age group 30 years or above. A similar finding was reported in one state of Brazil whereby the seroprevalence of HBV markers increased significantly from 8.7% in women younger than 20 years to 50.7% for women older than 30 years (Bertolini *et al*., 2006). In studies conducted in Ethiopia, Rwanda and Mexico a similar pattern was observed with older women having an increased HBV prevalence (Desalegn *et al*., 2016b; Nyamusi *et al*., 2011; Vázquez-Martínez *et al*., 2003; Zenebe *et al*., 2014). This could be explained by the very fact that cumulative risk of HBV exposure increases with age.

Contrary to these findings, studies conducted in northern Uganda, Nigeria and Cameron reported a higher prevalence of HBV among women younger than 25 years compared to older women (Bayo *et al*., 2014; Fomulu *et al*., 2013; Mbaawuaga *et al*., 2008). These differences in HBV prevalence might indicate that other local confounding factors including behavioural, socio-cultural and socio-economic conditions might contribute to the varied HBV prevalence.

In the current study exposure to HBV infection seems to occur more frequently in illiterate women when compared to educated women. There was an inverse relationship between educational level and prevalence of both seromarkers with the frequency of anti-HBc decreasing from 29.5% in illiterate women to 16.6% in women who have achieved secondary or tertiary education. Likewise the prevalence of anti-HBs seromarker frequency also decreased from 16% in illiterate women to 11.6% in women whose educational level was secondary or above. Similar findings were reported by Seid *et al* (2014) in Ethiopia with women with no formal education had a prevalence rate as high as 25% decreasing to 1.2% among women who had secondary or above education (Seid *et al*., 2014).

The variations observed in HBV exposure by educational level was statistically significant with illiterate women being almost 1.5 times more exposed to HBV infection compared to women who have had secondary or higher education (aOR = 1.50: p = 0.001). In most instances illiteracy rates are influenced by low socio-economic status and poverty which increases the risk of HBV infections. As opposed to the findings in this study other studies from Ethiopia, Uganda, Congo and Cameron reported a non-significant association between educational level and HBV carriage (Angounda *et al.*, 2016; Bayo *et al*., 2014; Desalegn *et al.*, 2016a; 2016; Fomulu *et al*., 2013).

In this series the evidence of exposure to HBV among divorced or widowed women were higher with 46.7% of the women having the anti-HBc seromarker. But when it comes to the seroprevalence of anti-HBs seromarker women living with partners had the highest anti-HBs rate 16.5%. Seid *et al* (2014) in a study conducted in Ethiopia reported a comparable finding where widowed (22.2%), divorced (14.3%) and single (18.8%) women had a higher prevalence of HBV (Seid *et al*., 2014). A similar finding was reported in Mexico whereby unmarried or divorced women had higher prevalence of HBV seromarkers (Vázquez-Martínez *et al.*, 2003). But other studies in Ethiopia reported no difference in prevalence among pregnant women by marital status (Desalegn *et al.*, 2016a; Metaferia *et al*., 2016; Zenebe *et al.*, 2014).

In this series privately or self-employed women (29.9%) and unemployed women (19.6%) were reported to have the highest prevalence for anti-HBc and anti-HBs seromarkers respectively. In a study conducted in Bahir Dar, Ethiopia the highest HBV prevalence was reported among daily labourers (12.5%) and students (11.8%) (Zenebe *et al*., 2014). As this study did not assess the sexual habits of pregnant women, it will not be possible to make any inferences about risk behaviour of women that could expose them to HBV. However, in most instances the low economic status among these women makes them vulnerable to sexual exploitation and they will be exposed to unprotected sex thus making them vulnerable to STIs (Bayo *et al*., 2014). These data are significant because they may signal the need for the development of specific strategies to the guidance of these pregnant women in health services related to prevention, protection and transmission of STIs. Evidence of previous exposure to HBV infection was slightly higher among Muslim women (24.8%) than Christian women (23.3%) as indicated by the anti-HBc seromarker prevalence. Even though there was no statistically significant association observed between HBV exposure and religion of women, it is worth noting that regions with higher HBV exposure rates have a substantial Muslim population.

In the current survey multigravida women had a higher prevalence of anti-HBc and anti-HBs seromarkers with 25.8% and 15.1% respectively. Likewise the seroprevalence of both markers was once again higher among multiparous women with 27.5% for anti-HBc and 15.5% for anti-HBs seromarkers. Multigravida and multiparous women are obviously sexually active which might indicate an increased risk of exposure to HBV infection. Moreover, majority of the multigravida and multiparous women are from the age group 30 years or above which have the highest prevalence for both seromarkers in the current study. This could be explained by the very fact that cumulative risk of HBV exposure increases with age.

However it is not clear how far these exposures contribute to burden of HBV infection in sub-Saharan African countries as most HBV infections are transmitted at early childhood (Kew, 1996, 2008; Kramvis 7 Kew, 2007). Similar findings of higher HBV carriage among expectant women with multiple pregnancies were reported in Ethiopia, Congo and Nigeria (Angounda *et al.*, 2016; Desalegn *et al*., 2016a; Mbaawuaga *et al*.,

2008; Metaferia *et al*., 2016). However, contrary to this higher HBV prevalence rates were reported among nulliparous women 14% in Uganda (Bayo *et al*., 2014). This might indicate other local confounding factors could have a bearing on HBV exposure which was clearly indicated in the Ugandan study with young women being vulnerable to sexually transmitted infections and possible transmission of HBV during childhood as most women live in internally displaced over crowded campus (Bayo *et al*., 2014)

Currently there are ten genotypes of HBV circulating worldwide designated A to J (Zhang *et al*., 2016). Increasing evidence shows that HBV genotypes and subgenotypes display distinct geographic distributions and impact HBV transmission patterns, clinical course and treatment outcomes (Kao, 2002; Mahtab *et al*., 2008; McMahon, 2009; Schaefer, 2005; Zhang *et al*., 2016). To date, HBV genetic diversity and genotype distribution pattern in Eritrea has not been investigated. Accordingly one of the main objective of this study was to determine the molecular epidemiology and genetic diversity of HBV in Eritrea. To this end the study assessed HBsAg positive samples from the 2016 ANC sentinel surveillance to determine the molecular epidemiology of HBV in Eritrea.

In the current study 120 HBsAg seropositive samples were targeted by PCR and 68 sample were positive for nuclear acid testing (NAT). The remaining 52 (43%) samples could not amplify specifically; these were considered to be either false-positives or had very low DNA undetectable by PCR. Furthermore, though the samples were stored at -80^0c, the recurrent blackouts in Eritrea might have affected the integrity of the samples and some samples might have deteriorated significantly losing the HBV DNA.

Out of the 68 NAT positive samples 42 samples were successfully sequenced and phylogenetically analysed. Results of the phylogenetic analysis revealed presence of genotypes A (77.8%), D (20%) and E (2.2%). This is consistent with previous studies of HBV genotype distribution in neighbouring countries and other regions of Africa (Hundie *et al*., 2016a; Hundie *et al*., 2016b; Kramvis and Kew, 2007; Ochwoto *et al*., 2016; Yousif *et al*., 2013). For instance Yousif *et al* reported genotypes A, D and E circulating in Sudan and similar finding were reported in a number of studies conducted in Kenya (Mwangi *et al*., 2008; Ochwoto *et al*., 2016; Yousif *et al*., 2013).

Genotype A was the predominant genotype reported in the current study with almost 78% of the strains, alike most sub-Saharan African countries (Kramvis & Kew, 2007).

Geographically genotype A was the predominant genotype found to circulate in all the six regions of Eritrea and in two regions namely Anseba and Northern Red Seait was the only genotype isolated. Similar pattern of genotype A dominance is reported in neighbouring countries including Ethiopia, Somalia, Kenya and Uganda (Hundie *et al*., 2016a; Kramvis and Kew, 2007; Ochwoto *et al*., 2016). But in other neighbouring countries including Sudan, Yemen and Egypt genotype D is the most prevalent genotype (Khaled *et al*, 2010; Sallam & Tong, 2004; Yousif *et al*., 2013). This places Eritrea at the middle of the genotype A and D geographical distribution in Africa and part of the Middle East.

HBV genotype A is the most widely distributed genotype with at least four subgenotypes designated A1, A2, quasi-subgenotype A3 and A4 circulating throughout the world (Kramvis & Kew, 2007; Pourkarim *et al*., 2011). In Africa subgenotypes A1, A4 and quasi-subgenotype A3 are the prevalent subgenotypes with A2 being predominantly reported from Europe and North America (Pourkarim *et al*., 2011). In this series all genotype A strains isolated belong to subgenotype A1, the main African A subgenotype. This finding once more is in concordance with what is reported among sub-Saharan African countries (Hundie *et al*., 2016a; Kew *et al*., 2005; Kramvis & Kew, 2007; Ochwoto *et al*., 2016). The A1 subgenotypes from Eritrea cluster in both Asian and African clade, with almost 94% of the strains clustering with the Asian clade with sequences from Somalia, Ethiopia, Sudan, UAE, Bangladesh and the Philippines. Nevertheless majority of the subgenotype A1 Asian clade strains from Eritrea clustered separately and distantly from the subgenotype A1 Asian clade reference sequences. This might indicate the Eritrean strains are more distinct thus suggesting a distant link with the reference sequences.

In the current study two strains clustered with subgenotype A1 African clade reference sequences from Congo, Rwanda and Haiti (Hannoun *et al*., 2005; Hubschen *et al*., 2009). This signifies that the Eritrean A1 isolates are more divergent as both the Asian and African subgenotype A1 clades circulate within the country. Similar findings were

reported in neighbouring countries including Ethiopia and Kenya among others (Hundie *et al*., 2016a; Ochwoto *et al*., 2016).

In this series genotype D was the second most prevalent genotype in Eritrea circulating in four out of the six regions. This genotype has been found universally, but predominates in Europe, North Africa, and Middle East (Kramvis & Kew, 2007). According to Hundie *et al* genotype D is the second most prevalent genotype circulating in Ethiopia and similar findings were reported by different authors in Kenya (Hundie *et al*., 2016b; Mwangi *et al*., 2008; Ochwoto *et al*., 2016). But in other neighbouring geographical locations including Sudan, Egypt and Yemen genotype D is the predominant genotype (El *et al*., 2010; Sallam & Tong, 2004; Yousif *et al*., 2013).

Compared to findings from neighbouring countries the isolates from Eritrea did not show diverse genotypic heterogeneity segregating into two subgenotypes D2 and D10. However findings from Ethiopia reported at least five subgenotypes D1, D2, D4, D6 and the newly isolated D10 (Hundie *et al*., 2016b). Similar results were reported in Sudan with subgenotypes D1, D2, D3, D4 and D6 found to be circulating in the country (Yousif *et al.*, 2013). This finding from Eritrea is surprising considering geographically the country is located at an important crossing point between Africa and the Middle East. Moreover, till 1991 Eritrea was considered a province of Ethiopia and the history of Eritrea is crisscrossed by colonizing forces from different nations including Turkey, Egypt, Italian and British. Therefore looking into available evidence associating human migration with introduction and dissemination of HBV genotypes and subgenotypes one expects a diverse genotypic heterogeneity among HBV isolates from Eritrea (Andernach *et al*., 2009; Kramvis 7 Kew, 2007).

Almost 90% of genotype D isolates from Eritrea belong to subgenotype D2, which is most prevalent in Eastern Europe including Russia (Tallo *et al*., 2008). But four of the D2 strains clustered separately away from the reference sequences from Africa, Asia and Europe. This might indicate the Eritrean strains are more distinct thus suggesting a distant link with the reference sequences. A single strain of D2 (ERDK67) from the Red Sea coasts closely formed a cluster with an isolate from central Ethiopia but two

more isolates (ERGB13 and 09) from western low lands of Eritrea formed a distant loose cluster. Hundie *et al* in 2016 proposed a novel subgenotype D10 circulating in Ethiopia and a single isolates from the Red sea coastal area (ERDK63) closely clustered with a D10 reference sequence from Ethiopia (accession no. KX357636) indicating that this novel subgenotype also circulates in Eritrea (Hundie *et al*., 2016b).

Findings from the African continent indicated that genotype E is most prevalent and exclusively found in Western and Southern Africa (Kramvis & Kew, 2007; Mulders *et al*., 2004; Odemuyiwa *et al*., 2001). In this study one isolate from zoba Gash Barka located in western Eritrea formed a sub-cluster with genotype E reference sequences from Angola (accession no. KF849721). In a study undertaken in Italy among immigrants Scotto *et al* (2010) reported that out of 65 isolates of genotype E identified almost 50% (33) were isolated from Eritrean immigrants (Scotto *et al*., 2010). But in another similar study in Italy among immigrants reported that none of the genotype E strains isolated in the study were from Eritrean immigrants (Palumbo *et al*., 2007). This difference in the findings might be due to the fact that most of the immigrants from Eritrea travel by road traversing many African countries including Sudan, Chad and other Western and Northern African countries to reach Europe. In most of the countries on the migrants' route genotype E is the most prevalent genotype, therefore there is a possibility for this migrants to pick the infection during their journey.

In countries were multiple HBV genotypes are prevalent recombination between HBV genotypes is common. Recombinants of genotypes D/E, A/E, A/D and even A/D/E were reported in many African countries and countries neighbouring Eritrea (Hundie *et al*., 2016b; Kramvis and Kew, 2007; Ochwoto *et al*., 2016; Yousif *et al*., 2013). In this series recombination of HBV genotypes was not analysed and this is a limitation of the study which should be addressed in subsequent studies.

5.1. Limitations of the study

- The study was not calibrated to measure potential risk factors for HBV infection among the study participants. This might be a potential limitation of the primary study given the difference in prevalence reported in the six regions and among the different socio-demographic and clinical characteristics.

- Plans to quantify HBV DNA levels in the current study didn't materialize due to break down of Cobas analyser at the National Health Laboratory of Eritrea. This had limited the data generated from the current study in appreciating the association of HBV genotypes and level of circulating HBV DNA as it had a significant impact on infectivity of the study subjects influencing MTCT.
- The limited number of amplified and sequenced samples coupled with the design of the study as it focused only among pregnant women had influenced the interpretation of the genotype results in relation to socio-demographic characteristics.

5.2. Conclusions

The mean age of the women was 26.7 ±5.9 years and findings of the socio-demographic data showed that 92.6% of the women were married with 88.4% being housewives. Approximately 70% of the women had attended formal education. The mean parity and gravidity of the women was 2.3±2.1 and 3.4±2.2 with 56.6% and 78.5% of the women being multiparous and multigravida respectively. The HBV exposure rates and the anti-HBs rates varied among the ANC attendees by socio-demographic and clinical characteristics. These variations in prevalence among the study subjects might indicate an underlying difference in exposure to HBV risk factors.

The overall prevalence of total anti-hepatitis B core and anti-hepatitis B surface antibodies markers were 25.8% (1241) and 14.2% (706) respectively. Collating the findings of the anti-HBV seromarkers from this study with the HBsAg result of the primary study revealed that approximately 30.5% of the study participants were positive for one or more of HBV seromarkers. This findings on HBV seromarkers indicate a potential substantial exposure to the virus among the study participants. It thus suffices to conclude that vaccination remains the best possible tool to mitigate risks of HBV infection.

This study provided the first description of HBV genetic diversity in Eritrea with a genotypes A, D and E circulating in the country. Subgenotype A1 was predominate (32/42) found to circulate in all the six regions of Eritrea. Genotype D (9/42) is the second most prevalent genotype and eight (8/9) of genotype D isolates from Eritrea

belong to subgenotype D2. Genotype D is reported in four out of the six regions. A single strain of genotype E is isolated in the western low lands of Eritrea. This demonstrates that there could be a high genetic diversity of HBV in Eritrea. Furthermore, majority of the Eritrean strains clustered separately and distantly from the reference sequences from the GenBank indicating a possible distant link.

5.3. Recommendations.

- Looking into variations observed in HBV exposure rates across the women based on differences in socio-demographic and clinical characteristics, it is thus recommended further investigations taking into account behavioral and cultural practices that could lead to HBV infection should be considered.
- From the results of anti-HBc prevalence, it is recommended that that the existing child vaccination program, currently available in Eritrea, should be strengthened and maintained. Moreover, considering the low result of vaccine induced anti-HBs and high proportion of women being negative for any HBV seromarkers, catch up vaccination programs for students and adult women of reproductive age should be considered.
- Looking into the diversity of HBV genotypes reported and considering the few sample used alongside the limitations of molecular methods used in the current study, it is recommended further investigations with larger sample size and employing cloning and full genome sequencing techniques. In addition, as this study was conducted on ‘healthy’ pregnant mothers from ANC clinics, it would be vital to find out the molecular diversity in clinical settings.

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APPENDICES

Appendix I. Ethical clearance (Kenya)

UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
P O BOX 19676 Code 00202
Telegrams: varsity
Tel:(254-020) 2726300 Ext 44355

KENYATTA NATIONAL HOSPITAL APPROVED
23 FEB 2017
KNH/UoN-ERC
P.O. Box 20723-00202

KNH-UON ERC
Email: uonknh_erc@uonbi.ac.ke
Website: http://www.erc.uonbi.ac.ke
Facebook: https://www.facebook.com/uonknh.erc
Twitter: @UONKNH_ERC https://twitter.com/UONKNH_ERC

KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP, Nairobi

Ref: KNH-ERC/A/58

23rd February 2017

Nahom Fessehaye Seulu
Reg. No.TM300-6423/2015
College of Health Sciences
J.K.U.A.T

Dear Fessehaye

Revised research proposal: "Prevalence of *Anti-HBV* Seromarkers and Molecular characterization of *Hepatitis B Virus* among *HBsAg* Positive Antinatal Care Attendee in Eritrea" (P965/12/2016)

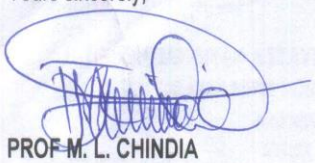
This is to inform you that the KNH- UoN Ethics & Research Committee (KNH- UoN ERC) has reviewed and **approved** your above revised proposal. The approval period is from 23rd February 2017 – 22nd February 2018.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH-UoN ERC before implementation.
c) Death and life threatening problems and serious adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH-UoN ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH- UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Clearance for export of biological specimens must be obtained from KNH- UoN ERC for each batch of shipment.
g) Submission of an *executive summary* report within 90 days upon completion of the study. This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/ or plagiarism.

For more details consult the KNH- UoN ERC website **http://www.erc.uonbi.ac.ke**

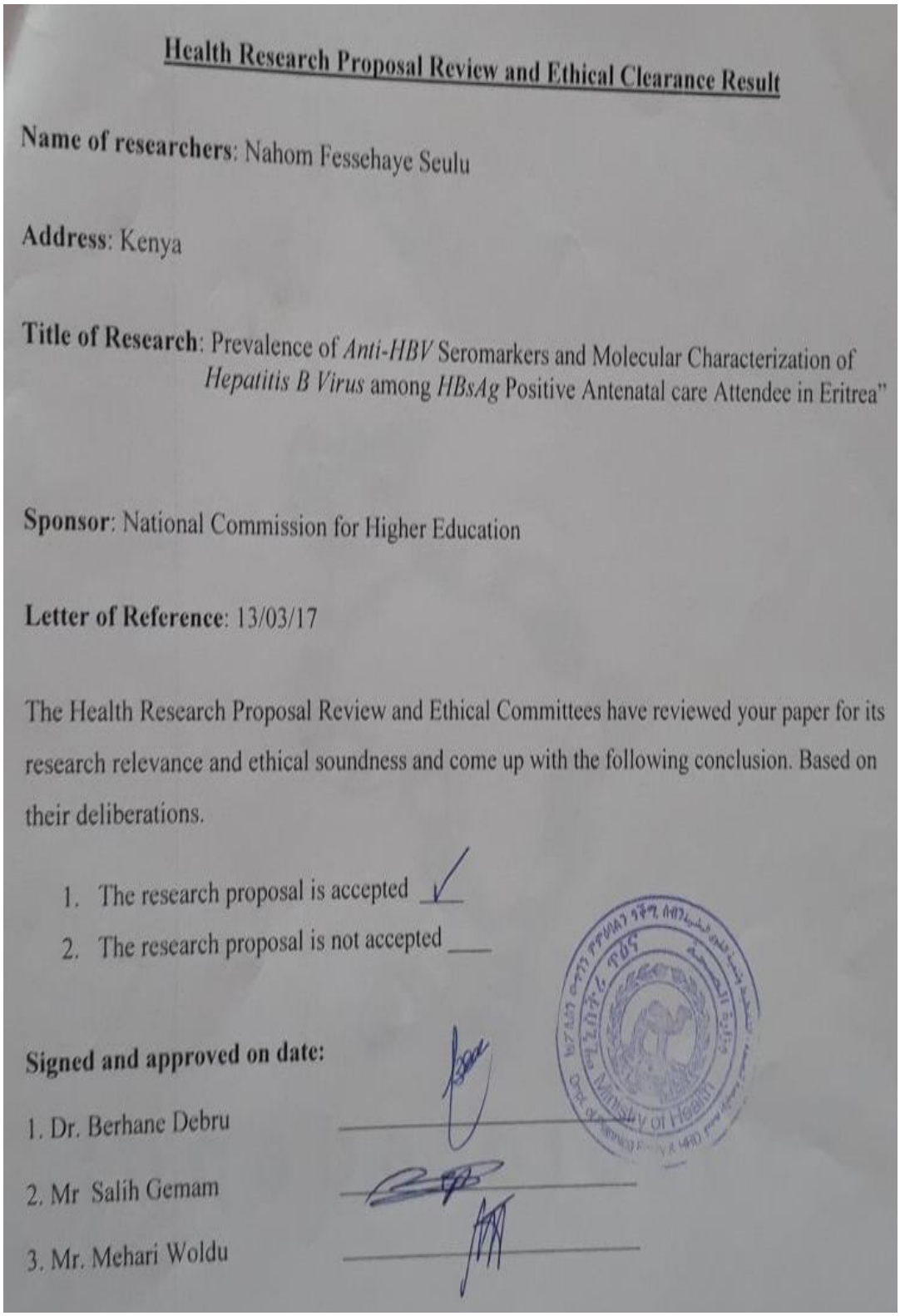
Yours sincerely,



PROF M. L. CHINDIA
SECRETARY, KNH-UoN ERC

c.c. The Principal, College of Health Sciences, UoN
The Director, CS, KNH
The Assistant Director, Health Information, KNH
The Chair, KNH-UoN ERC
Supervisors: Dr. Eddy Odari (JKUAT), Prof. Joseph Kangangi Gikunju (JKUAT)

Appendix II: Ethical clearance (Eritrea)



Health Research Proposal Review and Ethical Clearance Result

Name of researchers: Nahom Fesshaye Seulu

Address: Kenya

Title of Research: Prevalence of *Anti-HBV* Seromarkers and Molecular Characterization of *Hepatitis B Virus* among *HBsAg* Positive Antenatal care Attendee in Eritrea"

Sponsor: National Commission for Higher Education

Letter of Reference: 13/03/17

The Health Research Proposal Review and Ethical Committees have reviewed your paper for its research relevance and ethical soundness and come up with the following conclusion. Based on their deliberations.

1. The research proposal is accepted ✓
2. The research proposal is not accepted ___

Signed and approved on date:

1. Dr. Berhane Debru _____
2. Mr Salih Gemam _____
3. Mr. Mehari Woldu _____

Appendix III: Questionnaire

IDENTIFICATION	CODING CATEGORIES
Record Number (Zoba/Facility/Client)	☐☐☐
Zoba MAAKEL.....1 DEBUB.....2 ANSEBA.....3 GASH BARKA.....4 NRSZ.....5 SRSZ……..6	☐☐
Health facility	☐☐☐

Maakel	**Anseba**	**Northern Red Sea**
010=Semenawi Asmara H.C	070=Joko MCH	130=Amatere MCH Hosp
020= Akria HC	071=Hagaz HC	131=Kutmia HS
030=Serejeka HC	072=Megarih HS	132=Ghinda Hospital
031=Zagir HS	073=Bloko HS	140= Shieb HC
032=Weki HS	074=Waliko HS	141=Foro HC
033=Geshnashem HS	080=Geleb HS	142=Robrobia HS
034=Embaderho HS	081=Elabered HC	143=Ghelealo HC
035=Beleza HS	082=Kermed HS	
036=Azien HS	083=Hashishay HS	
037=Adi-Shaka HS	084=Fledarb HC	
Debub	**Gash-Barka**	**Southern Red Sea**
040=Mendefera Hosp	090=Baraentu Hosp	150=Assab MCH Hospital
0.41=Mendefera MCH	100=Agordat MCH	151=Tio Mini Hosp
050=Areza HC	110=Haycota HC	152=Bahti-Meskerem Clinic
051=Zban Debri HS	111=Mulki HC	153=Edi Community Hospital
052=Maidma HS	112=Mogolo HC	
053=Adi Guroto HS	113=Guluj HC	
054=Adi Gulti HS	120=Tesseney MCH	
060=Dekemhare MCH		

- Please complete one form to each **pregnant woman 15 to 49 years of age** who is attending **ANC for the first time for the current pregnancy**.
- Make sure that this form is **sent along with the serum sample** to the **National Health Laboratory**

NO.	QUESTIONS AND FILTERS	CODING CATEGORIES	SKIP
1	Date client enrolled (dd/mm/yyyy)	☐☐ ☐☐ ☐☐☐☐	
2	Age of Respondent (Age range 15-49 Years only)	MOTHER'S AGE IN COMPLETED YEARS ☐☐	
3	Gravida, (Write 01 if this is the women's first pregnancy, 02 if she has had one prior pregnancy,03 if she has had two prior pregnancies, etc.	☐☐	
4	Parity (Write 00 if this is the women's first pregnancy, 01 if she has had one previous birth, 02 if she has had two previous births, etc.)	☐☐	
5	Marital Status	Married …….1 Single .....2 Living with partner ……..3 Widowed .....4 Divorced/Separated …….5	
6	Nationality	Eritrean ……1 Ethiopian ……2 Other .....3 Specify_____	

7	Religion	Orthodox ……...1 Muslim ……2 Catholic .....3 Protestant .....4 Other …….....5 Specify_____	
8	Ethnicity	Tigrigna ……1 Tigre ……..2 Blen .....3 Saho .....4 Afar ...……5 Kunama ..……..6 Nara ……...7 Rashida ……..8 Hidarb ……9	
9	What is the <u>highest level</u> of Education attained:	Primary.....1 Middle.....2 Secondary.....3 Higher (Post-Secondary).....4	
10	Zoba or Place of usual residence	Maakel.....1 Debub.....2 Anseba.....3 Gash Barka.....4 NRS.....5 SRS..……6 Other……..……..7 Specify_____	
11	Town/village of usual residence	Name Of Town/Village:	

12	Main occupation of respondent	House Wife.....1 Unemployed.....2 Bar/Hotel/Tea Shop Worker.....3 Daily Labourer.....4 Military/National Service.....5 Student…….6 Merchant……..7 Farmer……8 Government office……..…9 Other…….……10 Specify_____	

Appendix IV: Published Manuscript

MedCrave
Step into the World of Research
Journal of Human Virology & Retrovirology

Prevalence of Hepatitis B Virus Infection and Associated Seromarkers among Pregnant Women in Eritrea

Abstract

Background: The global burden of chronic Hepatitis B infection is estimated at more than 240 million. Early HBV transmission and especially mother-to-child transmission (MTCT) contributes for more than one third of those chronic cases. Therefore understanding the epidemiology of HBV infection among pregnant women is critical to prevent MTCT. The epidemiology of HBV infection within the general population of many sub-Saharan African countries is documented but there is very limited data among pregnant women. In this study, the sero-prevalence of HBV markers was assessed among women seeking antenatal care within different health facilities in Eritrea.

Methods: This study was conducted within the framework of the 2016 national antenatal care (ANC) sentinel surveillance for HIV infection. A total of 5009 participants from a selected 16 ANC sites were screened for HBV Seromarkers. The Seromarkers screened using Enzyme Linked Immunosorbent Assay (ELISA) technique included; HBsAg, HBeAg, anti-HBe, anti-HBc and anti-HBs. The data generated by the serological testing were collated to the socio-demographic characteristic which was generated by use of questionnaires.

Results: The mean age of the women was 26.7 ±5.9 years. Results of the serological markers showed that 163 (3.2%) were positive for HBsAg indicating an active infection and 7(3.9%) positive for HBeAg indicating an increased infectivity. It was noted that 35 (17.4%) of the HBsAg positive women also presented with anti-HBe. The prevalence of anti-HBc and anti-HBs Seromarkers among the study participants was 1241 (25.8%) and 706 (14.2%) respectively. The prevalence of HBV showed marked difference among the zobas (regions) ranging from 2.1% to 7.5%. Results of the socio-demographic data showed that 92.7% of the women were married with 88.5% being housewives. Approximately 68% of the women had attended formal education.

Conclusion: The results of this study show a potential for vertical transmission within the 3.2% population determined. Childhood vaccination against HBV therefore remains key to prevention and mitigation of HBV in the Eritrean population.

Keywords: Hepatitis B virus; Seroprevalence; ELISA, Pregnant women; Antenatal care; Eritrea

Research Article

Volume 6 Issue 1 - 2018

Nahom Fessehaye[1]*, Araia Berhane[2], Hagos Ahmed[3], Salih Mohamed[4], Freweini Tecle[4], Joseph Gikunju[1] and Eddy Odari[1]

[1]School of Biomedical Sciences, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya
[2]Communicable Diseases Control Division, Ministry of Health, Asmara, Eritrea
[3]National Statistics Office, Asmara, Eritrea
[4]National Health Laboratory, Asmara, Eritrea

*Corresponding author: Nahom Fessehaye, School of Biomedical Sciences, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya, P.O. Box 62 0000-00200; Tel: +254-792639979; Email: abynabi27@gmail.com

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Abbreviations: 95%CI: 95% Confidence Interval; ANC: Antenatal Care Attendee; aOR: Adjusted Odds; Ratio; cOR: Crude Odds Ratio; HBV: Hepatitis B Virus; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B Surface Antigen; Anti-HBc: Hepatitis B Core Antibody; Anti-HBe: Hepatitis Be Antibody; Anti-HB: Hepatitis B Surface Antibody; HIV: Human Immunodeficiency Virus; MoH: Ministry of Health; NHL: National Health Laboratory; WHO: World Health Organization.

Introduction

WHO estimates the burden of Hepatitis B virus (HBV) infection at 2 billion, with more than 240 million patients developing chronic infection [1]. Annually 686,000 patients die as a result of HBV-related liver complications such as cirrhosis and hepatic carcinoma 1. Almost one third of those who develop chronic HBV infections acquire the infection via vertical transmission or horizontally during early childhood [2]. A mathematical projection on the global burden of HBV for children born in the year 2000 estimated that without HBV vaccine in the course of their life time an estimated 64 million will develop HBV infection with 1.4 million dying due to HBV related complications [3]. Eritrea is geographically located in sub-Saharan Africa, a region considered endemic to HBV infection with an intermediate to high prevalence [1]. The epidemiology of HBV infection in Eritrea is not well documented and there are only limited studies conducted to explore the extent of the problem. A study conducted among blood donors reported an intermediate prevalence of HBsAg Seromarkers of 2.6% [4]. The available HBV prevalence data from the region shows a varied prevalence pattern among countries and within each country. In Ethiopia different studies have been conducted among pregnant women documenting an HBsAg prevalence ranging from 3.4-7.8% [5-9]. Similarly a study

Appendix V: Blood sample testing using Antisurase B-96 ELISA

Materials Needed

- ELISA reader
- Automated microplate washer
- Microplate mixer
- ELISA Antisurase B-96 test kit
- Distilled water
- Disposable pipette tips
- Timer
- Incubator (37^0c)
- Manual or automatic pipettes capable of delivering 20µl, 50µl, 100µl and 1000µl

Procedure

1. Bring all reagents and specimens to room temperature (+20 to 300c) before testing. Adjust incubator to $+37\pm1^0$c.
2. Open the aluminum bag and take out the microplate with required number of strips
3. Reserve one well blank and pipette 50µl of each control or specimen into appropriate wells (3 negative controls and 2 positive controls)
4. Add 50µl of HBsAg peroxidase solution to each well except the blank and gently tap the plate
5. Cover the strips with a plate sealer. Incubate at $+37\pm1^0$c for 60 minutes
6. During incubation, dilute the concentrated washing solution 1:20 with distilled water
7. Wash each well with the diluted wash solution six times and then blot dry by pressing plate onto absorbent tissue
8. Pipette 50µl of TMB substrate solution A into each well including the blank well
9. Pipette 50µl of TMB substrate solution B into each well including the blank well. Carefully mix well
10. Cover the plate with a fresh plate sealer. Incubate at room temperature for 30 minutes

11. Stop the reaction by adding 100µl of 2N H_2SO_4 to each well including the blank well and mix completely
12. Put the plate in the microplate reader and read (within 30 minutes after step 11) the absorbance of the solution in the wells at 450nm reading wavelengthwith 620-690nm reference wavelength.

Result: Interpretation of result is based on the photometric reading data.

Abbreviations:

N= the mean absorbance of the negative control, N must be ≤0.2 otherwise test is invalid

P= the mean absorbance of the positive control, P must be ≥0.5 otherwise test is invalid

Calculation of cut-off value:

Calculate P-N value, the P-N value must be ≥ 0.3 otherwise the test is invalid

Cut of value = N + 0.025

Retest range = Cutoff value ± 10%

Interpretation of results

1. Specimen with absorbance values less than (0.9 x Cut of value) are considered Non-Reactive and are considered Negative for Anti-HBs
2. Specimen with absorbance values greater than (1.1 x Cut of value) are considered Reactive and are considered positive for Anti-HBs
3. Specimens with absorbance value within the retest range shall be repeated in duplicate and interpreted above.
4. Specimen with any repeat result in the retest range are reported as “Indeterminate”.

Appendix VI: Blood sample testing for anti-HBc total ELISA (DIAsource ImmunoAssays)

Materials Needed

- ELISA reader
- Automatic microplate washer
- Microplate mixer
- Anticorase B-96 test kit
- Distilled water
- Disposable pipette tips
- Timer
- Incubator (37^0c)
- Manual or automatic pipettes capable of delivering 20µl, 50µl, 100µl and 1000µl

Procedure

1. Bring all reagents and specimens to room temperature (+20 to 30^0c) before testing. Adjust incubator to $+37\pm1^0$c.
2. Open the aluminium bag and take out the microplate with required number of strips
3. Pipette 50µl of each specimen into the wells (leave 7 wells for control and blank). Pipette 50µl of positive control into each of two wells, 50µl of negative control into each of three wells and leave two blank, following addition of the sample
4. Add 50µl of anti-HBc peroxidase solution to each well except the blank and gently tap the plate
5. Cover the strips with a plate sealer. Incubate at $+37\pm1^0$c for 60 minutes
6. During incubation, dilute the concentrated washing solution 1:20 with distilled water
7. Wash each well with the diluted wash solution six times and then blot dry by pressing plate onto absorbent tissue
8. Pipette 50µl of TMB substrate solution A into each well including the blank well
9. Pipette 50µl of TMB substrate solution B into each well including the blank well. Carefully mix well
10. Cover the plate with a fresh plate sealer. Incubate at room temperature for 30 minutes

11. Stop the reaction by adding 100µl of 2N H_2SO_4 to each well including the blank well and mix completely
12. Put the plate in the microplate reader and read (within 30 minutes after step 11) the absorbance of the solution in the wells at 450nm reading wavelengthwith 620-690nm reference wavelength.

Result: Interpretation of result is based on the photometric reading data.

Abbreviations:

N= the mean absorbance of the negative control, N must be ≥ 0.4 otherwise test is invalid

P= the mean absorbance of the positive control, P must be ≤ 0.1 otherwise test is invalid

Calculation of cut-off value:

Calculate N-P value, the N-P value must be ≥ 0.3 otherwise the test is invalid

Cut of value =0.4 N + 0.6P

Retest range = Cutoff value ± 10%

Interpretation of results

1. Specimen with absorbance values less than (0.9 x Cut of value) are considered Non-Reactive and are considered Negative for Anti-HBc
2. Specimen with absorbance values greater than (1.1 x Cut of value) are considered Reactive and are considered positive for Anti-HBc
3. Specimens with absorbance value within the retest range shall be repeated in duplicate and interpreted above.
4. Specimen with any repeat result in the retest range are reported as “Indeterminate”.

Appendix VII: DNA extraction using QIAGEN extraction kit

Equipment and Supplies

1. QIAGEN consumables
2. Water bath (pre-set to 56°C)
3. Centrifuge
4. Vortex (setting pulse)
5. 1.5ml microcentrifuge tube
6. Filtertips (1000µL, 200µL, 100µL)
7. Pipettors (1000µL, 200µL)
8. Gloves (Medium, Large)

Reagents

1. Plasma Samples
2. QIAGEN Protease
3. QIAGEN Kit Buffers (AL: Lysis Buffer, AW1: , AW2: and AE: Elution Buffer)
4. Analar Ethanol

Pre-Extraction Steps

1. Equilibrate 1) Samples 2) Buffer AE to room temperature
2. Heat water bath or set heating block to 56°C
3. Remove any crystals from Buffer AL by incubating at 56°C
4. Prepare Buffer AW1, Buffer AW2 and QIAGEN Protease according to manufacturer instruction

Procedure

1. Pipet 20 µl QIAGEN Protease (or proteinase K) into the bottom of a 1.5 mlmicrocentrifuge tube.
2. Add 200 µl sample to the microcentrifuge tube. Use up to 200 µl whole blood,plasma, serum, buffy coat, or body fluids, or up to 5 x 106 lymphocytes in200 µl PBS.
3. Add 200 µl Buffer AL to the sample. Mix by pulse-vortexing for 15 s.
4. Incubate at 56°C for 10 min.

5. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
6. Add 230 µl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
7. Carefully apply the mixture from step 6 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g(8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.*
8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.
9. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2without wetting the rim. Close the cap and centrifuge at full speed (10,000 x g; 14,000 rpm) for 3 min.
10. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
11. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 60 µl Buffer AE or distilled water. Incubate at room temperature (15–25°C) for 5 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min.
12. Store the DNA extract at -80^0c for amplification.

Appendix VIII: HB test result registration worksheet

Prevalence of anti-HBV Seromarkers and Molecular Characterization of Hepatitis B Virus among Antenatal Care Attendee in Eritrea
Anti-HBc test work sheet

Operator: _____ Date: _____

Signature: _____ Lot No: _____

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

Ncx: _____ Pcx: _____

Cut of value: _____

Verified by: _____