Sudan University of Science and Technology

College of Graduate Studies

Sero-Detection of Hepatitis E Virus (HEV) among Pregnant Women Attending Khartoum North Hospital in Khartoum State

الكشف المصلي لفيروس التهاب الكبد الوبائي النوع (هـ) وسط النساء الحوامل اللائي يترددن على مستشفى الخرطوم بحري- ولاية الخرطوم

A Dissertation Submitted in Partial Fulfillment for the Requirements of M.Sc. degree in Medical Laboratory Science (Microbiology)

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الآيـــــة

قال تعالى:

﴿ اللَّهُ لا إِلَهَ إِلَّا هُوَ الْحَيُّ الْقَيُّومُ لا تَأْخُذُهُ سِنَةٌ وَلا نَوْمٌ لَهُ مَافِي السَّمَوَاتِ وَمَا فِي الأَرْضِ مَنْ ذَا الَّذِي يَشْفَعُ عِنْدَهُ إِلَّا بِإِذْنِهِ يَعْلَمُ مَا بَيْنَ أَيْدِيهِمْ وَمَا خَلْفَهُمْ وَلا يُحِيطُونَ بِشَيْءٍ مِنْ عِلْمِهِ إِلَّا بِمَا شَاءَ وَسِعَ كُرْسِيُّهُ السَّمَوَاتِ وَالأَرْضَ وَلا يَئُودُهُ حِفْظُهُمَا وَهُوَ الْعَلِيُّ الْعَظِيمُ ﴾.

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DEDICATION

To my precious loving family

To my husband and my little pretty girl

To my best friends

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First of all, thanks to**ALMIGHTY ALLAH**for blessing me with good health, wellbeing, strength and patience to carry out and complete this research work.

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ABSRACT

HEV infection in pregnant women is more common and fatal in the third trimester and the incidence of viral hepatitis E is known for being the cause of major outbreaks of waterborne hepatitis in Africa.

This descriptive, cross- sectional, hospital based studywas aimed to detect HEV among pregnant women attending Khartoum North Hospital during the period from February to December 2019.

A total of 90 subjects (n=90) were included in this study with age ranged from 19-42 years and the mean age was 30.5±5S.D and mostly in third trimester.

Blood samples were collectedand tested for total anti-HEV, anti-HEV IgM and IgG antibodies by Enzyme Linked ImmunosorbantAssay (ELISA).

Out of the 90 pregnant women who took part in the study, 36 (40%) were found positive for HEV and 5/90 (5.6%) were positive for HEV IgM antibodies, while 36/90 (40%) were positive for HEV IgG.

Regarding age groups, there were13(14.4%)in age group 19-26 years, 11(12.3%) in age between 27 to 34 years were positive for HEV and 12(13.3%) in age group from 35 to 42 years. There was no significant association (*P*=0.833) between age and HEV result.

Concerning trimester,13(14.5) were found positivefor HEV in third trimester and 11(12.2%),12 (13.3%) were in first and second trimester respectively with significant association (*P*=0.051) between them.

Relating to source of drinking water, 25 (28.8%) were positive for HEV antibodies in pregnant women drink from non filtered water and 11(12.2%) was positive they drink from filtered water and there no significant association (*P*=0.926) between them.

Regarding education level there were20(22.2%) HEV positive in educated women and 16(17.8%) in non- educated women and there was no significant association (*P*=0.482) between them.

HEV was associated with previous miscarriage in which about 24% were HEV IgGpositive.

From the above findings we concluded that, there was high percentage of HEV infection among pregnant women attending Khartoum North Hospital.

ملخص الأطروحة

تعد الإصابة بالتهاب الكبدي الوبائي النوع هـ عند النساء الحوامل أكثر شيوعًا ومميتة في الثلث الثالث من الحمل ، ومن المعروف أن الإصابة بالتهاب الكبد الفيروسي النوع هـ هي السبب في تفشي التهاب الكبد الوبائي المنقول بواسطة الماء في إفريقيا.

هدفت هذه الدراسة الوصفية المستعرضة المستندة إلى المستشفى إلى الكشف عن فيروس التهاب الكبد النوع هـ بين النساء الحوامل اللائي يحضرن مستشفى الخرطوم الشمالي خلال الفترة من فبراير إلى ديسمبر 2019.

تم تضمين 90 من المشاركات (ن = 90) في هذه الدراسة تراوحت اعمارهن بين 19–42 سنة وكان متوسط العمر 30.5 ± SD5 ومعظمهم في الثلث الثالث. تم جمع عينات الدم واختبارها من أجل الأجسام المضادة من النمط IgM و IgG المضاد لفيروس التهاب الكبد النوع هـ بواسطة اختبار الروز المناعي الانزيمي .

من بين 90 من النساء الحوامل اللائي شاركن في الدراسة ، وجدت 36 (40 ٪) إيجابية لفيروس التهاب الكبد النوع هـ و 90/5 (5.6 ٪) كانت إيجابية للأجسام المضادة من النمط IgM ، في حين أن 90/36 (40 ٪) كانت إيجابية لـ لأجسام المضاده من النمط IgG.

فيما يتعلق بالفئات العمرية ، كان هناك 13 (14.4 ٪) في الفئة العمرية 19–26 سنة ، و 11 (12.3 ٪) في سن ما بين 27 إلى 34 سنة كانت إيجابية و 12 (13.3 ٪) في الفئة العمرية من 35 إلى 42 سنة. لم يكن هناك ارتباط كبير (P = 0.833) بين العمر والنتيجه .

فيما يتعلق بمراحل الحمل ، تم العثور على 13 (14.5) ايجابية فيالثلث الثالث و 11 (12.2 ٪) ، 12 (13.3 ٪) في الثلث الأول والثاني على التوالي مع وجود ارتباط كبير (P = 0.051) بينهما .

فيما يتعلق بمصدر مياه الشرب ، فإن 25 (28.8 ٪) كانت إيجابية في النساء الحوامل اللائي يشربن من المياه غير المفلترة و 11 (12.2 ٪) كان إيجابيا لدى اللائي يشربون من الماء المصفى وليس هناك علاقة معنوية (P = 0.926) بين معهم.

فيما يتعلق بمستوى التعليم ، تم العثور على 20 (22.2 ٪) من فيروس نقص التهاب الكبد الوبائي النوع هايجابي لدى النساء المتعلمات و 16 (17.8 ٪) في النساء غير المتعلمات وليس هناك ارتباط كبير (P = 0.482) بينهما.

ارتبط فيروس التهاب الكبد الوبائي النوع هـ بالإجهاض السابق حيث كان حوالي 24 ٪ ايجابيا للاجسام المضاده من النمط IgG.

من النتائج المذكورة أعلاه خلصنا إلى أن هناك نسبة عالية من عدوى فيروس التهاب الكبد الوبائي النوع هـ بين النساء الحوامل اللائي يرتدين مستشفى الخرطوم الشمالي.

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ABBREVIATIONS

ALT	Alanine Aminotransferase
AP	Alkaline Phosphatase
AST	Aspartate Aminotransferase
EDTA	Ethylene Diamine Tetra-acetic Acid
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbant Assay
ESLD	End-stage Liver Disease
GBS	Guillain–Barré syndrome
GT	Gamma-glutamylTransferase
HEV	Hepatitis E Virus
LAMP	Loop Mediated Isothermal Amplification
NANBH	Non-A, Non-B Hepatitis
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
RT-PCR	Real Time Polymerase Chain Reaction
SPSS	Statistical Package for Social Science
UTR	Untranslated Region
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

CHAPTER I

1. INTRODUCTION

1.1. Introduction

Hepatitis E virus (HEV) is a small non-enveloped, positive-sense single-stranded RNA virus (Haldipur*et al.*, 2018).It has been classified as the single member of the genus Hepevirus and has a similar structure to the viruses of the Caliciviridae and Tombusviridae families (Abebe*et al*., 2017).

It is a major public health problem, especially in resource limited countries, in an annual estimate in 2005, there had been 20.1million HEV infections, resulting in 70000 deaths and 3000 intrauterine fetal deaths and a possibility of 0.019 and 0.198 mortality in symptomatic illness for non-pregnant and pregnant patients, respectively (Rayis *et al*., 2013).

Generally it is an enterically transmitted viral hepatitis with asymptomatic or acute self-limited manifestations(Abebe*et al*., 2017).

Epidemiological and clinical studies have suggested that vertical transmission of HEV may frequently happen in HEV infected pregnant women (Shinde*et al*.,2014).

Although most of HEV infections are mild or subclinical, the infection in pregnant women is particularly severe in high endemic countries. It has been reported that a significant proportion of pregnant women with hepatitis E may progress to fulminant hepatitis during epidemics, especially in the third trimester (Gu *et al*., 2015).

It is widely believed that HEV infection in pregnant women is confined to developing countries due to lack of safe water supply and epidemics of HEV with severe consequences in pregnant women have been recognized for many decades (Shalimar and Acharya, 2013).

The importance of HEV infection during pregnancy as a health dilemma is well known, but most of the time this importance is neglected maybe due to anomalous observations on hepatitis E complications among pregnant women in different parts of the world (Farshadpour*et al.*, 2018).

Infection in pregnant women is more common and fatal in the third trimester (Musa *et al.*,2016).

The maternal mortality rate of HEV infection during pregnancy can reach to 20±25% accompanying with prenatal or neonatal complications such as jaundice (Izopet et al., 2017).

HEV infection during pregnancy frequently leads to miscarriage, preterm delivery and poor neonatal survival, stillbirth and neonatal death (Pisanic*et al.*, 2017).

HEV infection in most patients follows a self-limited course;however, 20% to 30% mortality is seen in infected pregnant women (Haldipur*et al.*,2018).

Over the past 20 years, HEV has been considered an imported disease in developed countries, but there is evidence that autochthonous HEV infection is under recognized, despite a steadily increasing incidence (Capai, Charrel and Falchi, 2018).

The first reported cases of HEV infection in Sudan occurred in 1992 since then several larger out breaks have been observed, particularly in refugee camps in the Darfur region, furthermore, all of these outbreaks have been shown to be associated with high mortality rates in pregnant women (Eldum*aet al*., 2016).

1.2. Rationale

Because of HEV is highly endemic in several African countries including Sudan with high mortality rate among pregnant women and from an epidemiological point of view, hepatitis E is an old infection in Sudan, but only recently has its importance as public health concern been considered from research and public health standpoints (kim *et al*.,2014).

As such, there is still a long road ahead to clarify the real burden of HEV infection in pregnant women in Sudan.This study aimed to determine the infection status of HEV in pregnant women in Sudan and the obtained data could be helpful in order to manage crises and relapses of patients in order to control the HEV infection and improve vaccination which will minimize HEV infection.

1.3. Objectives

1.3.1. General Objective

To detect HEV serologically among pregnant women attending Khartoum North Hospital in Khartoum State.

1.3.2. Specific Objectives:

1.To detect anti-HEV IgM antibodies among pregnant women in Khartoum State by ELISA.

2. To detect anti-HEV IgG antibodies among pregnant women in Khartoum State by ELISA.

3. To determine the possible risk factors (e.g. age, trimester, source of drinking water and level of education) associated with HEV infection.

4. To identify the association between previous miscarriage and HEV infection.

CHAPTER TOW

LITRETURE REVIEW

CHAPTER II

2. LITRETURE REVIEW

2.1. Pregnancy

Pregnancy is the state of carrying a developing embryo or fetus within the female body (Abebe*et al*., 2017). This condition can be indicated by positive results on an over the counter urine test and confirmed through a blood test, ultrasound, detection of fetal heartbeat, or an X-ray.Moreover, pregnancy lasts for about nine months, measured from the date of the woman's last menstrual period (LMP) (Racicot *et al.*, 2017).

It is conventionally divided into three trimesters, each roughly three months long (Kourti s*et al*., 2014). In each trimester, the fetus will meet specific developmental milestones, the first trimester lasts for the first 12 weeks of the pregnancy and is crucial for the baby's development (Bhutta*et al.*,2010).

2.1.1. Viral infection during pregnancy

Viral infections during pregnancy have long been considered benign conditions with a few notable exceptions, such as herpes virus,HIV and hepatitis (Hodgins *et al.*, 2016).

The recent Ebola outbreak and other viral epidemics and pandemics show how pregnant women suffer worse outcomes (such as preterm labor and adverse fetal outcomes) than the general population and non-pregnant women (Silasi*et al.*, 2015). New knowledge about the ways of the maternal-fetal interface and placenta interact with the maternal immune system may explain these findings;immunologic changes during pregnancy promote the maintenance of the fetus in the maternal environment by suppression of T cell–mediated immunity, rendering pregnant women more susceptible to viral infections like HEV infection (Abebe *et al*.,2017).

During pregnancy, levels of progesterone, estrogen, and human chorionic gonadotropin increase as pregnancy advances,these hormones play a considerable role in altering immune regulation and increasing viral replications (Katz *et al.*, 2013).

Once thought to be “immunosuppressed”, the pregnant woman actually undergoes an immunological transformation, where the immune system is necessary to promote and support the pregnancy and growing fetus. When this protection is breached, as in a viral infection, this security is weakened and infection with other microorganisms can then propagate and lead to outcomes (Ilekis *et al.*,2016).

Viruses can gain access to the decidua and placenta by ascending from the lower reproductive tract or via hematogenous transmission; so, viral tropism for the decidua and placenta is then dependent on viral entry receptor expression in these tissues as well as on the maternal immune response to the virus (Lumbiganon *et al.*, 2014).

These factors vary by cell type and gestational age and can be affected by changes in the utero environment and maternal immunity.Some viruses can directly infect the fetus at specific times during gestation, while some only infect the placenta and both scenarios can result in severe birth defects or pregnancy loss (Etheredge *et al.*, 2015).

Viral infections in pregnancy are major causes of maternal and fetal morbidity and mortality (Capai *et al.*, 2018).

2.2. Hepatitis E Virus

Hepatitis E virus (HEV) is the causative agent of hepatitis E in humans worldwide. According to 2018 data from the World Health Organization (WHO), there are 20 million HEV infections each year, leading to about 3.3 million symptomatic cases, with approximately one-third of the world's population having been exposed to HEV (He *et al.*,2018).

It was identified as an epidemic of non-A, non-B hepatitis (NANBH) from Kashmir, India in 1978 (Deroux *et al.*, 2014).

In the last 36 years since the discovery of the disease, major advances have occurred in relation to its causative agent, the host range in the animal kingdom, epidemiology and modes of spread (Girones *et al.*, 2014).

HEVinfections are ubiquitous in developing countries as a cause of epidemic and endemic acute hepatitis,however, the disease is now encountered in developed countries as well (Khuroo *et al.*, 2016).

2.2.1. Classification

HEV strains belonging to the Hepeviridae family display extensive genetic diversity (Montpellier *et al.*,2018).

A taxonomic scheme was recently proposed to classify this family into two genera: Orthohepeviru sand Piscihepe virus.Orthohepevirus contains all mammalian and avian HEV strains and it divided into four species,OrthohepevirusA-D. OrthohepevirusAincludes four HEV major genotypes (1±4, or HEV-1 to HEV-4) (Ju *et al.*, 2019).

HEV-1 and HEV-2 are restricted to humans and transmitted through the consumption of contaminated water. While HEV-3 and HEV-4 have a wide host range including humans, swine, wild boars and other mammals, and are responsible for zoonotic transmission from animals to humans through the consumption of raw or undercooked meats in both developing and industrialized countries.Additional Orthohepevirus genotypes have been found in rabbits (HEV-3ra), wild boars in Japan (HEV-5 and HEV-6), and camels in the Middle East (HEV-7) and China (HEV-8). Other HEV species in the Orthohepevirus genus infect birds (Orthohepevirus B), rats, ferrets and minks (Orthohepevirus C) and bats (OrthohepevirusD) (luk *et al.*, 2018).

2.2.2.Structure

HEV is a non-enveloped virus of 27–34 nm in diameter. The 7.2-kb RNA genome encodes three open reading frames (ORF) which are translated into: (i) the ORF1 polyprotein,representingthe viral replicase, (ii) the ORF2 protein, corresponding to the viral capsid and (iii) The ORF3 protein, a small,hitherto poorly characterized protein (Gouttenoire *et al.*, 2018).

The HEV genome contains a 5' untranslated region (UTR), three open reading frames(ORFs) and a 3' UTR (Izopet et al., 2017).

Structure of a HEV-like particle (VLP) shows that each capsid protein contains 3 linear domains that form distinct structural elements: S, the continuous capsid; P1, 3-fold protrusions and P2, 2-fold spikes. The S domain adopts a jelly-roll fold commonly observed in small RNA viruses,the P1 and P2 domains both adopt barrel folds,each domain possesses a potential polysaccharide binding site that may function in cell receptor binding. Sugar binding to P1 at the capsid protein interface may lead to capsid disassembly and cell entry. Structural modeling indicates that native T3 capsid contains flat dimers, with less curvature than those of T1 VLP (Guu *et al.*, 2009).

HEV genome capped with 7-methylguanine at its 5'end and poly (A) at its 3'end. The genome has UTR's at the 5' end (27 nucleotides) and at the 3' end (65 nucleotides) and a conserved stretch (58-nucleotides) near its 5' end region within open reading frame 1 (ORF1), which fold in to stem loop and hairpin structures. HEV RNA replicates in to a genomic RNA of 7.2 kb and a bicistronicsubgenomic RNA of 2.2 kb. There are 3 ORFs in the genome namely ORF1, ORF2 and ORF3 (Khuroo *et al.*, 2016).

2.2.3. Replication

HEV lacks both a proper *in vitro* culture system and animal model and the life cycle of HEV remains poorly studied(Ju *et al.*, 2019).

It is assumed that HEV reaches the host through gut epithelial cells; attach to the surface of hepatocytes through heparin sulfate proteoglycans, binds to a receptor and enter the hepatocytes (Haldipur*et al.*, 2018).Once internalized, the virus is uncoated, releases RNA and non-structural proteins of the virus are translated,positive sense viral RNA is replicated in to negative sense RNA with help of RNA dependent RNA polymerase. Negative sense RNA become templates for 7.2 kb positive-sense RNA and 2.2 kb subgenocmic RNA,subsequent to this, pORF2 and pORF3 are formed with

the help of subgenocmic RNA as template. pORF2 protein along with genomic RNA assemble into the new virion while the pORF3 optimizes viral replication.The virion egressed from hepatocytes are coated with pORF3 and lipid layer. Both pORF3 and lipid layer are separated from virion after egress from hepatocytes (Haldipur *et al.*, 2018).

2.2.4. Transmission and epidemiology

The first retrospectively confirmed outbreak of Hepatitis E occurred in 1955-1956 in New Delhi, India and resulted in more than 29000 symptomatic jaundiced persons (Teshale, 2011). Since that time, many large outbreaks have occurred in Asia, Africa and Mexico. In addition, sporadic hepatitis E outbreaks commonly occur in developing countries of Asia and Africa as well as in industrialized countries.Although there is a distinct epidemiologic picture of HEV infection in North America, Europe and Japan (Nan and Zhang, 2016).

The HEV is transmitted mainly through the fecal-oral route due to fecal contamination of drinking water, this route accounts for a very large proportion of clinical cases with this disease. Other routes of transmission have been identified, but appear to account for a much smaller number of clinical cases. These routes of transmission include: ingestion of undercooked meat or meat products derived from infected animals (e.g. pork liver) transfusion of infected blood products and vertical transmission from a pregnant woman to her baby(Himmelsbach *et al.*, 2018).

2.2.5. Pathogenisity

The incubation period following exposure to HEV ranges from 2 to 10 weeks, with an average of5 to6weeks (Ju *et al.*,2019).

The infected persons excrete the virus beginning from a few days before 3 to 4 weeks after onset of the disease. Furthermore, in areas with high disease endemicity, symptomatic infection is most common in young adults aged 15–40 years and in these areas, infection does occur in children,but they often have either no symptoms or only a mild illness without jaundice which goes undiagnosed(Jeblaoui *et al.*, 2013).

Typical signs and symptoms of hepatitis include: an initial phase of mild fever, reduced appetite (anorexia), nausea and vomiting, lasting for a few days; some persons may also have abdominal pain, itching (without skin lesions), skin rash, or joint pain,jaundice (yellow color of the skin and whiteness of the eyes), with dark urine and pale stools; and a slightly enlarged, tender liver (hepatomegaly). These symptoms are often indistinguishable from those experienced during other liver

illnesses and typically last 1–6 weeks. In rare cases, acute hepatitis E can be severe, and result in fulminant hepatitis (acute liver failure); these patients are at risk of death (Ju *et al.*, 2019).

Fulminant hepatitis occurs more frequently when hepatitis E occurs during pregnancy(Himmelsbach *et al.*, 2018). Pregnant women with hepatitis E, particularly those in the second or third trimester, are at increased risk of acute liver failure, fetal loss and mortality(Salines *et al.*, 2017). Up to 20–25% of pregnant women can die if they get hepatitis E in third trimester. Cases of chronic hepatitis E infection have been reported in immunosuppressed people, particularly organ transplant recipients on immunosuppressive drugs, with genotype 3 or 4 HEV infection. These remain uncommon (Knegendorf *et al.*,2018).

2.2.6. Clinical features

The course and clinical presentation of HEV infection is highly variable and the mechanisms leading to the different clinical outcomes are only partially understood(Yamada *et al.*, 2009).

2.2.6.1. Acute Hepatitis

In humans, the acute form of the disease can be caused by strains belonging to four genotypes: HEV-1, HEV2, HEV3, and HEV-4. Symptoms are resembling those of hepatitis A. Clinical manifestations are similar in developing and industrialized countries (Festa*et al.*, 2014). The incubation period ranges from 15 days to nine weeks (mean 40 days)(Wang *et al.*, 2018).

The prodromal phase is quite variable and can manifest as asthenia, fever, and digestive disorders for several days, followed by an icteric phase of two weeks; accordingly, it is not surprising that most cases remain undetected at the acute stage. Hepatitis is caused by an immune reaction directed towards the infected hepatocytes. Acute cytolytic hepatitis is the most common symptom. In most cases, the outcome is favorable, and biological parameters normalize within three months,cholestatic forms occur in 20% of cases (Kumar *et al.*, 2011).

Routine laboratory testing usually detects an increase in alanine and aspartate aminotransferase (ALT, AST) levels, accompanied by an increase of alkaline phosphatase (AP), gamma-glutamyl-transferase (GT), and bilirubin levels. The ALT level increases usually between 1000–3000 IU/L, but extreme values can be seen. ALT elevation is commonly higher than AST elevation. Cases where ALT is normal

despite HEV RNA is detected during the acute stage have been described (Jeblaoui *et al.*, 2013).

In industrialized countries, symptomatic HEV infections mostly affect men older than 55 years and the mortality rate is 1–4%, which is higher than the mortality associated with acute hepatitis A (0.1–2.5%) (Gauss *et al.*, 2012).

However, these rates are likely overestimated, because they were calculated fromsymptomatic cases seen in hospitals. In the general population, the mortality rate ranges between 0.06–0.7% cases leading to death correspond to the acute forms, which can become fulminant (Festa *et al.*, 2014).

2.2.6.2. Fulminant Hepatitis

Occasionally 1–2% of cases, acute hepatitis can develop into fulminant hepatitis and it is frequent among people with underlying liver diseases in high income countries after HEV infection(Knegendorf *et al.*, 2018).

Cases have been reported in several industrialized countries: the first cases were reported in Italy, Spain, France and Japan.Despite clinical specificities, there would not be a correlation between the severity of the disease and the genotype (Jilani *et al.*,2007). However, a case study in France showed that infection with genotype 4 could be more severe (Anty *et al.*,2012).

2.2.6.3. Chronicity

Chronicity is defined as a persistent viremia at least three to six months after the diagnosis,AST and ALT are less elevated in patients who progress to chronic HEV infection; the mean ALT is 300 IU/L in chronic disease, and 1000 IU/L in acute disease, There is no correlation between the viral load and the risk of progression to fibrosis, Although the routes of infection (zoonotic transmission, consumption of infected products) do not differ between the general population and immunocompromised individuals, and the latter can also get infected via blood products or organ donation: transfusion and transplantation-associated cases have been described (Wang *et al.*, 2018).

The majority of HEV chronic infections is observed with HEV-3, probably because it is the most commonly circulating genotype in industrialized countries. However, chronic infections caused by strains belonging to ge2notype HEV-4 have been recently described, Rapid evolution towards cirrhosis and graft rejection were observed. Cases have been reported in several industrialized countries (Weller *et al.*, 2016).

2.2.6.4. Extra-hepatic Manifestations

Many types of extra-hepatic manifestations were reported in both acute and chronic infections, among others, thrombocytopenia, kidney injury, hemolytic anemia,and pancreatitis were described. Neurological signs are seen in 5% of cases,Guillain–Barré syndrome (GBS), neuralgic amyotrophyand encephalitis/meningoencephalitis/myositis were associated with acute forms (Deroux *et al*., 2014).

HEV superinfection can aggravate previous liver diseases caused by alcohol, hepatitis C, or hepatitis B viruses and it must be evoked in the presence of a brutal marked elevation of AST and ALT, or in the case of hepatic encephalopathy or renal impairment (Gerolami *et al.*, 2011).

2.2.7. Laboratory diagnosis

Serological analysis for HEV infection has been problematic and several issues need to be addressed while evaluating these tests. Some tests have problems while applying to different genotypes (Purdy and Khudyakov,2011).

Others perform poorly in immunocompromised persons and cross reactions with other viral infections have been reported. Several available assayshave been developed and evaluated by sera from patients with recent infections. These assays often have poor performance in sensitivity and specificity,assays developed and evaluated against WHO reference(Montpellier *et al.*, 2018).

Reagents give more predictable results, those either “indirect” ELISA or class capture ELISA technique whichgives better results (Koning*et al.*, 2013). Amongstthese, 2 assays for IgM anti-HEV marketed by the Beijing Wantai Biological Pharmacy (Wantai Rapid test) and Genelabs Diagnostics, Singapore (AssureTM) have high sensitivity and specificity. In routine clinical practice, acute HEV infection in immunocompetent patients can predictably be diagnosed by IgM anti-HEV,around 90% patients are reactive for IgM anti-HEV at 2 week of infection and stays for up to 5 months (Zhang *et al.*,2012).

In patients with immune deficiency disease, additional testing for HEV RNA is recommended in view of poor IgM response in this population. IgG anti-HEV testing is useful in seroprevalence studies and rising IgG titers may help in diagnosis of HEV infection in situations with poor IgM anti-HEV response (Khuroo *et al.*,2016).

Testing for IgG anti-HEV titers is essential for determining effectiveness of HEV vaccine,antibody titers of 2.5 WHO units/mL following vaccination or acute HEV infection isprotective. Testing for HEV RNA is useful in several situations which include: (1) donor screening; (2) diagnosis of HEV infections in patients with poor IgM response; (3) diagnosis of chronic HEV infection; and (4) evaluating response to antiviral drug therapy (Baylis *et al.*, 2011).

In house assays for HEV RNA detection may have limitations and needs to be standardized with WHO standard (genotype HEV-3a)(Montpellier *et al.*, 2018). Conventionally HEV RNA is detected in blood and other body fluids by real time-polymerase chain reaction (RT-PCR) and using primers from conserved segments of HEV(Khuroo*et al.*,2016). Another assay, the loopmediated isothermal amplification (LAMP) employs single tube, one step amplification of HEV RNA. The test is quick, reliable and needs no special equipment (Khuroo *et al.*,2016).

2.2.8. Treatment

There is no specific treatment capable of altering the course of acute hepatitis E. As the disease is usually self-limiting, hospitalization is generally not required,most important is the avoidance of unnecessary medications,Acetaminophen/Paracetamol and medication against vomiting should not be given (Mishra *et al.*,2016).

Treatment for hepatitis E infection can be justified by the chronic and persistent infections commonly caused by genotype 3 and involved with immunosuppressive or immunocompromised conditions (Montpellier *et al.*, 2018). However, hospitalization is required for people with fulminant hepatitis and should also be considered for symptomatic pregnant women. Immunosuppressed people with chronic hepatitis E benefit from specific treatment using ribavirin, an antiviral drug (Gill and Kurre,2019).

In some specific situations, interferon has also been used successfully therapies using ribavirin and pegylatedinterferon succeeded in establishing a sustained virologic response after 3–6months of treatment, with patients presenting a restoration of lymphocyte count (Gouilly *et al.*,2018).

Recently, a large followup study related the effect of ribavirin as amonotherapyfor recipients with prolonged HEVviremia thus; the recent findings suggestthat ribavirin is an antiviral therapy to treat HEV chronic infection in immunocompromised patients (Melgaço *et al.*, 2018).

2.2.9. Prevention and control

Currently there is no commercially available HEV vaccinein North America ((Montpellier *et al.*, 2018).

Hepatitis E vaccine using recombinant capsid protein has been shown in phase 2 and 3 clinical trials to be safe and effective in the general adult population (Kang *et al.*,2017).

The recombinant hepatitis E vaccine, the first prophylactic vaccine against HEV infection, was approved in China in December 2011(Fierro *et al.*,2016).It has not yet been approved in other countries (Kaushik *et al.*, 2017).

Prevention is the most effective approach against the disease. At the population level, transmission of HEV and hepatitis E disease can be reduced by:maintaining quality standards for public water supplies andestablishing proper disposal systems for human feces.On an individual level, infection risk can be reduced by: maintaining hygienic practices avoiding consumption of water and ice of unknown purity (Kaushik *et al.*, 2017).

2.2.10. HEV infection and pregnancy

The majority of clinical studies and cases in pregnant women come from developing countries (Central Africa and South East Asia, mostly) with genotype 1 and 2. In these highly endemic areas, mortality and vertical transmission rate is high and severe forms occurred (Germer *et al.*, 2017).

However, there are few cases reported during pregnancy in industrialized Western countries and the first case reported of a pregnant woman infected by HEV with genotype 3 in Europe was in a 41-years old woman living in France. This woman and her baby had no complications(Knegendorf *et al.*, 2018).

A prospective study in France showed that, out of the 315 pregnant women participating, HEV prevalence was 7.74%, HEV-3 and HEV-4 do not appear to cause fatal infections with fulminant hepatitis in pregnant women (Knegendorf *et al.*, 2018).

Hepatitis E infection during pregnancy in the third trimester, especially with genotype 1, is associated with more severe infection and might lead to fulminant hepatic failure and maternal death (Mejido *et al.*, 2019).

Although the mechanism of liver injury is not yet clear, it is possible that interplay of hormonal and immunologic changes during pregnancy, along with a high viral load of HEV, renders the woman more vulnerable (Mushahwar *et al.*, 2008).

2.3. Previous studies

Al-Tayeb and his collaegues in (2014) in Khartoum, Sudan found that; 41.1% (37/90) pregnant women were anti-HEV positive.

In 2016,Musa and others in Kasala,Sudan found that HEV IgG antibodies were 61.2% (57/93) of the women under study.

Abebe *et al.*(2017), in Ethiopia found Anti- HEV IgG antibody was detected in 122 (31.6%) women and two women (0.5%) were positive for anti-HEV IgM from the total 386 women.

Adjei and othersin 2009 in Ghana found the seropositive pregnant women was 64.40% (29 out of 45) tested for anti-HEV IgM whereas 35.60% (16 out of 45) tested positive for anti-HEV IgG.

In China, Gu (2015) found that, 3 (0.6 %) pregnant women were anti-HEV IgM positive and 55 (11.1 %) were IgG positive.

Junaid in 2014 in Nigeria found anti-HEV IgG and IgM was 42.7% and 0.9%, respectively in pregnant women.

CHAPTER THREE

MATERIALS AND METHODS

CHAPTER III

3. MATERIALS AND METHODS

3.1. Study Approach

Qualitative research.

3.2. Study design

This is a descriptive, cross- sectional, hospital based study.

3.3. Study area

This study was conducted in Khartoum North Hospital in Khartoum State.

3.4. Study duration

The study was carried out in the period from February2019 to December 2019.

3.5. Study population

Pregnant women.

3.6. Inclusion criteria

Sudanese pregnant women with symptoms of hepatitis and different age.

3.7. Ethical considerations

Approval to conduct this study was obtained from Scientific Research Committee, College of Medical Laboratory Science, Sudan University of Science and Technology. Participants were informed about the aims and the value of the study and informed consent was taken.

3.8. Sample size

The total sample size was 90 samples.

3.9. Data collection

Data was collectedby direct interview (questionnaire) with each participant (appendix 1).

3.10. Specimen collection

Ninety (n=90) blood specimens were collectedfrom each participantthrough venous puncture technique and blood was withdraw aseptically using syringe or vacuotainer closed system. Blood were allowed to clot and serum was separate by centrifugation 3000/ rpm for 15 minutes. Then sera stored at (-20°C) until performance of the test.

3.11. Enzyme Linked Immunosorbant Assay (ELISA)

3.11.1.Detection of Anti HEV (IgM) Antibody by ELISA

3.11.1.1. Procedure

The procedure followed the manufacturing's instructions (EUROIMMUN), in which100 µL of the calibrators, positive and negative controls and diluted patient sera

weredispensed into designated wells of the 96-well microtiter plate.After incubation at room temperature for30 minutes, washing was done 3 times by the working wash buffer.

Then 100 µL of the enzyme conjugate (peroxidase labelled anti-human IgG) was added in the all wells and incubated for 30 minutes at room temperature, then the wells were washed 3 timesusing working wash buffer.100 µLofchromogen\substrate solution was added into each of the microplate wells and incubated for 15 minutes at room temperature avoiding direct light.

Finally, the reaction was stopped by adding 100 µL of sulphuric acid to all wells and the optical density was read spectrophotometrically using ELISA reader at a wavelength of 450 nm as well as a reference wavelength of 620 nm and 650 nm within 30 minutes of adding the stop solution (appendix 2)

3.11.1.2. Calculation

Results were evaluated semi-quantitatively by calculating a ratio of the extinction value of each sample over the extinction value of the calibrator 2 according to the formula in the leaflet.

3.11.1.3. Interpretation of the results

Wells of samples with ratio greater than or equivalent to 1.1 were considered positive, while samples with ratio less than 0.8 were considered negative.

3.11.2. Detection of Anti HEV (IgG) Antibody by ELISA

3.11.2.1. Procedure:

The procedure was carried out according to guidance of manufacturing (EUROIMMUN) in which 100 µL of the calibrators, positive and negative controls and diluted patient sera were dispensed into designated wells of the 96-well microtiterplate.After incubating at room temperature for 30 minutes, washing was done 3 times by the working wash buffer.

Then 100 µL of the enzyme conjugate (peroxidase-labelled anti-human IgG) was added in the all wells and incubated for 30 minutes at room temperature, then the wells was washed 3 timesusing working wash buffer.100 µLof chromogen\substrate solution was added into each of themicroplate wells and incubated for 15 minutes at room temperature avoiding direct light.

Finally, the reaction was stopped by adding 100 µL of sulphuric acid to all wells and the optical density was read spectrophotometrically using ELISA reader at a

wavelength of 450 nm with a reference wavelength of 620 nm and 650 nm within 30 minutes of adding the stop solution (appendix 3).

3.11.2.2.Calculation

Results were evaluated semi-quantitatively by calculating a ratio of the extinction value of each sample over the extinction value of the calibrator 2 according to the formula in the leaflet.

3.11.2.3. Interpretation of the results:

Wells of samples with ratio greater than or equivalent to 1.1 were considered positive, while samples with ratio less than0.8 were considered negative.

3.12. Data analysis

The data were analyzed and presented using Statistical Package for Social Science (SPSS) software version 23for windows.

Frequencies were presented in form of tables and figures andsignificant of differences was determined using Chi-square test. Statistical significance was set at *P-value<0.05.*

CHAPTER FOUR

RESULTS

CHAPTER IV

4. RESULTS

4.1. Distribution of pregnant women according to age groups and trimester

A total of 90blood specimens were collected from pregnant women withage ranged from 19 to 42 yearswith mean age of 30.5 ±5S.D.Age was divided into three groups as follow:40(44.4%) in age group 19-26 years,27(30.1%) in age between 27 to 34 years and 23(25.5%) in age group 35-42 years.

They were in different trimester of pregnancy, in which26(29%) in first, 26(29%) in second and 38(42%) in third trimester.

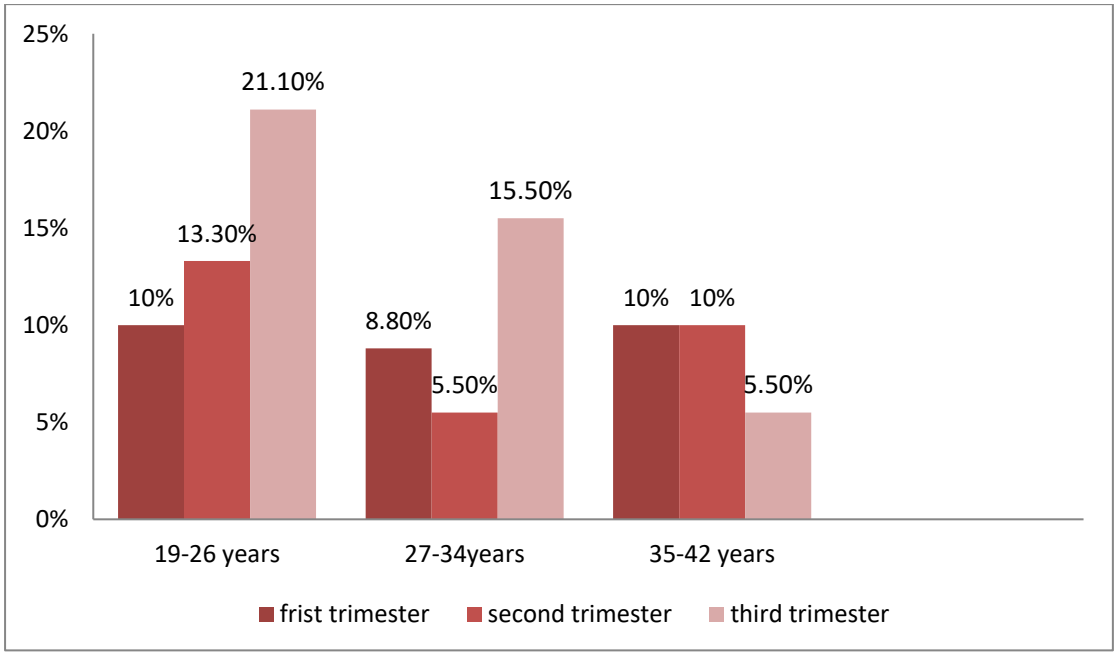

Figure 4.1: Distribution of pregnant women according to age groups and trimester of pregnancy

4.2. Distribution of pregnant women according to education

Figure 4.2 demonstrate that54(60%) were educated and 36(40%) non-educated pregnant women.

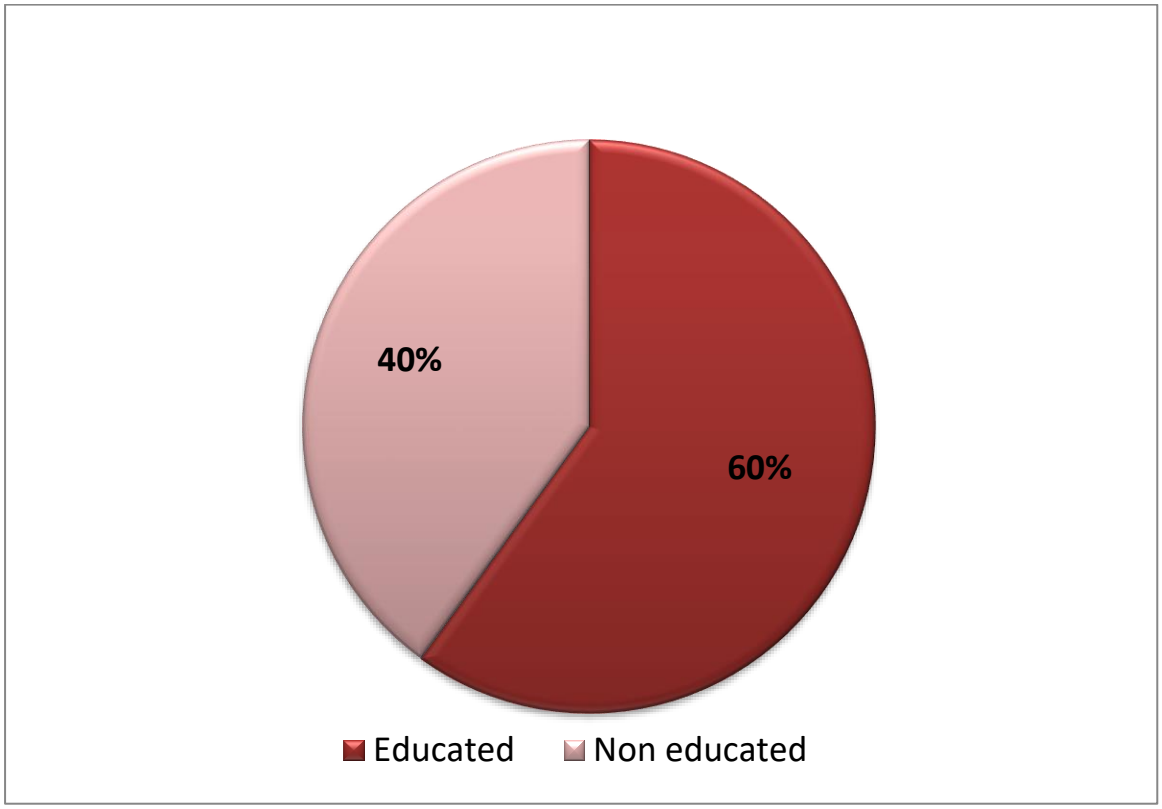

Figure 4.2: Distribution of pregnant women according to education

4.3. Frequency of HEV antibodies among pregnant women

Out of 90 pregnant women 36 (40%) were found positive for HEV.

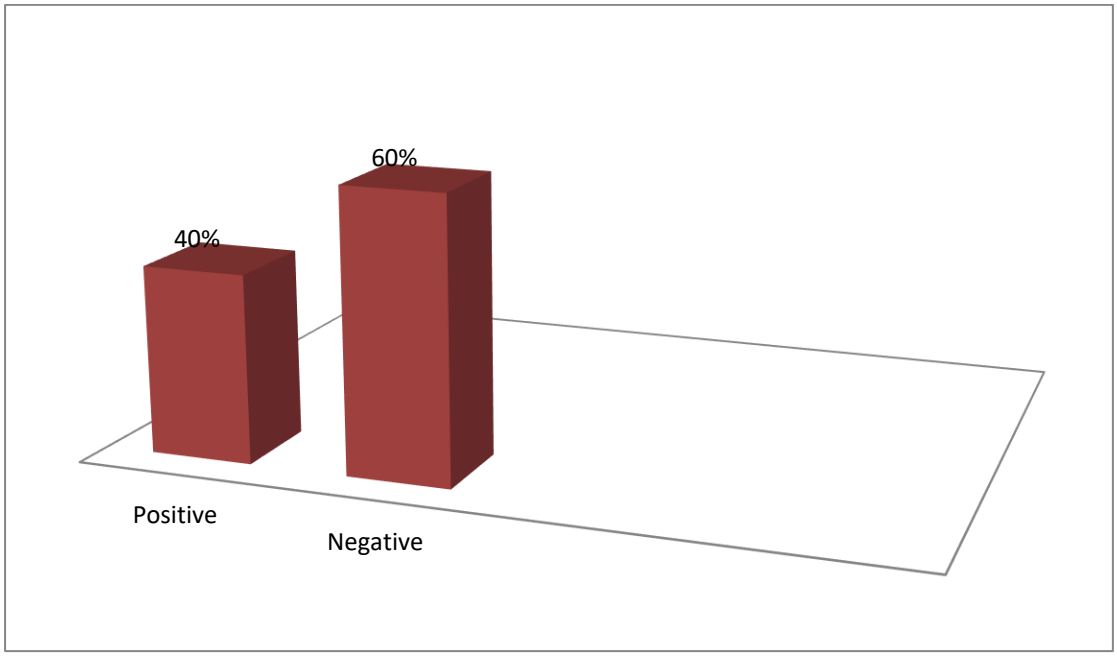

Figure 4.3: Frequency of HEV antibodies among pregnant women

4.4.Frequency of anti-HEV IgM antibodies among pregnant women

Out of 90 pregnant women,5(6%) were found positive forIgM

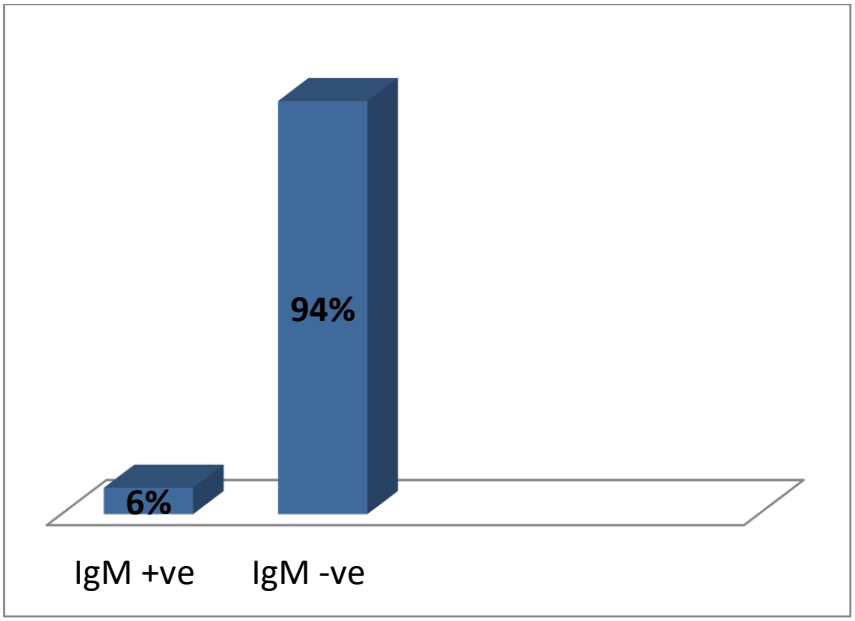

Figure 4.4:Frequency of anti-HEV IgM antibodies among pregnant women

4.5. Frequency of anti-HEV IgG antibodies among pregnant women

Out of ninetypregnant womenthere were 36 (40%) positive forIgG

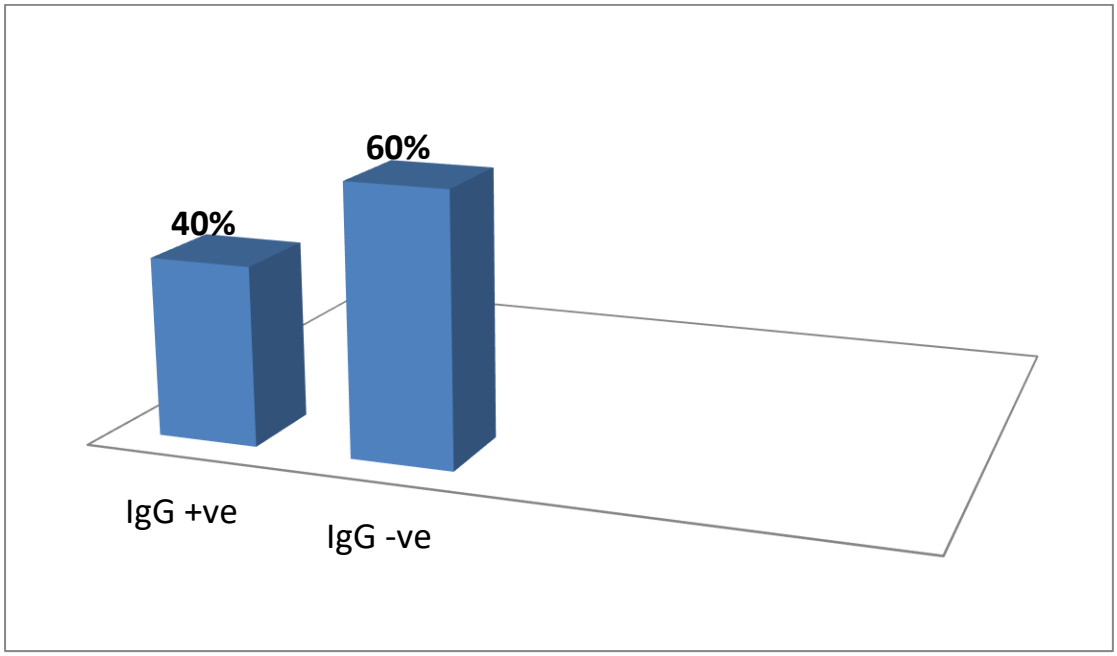

Figure 4.5:Frequency of anti-HEV IgG antibodies among pregnant women

4.6. The associationbetween age groupsand HEV infection among pregnant women

Table 4.1 displayed that,13(14.4%) were in age group 19-26 years,11(12.3%) in age between 27 to 34 years were positive for HEV and 12(13.3%) in age group from 35 to 42 years. There was no significant association (*P*=0.833) between age and HEVinfection.

Table 4.1:The association between age groups and HEV infection among pregnant women

HEV result	Age Groups			Total	*P-value*
	19-26 years	**27-34 years**	**35-42 years**		
Positive	13(14.4%)	11(12.3%)	12(13.3%)	36(40%)	0.833
Negative	20(22.3%)	19(21%)	15(16.6%)	54(94%)	
Total	33(36.7%)	30(33.3%)	27(30%)	90(100%)	

4.7.The association between trimester and HEVinfection among pregnant women

Table 4.2demonstrated that;13(14.5) were found positive HEV in third trimester and 11(12.2%),12 (13.3%) in first and second trimester respectively with significant association (*P*=0.051) between trimester and HEV.

Table 4.2: The association between trimester and HEV infection among pregnant women

HEV results	Trimester			Total	*P-value*
	First	Second	Third		
Positive	11(12.2%)	12 (13.3%)	13(14.5)	36(40%)	0.051
Negative	20(22.22%)	17(18.88%)	17(18.80%)	54(60%)	
Total	31(34.42%)	29(32.2%)	30(33.3%)	90(100%)	

4.8. The association betweensource of water drinking and HEV infection

Table 4.3 demonstrate that 25(28.8%) were positive forHEV antibodiesin pregnant women drinking from non-filtered water and 11(12.2%) was positivepregnant women drinking fromfiltered water and there no significant association(*P*=0.926) between the source of infection and HEV infection.

Table 4.3: The association between drinking water supply andHEVinfection

HEV results	**Source of water drinking**		**Total**	***P-value***
	Non-filtered water	**Filtered water**		
Positive	25(28.8%)	11(12.2%)	36(40%)	0.926
Negative	37(41%)	17(19%)	54(60%)	
Total	62(69%)	28(31%)	90(100%)	

4.9. The association between education and HEV infection

Table 4.4showed that 20(22.2%) were HEV positive in educated women and 16(17.8%) in non-educated women and there was no significant association (*P*=0.482) between education and HEV infection.

Table 4.4: The association between education and HEV infection

HEV results	Education		Total	*P-value*
	Educated	Non-educated		
Positive	20(22.2%)	16(17.8%)	36(40%)	0.482
Negative	34(37.8%)	20(22.2%)	54(60%)	
Total	54(60%)	36(40%)	90(100%)	

4.10. The association betweenprevious miscarriage and the result of IgG among pregnant women

Table 4.5 showed that, out of 36positivecases there were 22 (24%) anti-HEV IgG antibodiespositive pregnant women with previous miscarriage and there was significant association (*P*=0.000) between previous miscarriage and the result of IgG.

Table 4.5: The association between previous miscarriage and the result of IgG among pregnant women

IgG results	Miscarriage		Total	*P-value*
	Yes	No		
Positive	22(24%)	14(16%)	36(40%)	0.000
Negative	9(10%)	45(50%)	54(60%)	
Total	31(34%)	59(66%)	90(100%)	

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMENDATIONS

CHAPTER V

5.DISCUSSION, CONCLUSION AND RECOMENDATIONS

5.1. Discussion

This study found that; 36/90(40%) of pregnant women were anti- HEV positive which was similar to those obtained by Niguse *et al*. (2018) in Ethiopia (43.4%) and Junaid *et al*. (2014) in Nigeria (42.7%), and higher than Obiri-Yeboah *et al*.(2018) in Ghana (12.3%), Adjei *et al*. (2009) in Ghana 28.66% (45/157) and Renou *et al*. (2014) in france (7.74%) and lower than Boccia *et al.* (2006) in Darfur State, Sudan 95% (19/20).

And 5/90 (5.6%) of pregnant women were anti- HEV IgM positive,indicating recent infection which was similar to those obtained by Rui *et al*. (2018) in China 3.6%.

This finding was higher than that obtained by Obiri-Yeboah *et al*.(2018) in Ghana (0.2%), Niguse *et al*. (2018) in Ethiopia (0.9%) , Abebe*et al*. (2017) in Ethiopia (0.5%), Farshadpour and his colleagues (2018) in Iran (0.83%), Gu *et al.* (2015) (0.6%)in China and Junaid *et al*. (2014) in Nigeria (0.9%).

The rate of seropositivity revealed by this study was lower than those reported by Adjei *et al*. (2009) in Ghana 64.40% (29/45).

Reason for these differences could be due to difference in level of hygiene, educational status, social status, endemicity of virus, different lifetime exposures of the participants to HEV.

The frequency of HEV IgG antibodies among pregnant women was 36/90(40%)that was similar to those obtained by Al-Tayeb*et al*. (2014) in Khartoum, Sudan (41.1% (37/90), Niguse *et al*. (2018) in Ethiopia (42.4%) , Junaid *et al*. (2014) in Nigeria (42.7%) and Adjei *et al*. (2009) in Ghana 35.60% (16/ 45).

The above frequency was higher than that found by Ismail *et al*. (2020) in Lebanon (0.22%), Rui *et al*. (2018) in China 21.8%, Abebe*et al*. (2017) in Ethiopia (31.6%), Obiri-Yeboah *et al*.(2018) in Ghana (12.2%), Huang *et al*. (2013) in China (10.2%) and Adjei*et al*. (2009) in Ghana (24.7%), Also Gu *et al*. (2015) in China (11.1%).

The rate of seroposssitivity of IgG antibodies revealed by this study was lower than those reported by Musa *et al*. (2016) inSudan which found that;anti-HEV IgG antibodies was detected in 61.2% (57/93), Mohamed *et al*. (2017) in Egypt (67.6%) , Gu *et al*. (2015) in China detect 55 (11.1%) positive for IgG , Stoszek *et al*. (2006) in Egypt (84.3%) and Adjei *et al*. (2009) in Ghana 64.40% (29/45).

These variationsof results may be attributed also to high pressure of water inside the network which supplies Sudan area. During the season, this pressure broke down and corrodes old metallic pipes and become a major source of contamination by feces.

The seropositivity was higher among the age group 19-26 years (14.4%), 12.3% were found positive among age group 27-34 years and 13.3% were positive among age group 35-42 years.However, there was no statistically significant correlation between age groups and HEV infection, this was matched to the result obtained by Al-Tayeb*et al*. (2014) in Sudan The seroprevalence was highest 45.9% among pregnant women 16 - 24 years age, followed by 35.1% in 25 – 33 year group, then 19.0% in 34 - 42 year group.

Further more, noted that the high positive rates of HEV infection among pregnant women was in third trimesters(13(14.5%)) then in the second followed by first trimester 12(13.3%),11 (12.2%) respectively. This result was agreed with Musa *et al.*(2016) in Sudan (62.5%), Al-Tayeb *et al*.(2014) in Sudan (48.7%) and Adjei *et al*. (2009) in Ghana (30.25%) the high rate of infection in third trimester.

There was significant association between trimester and HEV infection.

Regarding level of education, 22.2% of educated pregnant women were positive for HEV that was compatible with result obtained by Adjei*et al*. (2009) in Ghana (28.05%) and Junaid *et al*.(2014) in Nigeria(32.4%). Moreover, there was no significant association between education and HEV infection.

About source of water drinking, 28.8% of pregnant women who drank from non-filtered water were positive for HEV, this harmonized with result of Musa *et al.* (2016) in Sudan(71.9%) drank from water system supply.

In the current study, HEV was associated with previous miscarriage, in which24%of pregnant women were positive for HEV;this was agreed with Musa *et al.* 2016 in Sudan (36.8%).

5.2. Conclusion

The findings of the present study conclude that; the frequency of infection of HEV was high among pregnant women attending Khartoum North Hospital.

There was significant association between HEV infection and trimester and also with previous miscarriage.

There were no significant association between HEV infection and age, level of education and source of drinking water.

5.3. Recommendations

Large sample size with more accurate tests (such as PCR) should be used to determine the rate of infection accurately.

Screening programfor HEV is highly recommended as part of the routine test for pregnant women.

Specific programs and strategies for HEV vaccination should be developed.

Systematic application of hygiene measures is highly recommended to avoid exposure to the virus.

Ultimately prevention of transmission of virus by good sanitation and boiling drinking water which are the best approaches to reduce morbidity of HEV infection and a number of other waterborne pathogens.

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APPENDIXES

APPENDIX 1

Sudan University of Science&Technology

College of Graduate studies

Sero-Detection of Hepatits E Virus (HEV)among PregnantWomen AttendingKhartoum North hospital in Khartoum State- 2019

No: ……**Age:** ……

Education: Yes ☐ No ☐

Trimester: First ☐ Second ☐ Third ☐

Medical history:

Miscarriage: Yes ☐ No ☐

Symptoms: Fever ☐ Vomiting ☐

Jaundice ☐ Nausea ☐

Loss of weight ☐ Abdominal pain ☐

Dark urine ☐ Light colored stool ☐

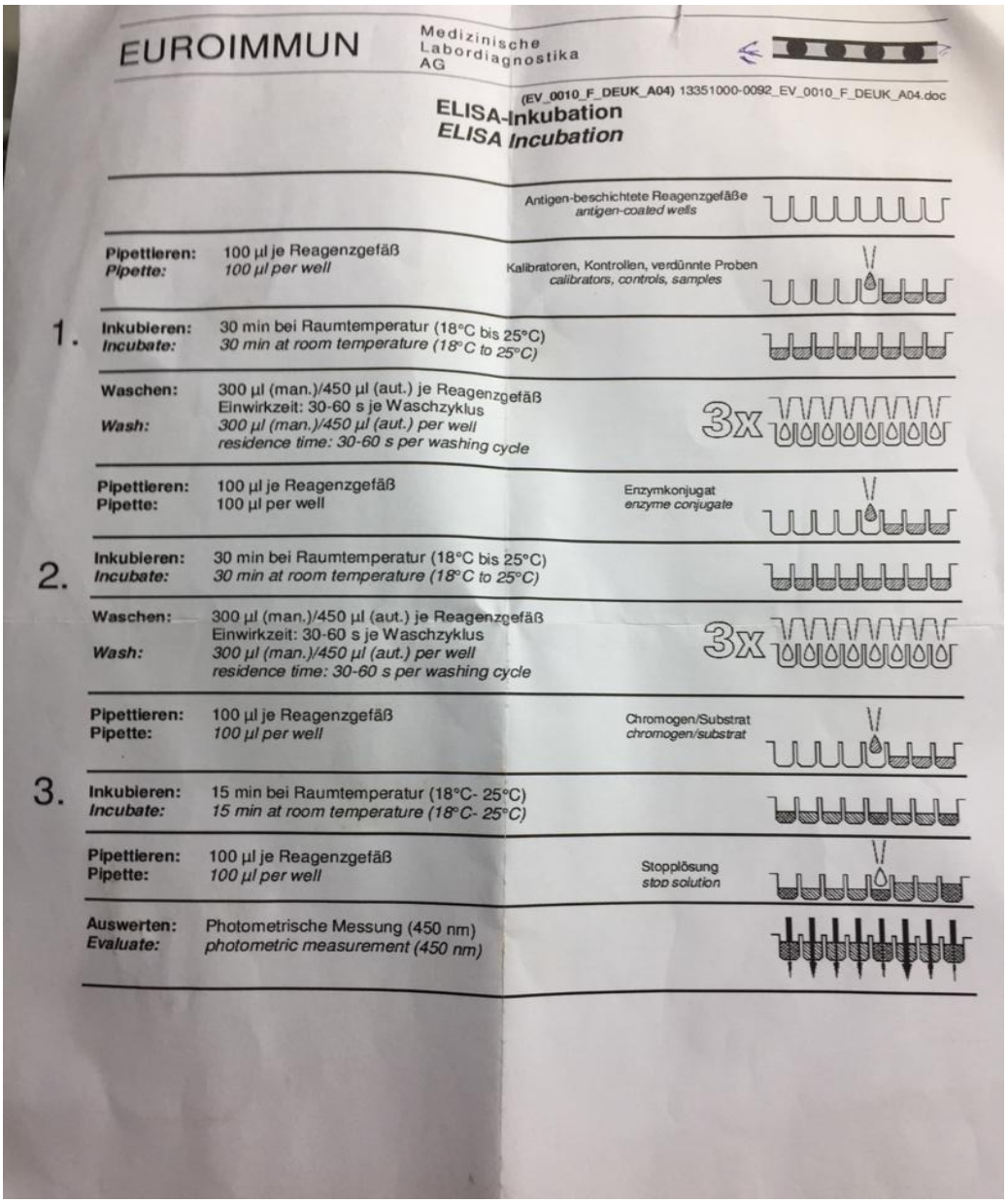
Drinking water sources: Non-filtered water ☐ Filtered water ☐

InvestigationResults:

HEV (IgM) antibodies: **+ve** ☐ **-ve** ☐

HEV (IgG) antibodies: **+ve** ☐ **-**ve ☐

APPENDIX 2



EUROIMMUN Medizinische Labordiagnostika AG

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ELISA-Inkubation
ELISA Incubation

Antigen-beschichtete Reagenzgefäße
antigen-coated wells

Pipettieren: 100 µl je Reagenzgefäß
Pipette: *100 µl per well*

Kalibratoren, Kontrollen, verdünnte Proben
calibrators, controls, samples

1. **Inkubieren:** 30 min bei Raumtemperatur (18°C bis 25°C)
Incubate: *30 min at room temperature (18°C to 25°C)*

Waschen: 300 µl (man.)/450 µl (aut.) je Reagenzgefäß
Einwirkzeit: 30-60 s je Waschzyklus
Wash: *300 µl (man.)/450 µl (aut.) per well*
residence time: 30-60 s per washing cycle

3x

Pipettieren: 100 µl je Reagenzgefäß
Pipette: 100 µl per well

Enzymkonjugat
enzyme conjugate

2. **Inkubieren:** 30 min bei Raumtemperatur (18°C bis 25°C)
Incubate: *30 min at room temperature (18°C to 25°C)*

Waschen: 300 µl (man.)/450 µl (aut.) je Reagenzgefäß
Einwirkzeit: 30-60 s je Waschzyklus
Wash: *300 µl (man.)/450 µl (aut.) per well*
residence time: 30-60 s per washing cycle

3x

Pipettieren: 100 µl je Reagenzgefäß
Pipette: *100 µl per well*

Chromogen/Substrat
chromogen/substrat

3. **Inkubieren:** 15 min bei Raumtemperatur (18°C- 25°C)
Incubate: *15 min at room temperature (18°C- 25°C)*

Pipettieren: 100 µl je Reagenzgefäß
Pipette: *100 µl per well*

Stopplösung
stop solution

Auswerten: Photometrische Messung (450 nm)
Evaluate: *photometric measurement (450 nm)*

APPENDIX 3

EUROIMMUN Medizinische Labordiagnostika AG

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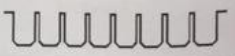
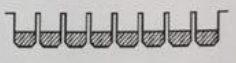
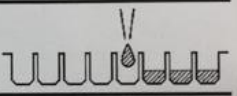
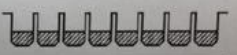
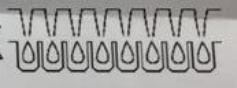
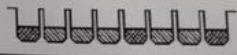
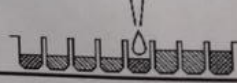
ELISA-Inkubation
ELISA Incubation

		Antigen-beschichtete Reagenzgefäße *antigen-coated wells*	
	Pipettieren: ***Pipette:***	100 µl je Reagenzgefäß *100 µl per well*	Kalibratoren, Kontrollen, verdünnte Proben *calibrators, controls, samples* 
1.	**Inkubieren:** ***Incubate:***	30 min bei Raumtemperatur (18°C bis 25°C) *30 min at room temperature (18°C to 25°C)*	
	Waschen: ***Wash:***	300 µl (man.)/450 µl (aut.) je Reagenzgefäß Einwirkzeit: 30-60 s je Waschzyklus *300 µl (man.)/450 µl (aut.) per well* *residence time: 30-60 s per washing cycle*	3x 
	Pipettieren: **Pipette:**	100 µl je Reagenzgefäß 100 µl per well	Enzymkonjugat *enzyme conjugate* 
2.	**Inkubieren:** ***Incubate:***	30 min bei Raumtemperatur (18°C bis 25°C) *30 min at room temperature (18°C to 25°C)*	
	Waschen: ***Wash:***	300 µl (man.)/450 µl (aut.) je Reagenzgefäß Einwirkzeit: 30-60 s je Waschzyklus *300 µl (man.)/450 µl (aut.) per well* *residence time: 30-60 s per washing cycle*	3x 
	Pipettieren: **Pipette:**	100 µl je Reagenzgefäß *100 µl per well*	Chromogen/Substrat *chromogen/substrat* 
3.	**Inkubieren:** ***Incubate:***	15 min bei Raumtemperatur (18°C- 25°C) *15 min at room temperature (18°C- 25°C)*	
	Pipettieren: **Pipette:**	100 µl je Reagenzgefäß *100 µl per well*	Stopplösung *stop solution* 
	Auswerten: ***Evaluate:***	Photometrische Messung (450 nm) *photometric measurement (450 nm)*