Sudan University of Science and Technology
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Serofrequency of Hepatitis E virus among Pregnant Women
Attending El Ribat University Hospital - Khartoum

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

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بسم الله الرحمن الرحيم

إِنَّا فَتَحْنَا لَكَ فَتْحًا مُّبِينًا (1) لِيُغْفِرَ لَكَ اللَّهُ مَا تَقَدَّمَ مِنْ ذَنْبِكَ وَمَا
تَأَخَّرَ وَيُعْمِمَ عَلَيْكَ وَيُهْدِيَكَ صِرَاطًا مُّسْتَقِيمًا (2) وَيَنْصُرِكَ اللَّهُ
نصيرًا عزيزًا (3)

صدق الله العظيم

سورة الفتح: الآيات (1-3)
Dedication

To who have been my constant sources of inspiration and guidance

Father,

Mother,

Lovely Daughter,

And Sisters
All praise belongs to ALMIGHTY ALLAH, the most merciful, the most beneficent and the most kind for giving me the strength and power to complete this work.

I would like to thank my supervisor Dr. Wafa Ibrahim Elhag, Deputy Dean Faculty of Medical Laboratory Sciences at AL-Neelain University, for helping me to conduct this research with providing any assistant requested, guidance and advice.

Also deep thanks to personnel of the Department of Obstetrician and Gynecologic, El Ribat University Hospital for their help during collection of clinical specimens.

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Abstract

Hepatitis E virus (HEV) is an important enteric human pathogen worldwide distribution. It can cause sporadic cases as well as large epidemics of acute hepatitis. Many studies proved that HEV infection in pregnancy leads to poor maternal and fetal outcome, especially in the third trimester of pregnancy.

The current study is descriptive cross-sectional study aimed to determine the prevalence of HEV among pregnant women attending El Ribat University Hospital in Khartoum, during the period from April to August 2015.

A total of 91 pregnant women were selected to participate in this study. Blood specimens were collected and serum was obtained then analysed for Anti HEV IgM by ELISA technique.

Data were collected by direct interviewing questionnaires including age, gestational age, history of previous abortion, educational status and occupational status.

Overall, the HEV IgM serofrequency rate among pregnant women was found to be 9 out of 91 (9.9%).

Most of studied populations were in age range (26-35 years), third trimester, had no past history of abortion, educated, and were housewives.

The result revealed that most of seropositive women (5(56%)) were in age group (15-25 years), (4(44.4%)) were in third trimester, (6(67%)) had no history of previous abortion, (7(78%)) among educated women, and (7(78%)) were housewives.

It is therefore necessary according to results of this study to incorporate HEV screening for pregnant women and further studies should be conducted to better understand of HEV and it is relation to risk factors.
فيروس التهاب الكبد هو مرض معدوي مهم يجعل الإنسان متضرر في أنحاء العالم. هذا الفيروس يمكن أن يسبب حالات متفرقة ضعف في ظهور وأنواع النباتات من التهاب الكبد الحاد. أثبتت العديد من الدراسات أن الإصابة بالتهاب الكبد الفيروسي (H) خلال فترة الحمل يؤدي لنتائج سيئة للأم والجنين، خاصة في الثلث الأخير من الحمل.

الدراسة الحالية هي دراسة وصفية مقطعية تهدف إلى تحديد انتشار الإصابة بالتهاب الكبد الفيروسي (H) بين النساء الحوامل اللائي تقدمن إلى مستشفى الرباط الجامعي في الخرطوم في الفترة من أبريل إلي أغسطس 2015، تم اختيار عدد 91 إمرأة حامل للمشاركة في هذه الدراسة، جمعت عينات الدم والأمراض التي تم الحصول عليها خلال الكشف عن الأجسام المضادة من نمط (الغلوبولين المناعي M) بواسطة طريقة المقاومة المفعولية المناعية للالنزم المرتب.

البحث: جمعت البيانات عن طريق المقابلات المباشرة وتضمنت العمر، مدة الحمل، حالات الإجهاض السابق، المستوى التعليمي، والحالة الوظيفية.

كشفت الدراسة أن العينات الإيجابية للأجسام المضادة نمط (الغلوبولين المناعي M) كانت بنسبة (9.9%) (9 من 91). بأغلب مجتمع الدراسة كان في الفترة العمرية (25-35 سنة)، في الثلث الثالث من الحمل، ليس لديه تاريخ إجهاض سابق، متعلمات وربات منازل.

أظهرت الدراسة أن العينات الإيجابية (56%) (منها كانت لنساء في الفترة العمرية (15-25 سنة)، (44.4%) في الثلث الثالث من الحمل، (67%) ليس لديهم تاريخ إجهاض سابق، (78%) من النساء المتعلمات وربات منازل.

وفقًا لنتائج هذه الدراسة من الضروري إدراج فحص الجسم المضادة للفيروس التهاب الكبد الوبائي (H) للحوامل وينبغي إجراء مزيد من الدراسات لفهم أفضل لالتهاب الكبد الفيروسي (H) وعلاقته بعوامل الخطر.
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CHAPTER ONE
INTRODUCTION
1. Introduction

Hepatitis E virus (HEV) infection is a major public health problem in developing countries, which could lead to an acute self-limiting hepatitis (epidemic or sporadic). It is the most common cause of fulminant hepatic failure in areas with endemic HEV infection (Teshale et al., 2010A).

In these areas, poor individual and public sanitation may lead to fecal-oral transmission of HEV and consequently large outbreaks may occur through contaminated water and foods. The endemic regions for this infection include large areas in Asia, Africa, the Mediterranean region, Mexico, and South America (Aggarwal and Naik, 2009).

The young adults and middle-aged populations are more likely to be infected than children and elderly persons (Begum et al., 2010). Although the disease is usually mild in general population, severe infection is more seen among pregnant women, which leads to a high rate of mortality in this population (Boccia et al., 2006).

The infection is associated with two distinct patterns of disease, in low income countries with poor sanitation and hygiene, HEV is a common cause of acute hepatitis, and is responsible for waterborne outbreaks and sporadic cases due to genotype 1 or 2 that exclusively infect humans. Disease has a high attack rate in young adults and is particularly severe among pregnant women where the mortality secondary to symptomatic infection was estimated tenfold higher than in men or non-pregnant women (Rein et al., 2012). In high income countries, HEV is responsible for sporadic cases due to genotypes 3 and 4 that also infect other animals, and zoonotic and food-borne transmission is suggested (Kamar et al., 2012). In these countries, the clinical presentation differs from disease in high endemic areas, including older age, more marked male predominance, higher frequency of underlying liver disease, and a lack of severe disease among pregnant women. Indeed, only few cases of hepatitis E during pregnancy have been reported (Andersson et al., 2008), and none with severe hepatitis. The role of nutritional, immunological, and genetic factors has been suggested in the pathophysiology of fulminant HEV during pregnancy in developing countries but the distinct clinical pattern between low and high income countries is still not understood. It may reflect differences in disease biology between different HEV genotypes but also a
reduced exposure to the virus because endemicity is low and undiagnosed asymptomatic infections in high income countries (Anty et al., 2012).

1.2 Rationale:
Hepatitis E (HEV) mostly causes a self limited disease in developing countries, but the nature of disease is more severe in pregnant women, due to hormonal changes (estrogen and progesterone) during pregnancy. The infection during pregnancy associated with poor foetal outcomes including abortion, premature delivery, and stillbirths (Tabatabi et al., 2014).

The mortality rate of pregnant women with HEV infection has been reported about 25%, which is much higher than general population (Mamun et al., 2009).

In Sudan, a high mortality rate was reported among pregnant women in an outbreak of HEV in Darfur and in eastern Sudan (Boccia et al., 2006).

Research on the seroprevalence of HEV is important for health policy makers as well as for the practicing clinicians and it will yield data necessary for developing preventive measures.

1.3 Objectives
1.3.1 General Objective
To determine serofrequency of HEV among pregnant women attending El Ribat University Hospital in Khartoum in the period from April to August 2015, using ELISA.

1.3.2 Specific Objectives
(i) To detect HEV IgM Antibodies.
(ii) To detect relation between the presence of HEV antibodies and other factors including age, gestational age, past history of abortion, level of education, and occupational status.
CHAPTER TWO

LITERATURE REVIEW
2. Literature Review

2.1 Discovery of HEV
Hepatitis E virus (HEV) was not recognized as a distinct human disease until 1980, when specific tests for antibody against hepatitis A were first applied to the study of epidemic waterborne hepatitis in India. The results showed that the epidemics were not epidemics of hepatitis A. Actually; very few epidemics of waterborne disease in developing countries of Asia and Africa have been linked to hepatitis A (Fujiwara et al., 2014). The first experimental evidence for the existence of an additional waterborne hepatitis agent was reported in 1983 (Feray et al., 2014), this form of non-A, non-B hepatitis came to be known as Enterically transmitted non-A non-B hepatitis (ET-NANB), and the agent of this disease was subsequently found to be the major cause of sporadic hepatitis cases in regions where the epidemic form was known to exist (Kamar et al., 2012).

2.2 Classification and Taxonomy of HEV
HEV was originally classified in the family Caliciviridae. However, because HEV genome does not share significant sequence homology with caliciviruses, the virus was subsequently declassified from the family Caliciviridae. Currently, HEV is placed in a sole genus Hepevirus within a new family Hepeviridae (Emerson et al., 2004). Currently, the species in the genus Hepevirus includes the four recognized major genotypes of HEV in mammalian species (genotype 1, 2, 3 and 4) (Meng, 2009). Recently, a novel strain of HEV was isolated from farm rabbits in China which appears to be genetically distinct from the four recognized mammalian genotypes, and thus probably represents an additional and fifth genotype within the genus Hepevirus (Zhao et al., 2009).

2.3 Morphology of HEV
HEV is a small and structurally simple RNA animal virus, the virion is non enveloped with a diameter of 27-34 nm, is composed entirely of viral protein and RNA. Electron microscopy (EM) analyses show spherical particles of possible icosahedral symmetry, with indefinite surface substructure, resembling the caliciviruses (Guu et al., 2009). Morphologically, HEV is similar to Norwalk virus, a member of the calicivirus family,
although the sequence of HEV most closely resembles the sequence of rubella virus, a
togavirus, and beet necrotic yellow vein virus, a plant furovirus (Li et al., 2005).

Image 1 Hepatitis E structure (Yamashita et al., 2009)

2.4 Genome and proteins

The hepatitis E genome consists of a linear, single-stranded, positive-sense RNA (that is,
mRNA) of approximately 7.5 kb containing a 3’ poly (A) tail and short 5’ and 3’
noncoding (NC) regions (Tyagi et al., 2005).

Three overlapping open reading frames (ORFs) exist, and all three coding frames are
used to express different proteins (Huang et al., 2007).

ORF1 (5 kb) is located towards the 5’ end of the genome and encodes a polyprotein of
about 1690 amino acids that probably undergoes post translational cleavage into multiple
nonstructural proteins required for virus replication, including a methyltransferase, a
putative papain-like cystein protease, an RNA helicase and an RNA-dependent RNA
polymerase (Sehgal et al., 2006).

ORF2 does not overlap with ORF1; it is located at the 3’-end of the genome and encodes
the principal and probably only structural protein. It is a capsid protein of 660 amino
acids (71 kDa) (Zhang et al., 2008).
ORF3 begins with the last nucleotide of ORF1; it overlaps extensively with ORF2 and is
the shortest of the open reading frames, encoding a small immunogenic 123 amino acid
phosphoprotein (14.5 kDa) which associates with the cytoskeleton, suggesting a possible
role in the assembly of virus particles (Takahashi et al., 2008).

2.5 Recent developments
In the last few years, there have been major advances in our understanding of the virus
and its structure, biology and molecular heterogeneity. In vitro systems using
complementary DNA clones that can transfect cultured cell lines, leading to replication of
viral RNA, expression of viral proteins and production of viable viral particles, have been
developed (Tanaka et al., 2007). Furthermore, in vitro cell culture systems for HEV, albeit relatively inefficient, have been developed. On the clinical front, occurrence of
persistent HEV infection in persons receiving immunosuppressive drugs, and those with
hematological diseases or HIV infection has been recognized and successful attempts at
drug therapy of such infection have been made. The most important advances include
development of two successful hepatitis E vaccines (Tanaka et al., 2009).

2.6 Mode of Transmission
The feco-oral route is the primary and most well documented mode of transmission. It is
more prevalent with HEV-1 and -2 and explains the endemicity and frequent outbreaks of
HEV-1 and -2 in developing countries (Alvarado et al., 2014).

In developed countries, some cases of vertical transmissions of HEV have been reported; however, transmission through breast milk has not been described (Mirazo et al., 2014).
HEV has recently been reported in homosexual men, which supports its sexual
transmission (Payne et al., 2013).

2.7 Clinical Presentation
Hepatitis E has variable clinical presentations and ranges from asymptomatic carriers to
fulminant hepatitis. As one would expect clinical manifestations to some extent depend
on the predominant genotype. In endemic areas where genotypes 1 and 2 are most
prevalent it primarily manifests as acute hepatitis. On the other hand in developed
countries genotypes 3 and 4 are more prevalent and patients are mostly asymptomatic
(Al-Shukri et al., 2013).
The incubation period is 3–8 weeks followed by a short prodromal phase. The symptomatic phase can last anywhere from days to several weeks (mean 4–6 weeks) (Hoofnagle et al., 2012). As with acute hepatitis from other etiologies, patients present with jaundice, right upper quadrant pain, and nondescript symptoms such as fever, asthenia, nausea, vomiting, and joint pains (Aggarwal, 2013). A wide range of extra hepatic manifestations have been attributed to HEV. Those associated with acute illness include rash and arthralgia, Guillain-Barre syndrome (Comont et al., 2014), myasthenia gravis, bilateral brachial neuritis, peripheral neuralgia with meningitis, seizures, nerve palsies, and pseudotumor cerebri (Belbezier et al., 2014).

2.7.1 Clinical presentations in hyperendemic areas

Frequent detection of anti-HEV antibodies among residents of high-endemic regions who do not recall prior acute hepatitis indicates that asymptomatic or in apparent HEV infection is common. During hepatitis E outbreaks, some persons show evidence of an icteric hepatitis (elevated liver enzymes with normal serum bilirubin) and HEV infection (HEV viremia and seroconversion) (Khuroo, 2010). Factors that determine disease severity are poorly understood. In animal studies, the viral inoculum dose determines severity of liver injury, and lower doses are associated with subclinical infection (Alvarado et al., 2014), the role of this factor in humans has not been studied. In areas where hepatitis E is common, HEV super infection can occur in patients with pre-existing chronic liver disease of viral or non-viral etiology, leading to superimposed acute liver injury and clinical presentation with acute or chronic liver disease. There is an evidence of recent HEV infection in nearly one-half of Indian patients with chronic liver disease and recent decompensation, such patients may be at a higher risk of a poor outcome.

In some patients, chronic liver disease had been clinically silent till the time of HEV super infection (Kumar et al., 2007).

Case-fatality rates of hepatitis E have been reported as 0.5% to 4%. However, these data are derived from hospitalized cases with more severe disease. In population surveys during disease outbreaks, much lower mortality rates of 0.07% to 0.6% have been observed (Boxall et al., 2006).
2.7.2 Clinical manifestations in areas with lower disease prevalence

In low-endemicity areas, the disease is most often recognized when serological tests are undertaken in patients with unexplained liver injury. Clinical illness in these patients is generally similar to that in high-endemicity regions, except that most patients have been middle aged or elderly men, who often had another coexistent disease (Borgen et al., 2008).

Common clinical presentations have included icteric hepatitis, an icteric illness with non-specific symptoms, and asymptomatic transaminase elevation (Dalton et al., 2008), some cases were initially suspected to have drug-induced liver injury. Prognosis of HEV infection appears to be worse in patients in these areas than those in high-endemicity areas, mainly because of their older age and higher frequency of coexistent illnesses (Dalton et al., 2007).

2.8 Prevalence

The highest prevalence of infection occurs in regions where low standards of sanitation promote the transmission of the virus (Sailaja et al., 2009).

The prevalence of antibody to HEV in suspected or documented endemic regions has been much lower than expected (3 - 26%) (Teshale et al., 2010A).

Screening of blood donors in central Europe and North America has shown prevalence of anti-HEV antibodies of 1.4 - 2.5%, in South Africa of 1.4%, in Thailand of 2.8%, in Saudi Arabia of 9.5% in Egypt of 24.0% (Echevarria, 2014).

The prevalence of antibody to HEV in non endemic regions (like the US) has been much higher than anticipated (1 - 3%) (Kuniholm et al., 2009).

2.8.1 Seroprevalence of HEV in developing countries

There are several studies that have examined the prevalence of antibodies against HEV in different population groups. However, the sero epidemiology of hepatitis E in developing countries is not uniform and often does not follow the pattern of clinical disease. Many studies have consistently observed that the prevalence of antibodies against HEV is low. In a study in Pune, India, researchers found that the prevalence of anti-HEV remained low until age 15 years at which point it slightly increased and peaked at around only 50% (Kuniholm et al., 2009).
There is no clear explanation for the relatively low prevalence of anti-HEV but it may be due to loss of serological evidence following natural infection (Christensen et al., 2008). On the contrary, serological data from Egypt have shown that anti-HEV could reach 100% with a very high prevalence even at a very young age (Faber et al., 2012).

2.8.2 Seroprevalence of HEV in developed countries
The discordance between seroprevalence and incidence of hepatitis E is unclear in developed countries. Despite the high seroprevalence in many European countries and the US, the occurrence of disease is generally low. As demonstrated by many studies, the anti-HEV prevalence in the general population is high and a number of studies have shown that the anti HEV prevalence among persons with close work contacts with pigs is even higher (Teshale et al., 2010B).

The HEV seroprevalence in most study populations is higher among older persons, generally increasing with age, but not different by gender (Drobeniuc et al., 2006).

In the US, in a nationally representative sample tested using the same assay, the HEV seroprevalence declined significantly during the period 1988-94 to 2009-10 from 21% to 10% (Wenzel et al., 2014). There is no clear explanation for this observed decline but a similar trend had been documented in Germany and Denmark (Teshale et al., 2010B).

2.9 Endemicity
Data on the endemicity of HEV infection have predominantly been collected in areas where outbreaks have been reported. As an exception, seroprevalence studies carried out in Egypt, where outbreaks of HEV have not been noted, showed rates of up to 60%, suggesting that most infections occurred early in life and were asymptomatic or mild (Gad et al., 2011).

Outbreaks have been reported from Algeria, Bangladesh, Borneo, China, Egypt, Ethiopia, Greece, India, Indonesia, Iran, Côte d’Ivoire, Jordan, Libya, Mexico, Myanmar, Nepal, Nigeria, Pakistan, southern Russia, Somalia, and eastern Sudan.

Most outbreaks have occurred following monsoon rains, heavy flooding, contamination of well water, or massive uptake of untreated sewage into city water treatment plants (Scotto et al., 2014).
2.10 Host immune response

Viremia in bile and serum and shedding of HEV in faeces reach their peak during the incubation period and keep constant levels in the acute phase of the disease, at the same time HEV antigens can be detected in the liver, although the period of infectivity after acute infection has not been determined, virus excretion in faeces has been demonstrated up to 14 days after onset of jaundice then disappears during the recovery phase (Mirazo et al., 2014).

Antibodies to HEV (IgM and IgG) develop at the time symptoms occur, usually before the development of jaundice. IgM anti-HEV precedes the IgG anti-HEV by a few days and viremia may persist after appearance of serum antibodies (Image 2) (Candido et al., 2012).

IgM anti-HEV titers decline rapidly during early convalescence 48 while IgG anti-HEV have been shown to persist for long periods of time (>14 yrs) and provide protection against subsequent infections (Bendall et al., 2010).

Image 2 Hepatitis E Virus infection, typical serologic course (Candido et al., 2012)

2.11 Chronic Hepatitis

It is usually caused by genotype 3, chronic infection secondary to genotypes 1 and 2 has not been documented (Arends et al., 2014), and one case of chronic HEV infection by genotype 4 has been reported in the literature (Geng et al., 2014).

Risk factors include immunosuppression, solid organ transplantation, HIV infection, hemodialysis, and hematological malignancies (Kamar et al., 2008).
Presence of chronic infection in immunocompromised patients carries a bad prognosis which if left untreated rapidly progresses to cirrhosis (10% in 2 years) and end-stage liver disease (Dalton et al., 2007).

2.12 Disease in pregnancy

Hepatitis E (HEV) mostly causes a self limited disease in developing countries, but the nature of disease is more severe in pregnant women due to many reasons: associated hormonal changes (estrogen and progesterone) during pregnancy, reduced expression of progesterone receptor and progesterone induced blocking factor, a higher IL-12/IL-10 ratio and down regulation of the P65 component of nuclear factor (NF-Kappa B) with a predominant T-helper type 2 (Th2) bias in the T-cell response along with host susceptibility factors, mediated by human leukocyte antigen expression (Kamar et al., 2012).

Also higher prevalence of folate deficiency in HEV in pregnant women of endemic areas, and a higher viral load in pregnancy due to the influence of sex hormones are some etiologies proposed for the worse prognosis of HEV infection in pregnancy (Andersson et al., 2008) which increased risk of prematurity, abortion, low birth weight, perinatal mortality (Navaneethan et al., 2008).

2.13 HEV in immunocompromised people

The unique characteristics of HEV genotype 3 infection is chronicity (persistence of HEV infection for at least 6 months) in persons who receive immunosuppressive therapy following solid organ transplantation (SOT) or persons with severe immunodeficiency from other causes (Fujiwara et al., 2014).

In solid organ transplant recipients, acute hepatitis E can progress to chronicity in up to 60% of infected patients (Kamar and Izopet, 2014).

Risk factors independently associated with chronic infection include heavy immunosuppression, reflected by a shorter time from transplantation to infection, lower CD2, CD3, CD4 and total lymphocyte counts as well as being on a tacrolimus versus a cyclosporine regimen (Halleux et al., 2012).

In one small study, about two third of SOT patients with acute hepatitis E progressed to chronic hepatitis E (Krain et al., 2014).
Solid organ transplant recipients are advised to avoid raw or undercooked pork and seafood to prevent HEV infection. A few small case series have shown that treatment with reduction of dose of immunosuppression therapy and ribavirin can result in a high rate of sustained virologic response. Although HIV infected patients are at risk for HEV infection, the number of acute infections is low and very few chronic cases were found thus far (Robbins et al., 2014).

A study of kidney transplant recipients that looked for chronic hepatitis E in India did not reveal chronic infection (Naik et al., 2013).

2.14 HEV in chronic liver disease patient

Persons with advanced liver disease, including cirrhosis, can develop acute hepatic failure when super-infected with HEV.

The data from developed countries is limited; there is a report of severe liver failure as a result of HEV infection of an undiagnosed case of cirrhosis (Crossan et al., 2014).

Hepatitis E was found to be the culprit in a number of studies where drug induced liver injury was erroneously diagnosed (Dalton et al., 2007).

The burden of HEV-induced acute liver failure in patients with pre-existing chronic liver disease is unknown (Davern et al., 2011).

2.15 Diagnosis

Clinically, hepatitis E is indistinguishable from hepatitis A, with elevated serum liver enzymes when hepatitis A has been ruled out, hepatitis E should be suspected, particularly in outbreaks of waterborne hepatitis occurring in developing countries, or with recent travel to endemic areas. HEV should be especially suspected in cases of fulminant hepatitis in pregnant women (Pischke and Wedemeyer, 2013).

Diagnostic methods are broadly classified into two types direct and indirect. The direct methods detect the virus, viral proteins, or nucleic acids in blood and stool samples by immune-electron microscopy and RT-PCR.

The indirect methods detect the anti-HEV IgM and IgG antibodies (Fujiwara et al., 2014).

Detection of anti-HEV IgM is considered diagnostic for acute infection. The presence of IgG antibodies points out to previous exposure to HEV (Arends et al., 2014). Anti-HEV IgM is detectable 4 days after the onset of jaundice and persists for up to 3–5 months.
Shortly after the appearance of IgM, IgG antibodies develop and peak at about 4 weeks after the onset of symptoms and persist for a variable period of 1 to 14 years after infection (Mirazo et al., 2014).

The detection of HEV RNA in biologic specimen (serum and/or stools) is the “gold standard” for the confirmation of acute HEV infection. HEV RNA can be detected in stools 1 week before and up to 6 weeks after the onset of symptoms and in serum for 3-4 weeks from the onset of illness (Arends et al., 2014).

The sensitivity of molecular tests for the detection of HEV RNA is dependent on how early the patient presents, timely collection of specimens along with its rapid transport, processing, and viral genotype inclusivity. Therefore, undetectable HEV RNA does not rule out recent infection (Vollmer et al., 2014).

PCR assays published so far have a high degree of performance variability. Therefore, World Health Organization (WHO) has recommended an international standard for HEV RNA detection and quantification that uses genotype 3a due to its worldwide distribution and its detection in chronic infections (Pavri et al., 2014).

Another nucleic acid amplification technique, the loop-mediated isothermal amplification (LAMP) assay, has been developed for the detection of HEV RNA. The LAMP assay is quicker than real-time PCR and does not need special equipment, making it ideal for resource limited areas (Kamar et al., 2011).

Insensitive and unspecific diagnostic tests for anti-HEV antibodies have made diagnosis challenging. In a study, only 13.3% of the samples, anti HEV IgM serology correlated to HEV polymerase chain reaction (PCR) positivity. This demonstrates an extremely low level of correlation with PCR confirmed HEV infections. Furthermore, false reactivity for anti-HEV IgM with Epstein-Barr virus (EBV) and cytomegalovirus (CMV), 33.3% and 24.2%, respectively, has been expressed in a study (Mirazo et al., 2014). This is a clinically important consideration because these viruses form the differential diagnosis for acute non-A, non-B hepatitis. Nonetheless, recently developed “point-of-care” assays for anti-HIV IgM are simple, rapid, highly sensitive, and specific, ideal for resource-limited areas. Recently, novel efficient cell cultures have been generated for HEV3 and HEV4 that permitted the propagation of HEV in fecal and serum samples (Echevarria, 2014).
Anti-HEV-IgG and -IgM are fairly reliable methods of diagnosis in immune-competent hosts. However, they are frequently false-negative in immunocompromised host, which imposes a diagnostic challenge (Hoofnagle et al., 2012). RT-PCR is recommended to diagnose HEV infection in this subset of patients. In this setting, HEV RNA detection and quantification also has a role in monitoring response to antiviral therapy and determining the genotype of HEV involved (Seo et al., 2012).

2.16 Background Studies

A study in Ethiopia was aimed to determine the seroprevalence and risk factors of HEV infection among pregnant women attending antenatal clinic (ANC) in Addis Ababa, Ethiopia from April 2014- January 2015. This study found a high seroprevalence rate of anti-HEV IgM among pregnant women 31.6 % (144/386) (Meseret, 2015).

Another study was conducted in India to determine seroprevalence of HEV in pregnant women attending Imam Khomeini general hospital in Ahvaz, India, in the period from January 2010 to January 2011. Overall, 5.26% (22/418) cases were positive for anti HEV IgM among pregnant women (Rasti et al., 2014).

Also a study was conducted in the Obstetrics and Gynaecology Outpatient Clinic of the KBTH, Accra, Ghana, to evaluate the prevalence of anti-HEV IgM among pregnant women seen between the months of January and May, 2008. This study indicate that Ghana is an endemic area for hepatitis E, with very high overall prevalence rates of HEV IgM 28.66% (45/157) (Adjei et al., 2009).

2.17 Prevention and Control

Acute hepatitis E is usually self-limiting and does not need treatment. Recent recognition of chronic HEV infection and the associated risk of progressive liver injury have led to attempts at antiviral treatment using pegylated interferon, ribavirin or both with fairly good results (Mallet et al., 2010). However, the published reports are mostly in the form of case reports or small case series. Whether these drugs will be useful in patients with FHF due to hepatitis E, or those with chronic liver disease and HEV super infection remains unclear. Teratogenicity of ribavirin may pose a problem for use during pregnancy. In view of the rapid downhill course of such patients, the temporal window of
opportunity for the drug to act and alter the outcome in such patients may also be limited (Kamar et al., 2010).

At present, no commercially available vaccines exist for the prevention of hepatitis E. However, several studies for the development of an effective vaccine against hepatitis E are in progress (Wedemeyer et al., 2012).

Recombinant vaccines:
A 55 kDa recombinant HEV-derived ORF2 protein has been used to vaccinate rhesus monkeys against different strains of hepatitis E. Although primates could still be infected, the vaccine protected them from the symptoms of disease.

Subunit HEV vaccines:
The direct intramuscular injection of purified plasmid DNA containing the full-length ORF2 of HEV has induced a prolonged humoral immune response (>12 months) to the expressed structural protein ORF2 in 80% and 100% of two separate groups of challenged mice, respectively.

Because swine HEV is immunologically cross-reactive with human HEV and their capsid genes are very conserved, swine HEV may prove useful as an attenuated vaccine for immunization against human hepatitis E through the “Jennerian” approach (Zhu et al., 2010).

The HEV vaccine which is in the most advanced stages of development is HEV 239. It is a Chinese manufactured vaccine that has a 94–100% efficacy in a phase III trial conducted on more than 100,000 Chinese soldiers (Zhu et al., 2010).

prevention of viral diseases remains the most important weapon for their control, as almost all HEV spread by the faecal-oral route, good personal hygiene, high quality standards for public water supplies and proper disposal of sanitary waste have resulted in a low prevalence of HEV infections in many well developed societies (Kamar et al., 2012).

For travelers to high endemic areas, the usual elementary food hygiene precautions are recommended, these include avoiding drinking water and eating uncooked shellfish, uncooked fruits or vegetables that are not peeled or prepared by the traveler (Dalton et al., 2008).
CHAPTER THREE

MATERIALS AND METHODS
3. Materials and Methods

3.1 Study design
This was descriptive and cross-sectional study.

3.2 Study duration
This study was conducted during the period from April to August 2015.

3.3 Study area
The study was conducted in El Ribat University Hospital in Khartoum.

3.4 Study population
All pregnant women attending El Ribat University Hospital during the study period were included.

3.5 Sample size
A total of 91 pregnant women were participated in this study.

3.6 Data collection
Data collected by direct interviewing questionnaires included age, gestational age, history of previous abortion, educational status, and occupational status, (Appendix1).

3.7 Ethical consideration
The ethical clearance was obtained from the Ethical Committee Board of Sudan University of Science and Technology and permission letter to collect specimen (Appendix 2), Informed consent was obtained from each pregnant lady after describing the goal of the study, any favourable outcome and potential risks that might be encountered.

3.8 Experimental Work
3.8.1 Collection of Specimen
Three ml of venous blood were collected from each participant under Aseptic condition into sterile plain container and allowed to clot at room temperature. The sera were obtained by centrifugation of the blood at 3000 rpm for 5 minutes. The serum was separated from the clot and transferred into new sterile labeled plain containers and stored at -20°C until used.
3.8.2 Specimens processing

Specimens were analysed for HEV IgM by Enzyme linked immunosorbent assay (ELISA), (Anti-HEV ELISA IgM, EUROIMMUN Medizinische Labordiagnostika AG, Germany) (Appendix 3).

3.8.2.1 Assay principle (Appendix 4)

3.8.2.2 Assay method

Preparation of the samples
The samples were diluted 1:101 with sample buffer (Appendix 5).

Numbering the wells
The strips needed were set in strip holder and sufficient number of wells including one blank (B), two calibrator (C1, C2), positive control (PC), and negative control (NC) were numbered.

Adding samples
Amount of 100µl of diluted samples, positive controls, negative controls and calibrators were added into their respective wells by using separate disposable pipette tip for each specimen, negative and positive controls to avoid cross contamination, and then mixed by taping the plate gently to avoid over flowing and contamination of adjacent wells in order to fully distribute the samples.

Incubation (1)
The plate was covered with plate cover and incubated for 30 minutes at room temperature (18-25°C).

Washing (1)
After the end of incubation the plate cover was removed and discarded. The wells were washed manually with 300µl of working strength wash buffer 3 times, wash buffer was left for 30 to 60 seconds per washing cycle, then the wells were emptied.

Adding HRP-conjugate
An amount of 100µl of HRP-conjugate was added into each of the microplate wells.

Incubation (2)
The plate was covered and incubated for 30 min at room temperature (18-25°C).

Washing (2)
After the end of incubation the plate cover was removed and discarded. The wells were washed with diluted washing buffer 3 times.

**Coloring**
An amount of 100µl of chromogen/substrate solution were added into each well (Appendix 6). The strips were covered with plate cover and incubated at room temperature for 15 minutes avoiding direct sun light. The enzymatic reaction between the chromogen solutions produced blue color in positive control and anti HEV positive sample wells.

**Stop reaction**
Amounts of 100µl of stop solution (0.5M Sulphuric acid) were added into each wells and mixed by tapped the plate gently, intensive yellow color developed in positive sample wells (Appendix 7).

**Measuring the absorbance**
Photometric measurement of the color intensity was calibrated with blank well and the absorbance was read at wavelength of 450nm and the reference wavelength between 620 nm and 650 nm within 30 minutes of adding the stop solution.

**3.8.2.3 Interpretation of the result**
Each micro plate has been considered separately when calculating and interpreting results of the assay, regardless of the number plates concurrently processed. The results are interpreted as a ratio of the sample OD (450nm) and cut-off value (CO).

**Calculation of cut-off value**
The extinction value of the calibrator defines the upper limit of the reference range of non infected persons (Cut-off) recommended by kit manufacture (EUROIMMUN), values above the indicated cut-off are to be considered as positive, those below as negative.

The ratio for each specimen was calculated as follow:

\[
\text{Ratio} = \frac{\text{Extinction of the control or patient sample}}{\text{Extinction of calibrator}}
\]

*Ratio < 0.8: Negative*
*Ratio ≥ 0.8 to < 1.1: Borderline*
*Ratio ≥ 1.1: Positive*
**Negative result**

Sample giving absorbance less than the cut-off value are negative for this assay, which indicate that no antibody to hepatitis E virus has been detected with this anti hepatitis E virus ELISA kit. The patient is probably not infected with hepatitis E virus.

**Border line**

Sample with absorbance OD greater or equal cut-off are considered borderline and retesting of those samples should be taken 7 days later and re-tested in parallel with the first patient. For duplicate determinations the mean of the two values should be taken.

**Positive result**

Sample giving an absorbance greater than or equal to the cut-off value are considered initially reactive which indicates that antibody to hepatitis E virus have probably been detected using this anti HEV ELISA kit.

**3.9 Data Analysis**

Data was entered and organized into Microsoft Office Excel 2007 data sheet, then transferred to statistical package program SPSS (version16). Descriptive analysis was performed for all variables where frequencies and percentages was used to express categorical variables, followed by detection the significance of frequencies distribution among each variable using the General trend analysis. Comparisons between categorical variables were performed using Cross-tabulations.
CHAPTER FOUR

RESULTS
4. Results

A total of 91 pregnant women who attended El Ribat University hospital in Khartoum were enrolled in this study during the period from April to August 2015, to determine serofrequency of HEV, and to detect relation between the presence of HEV and other factors (age, gestational age, history of previous abortion, level of education, and occupation).

The overall HEV IgM seroprevalence rate among pregnant women was found to be (9.9%) (9 out of 91) (Figure1).

Most of studied pregnant women 46(50.5%) were belonged to age group (26-35 years) however highest seropositivity 5(56%) observed among (15-25 years) (Table1), and most of them 37(41%) were in third trimester, also highest seropositivity 4(44.4%) was observed among this group (Table2).

Most of studied pregnant women 66(72.5%) had no history of previous abortion, also highest seropositivity 6(67%) was observed among this group (Table 3). 66(72.5%) of studied pregnant women were educated, and highest frequency of HEV seropositivity 7(78%) among educated women (Table 4), and 76(83.5%) of studied pregnant women were housewives, also highest seropositivity 7(78%) observed among this group (Table5).
Figure 1 Serofrequency of HEV IgM among pregnant women (n=91)
Table 1 Distribution of positive HEV IgM among pregnant women (n=91) according to their age groups

<table>
<thead>
<tr>
<th>Age groups (years) No (%)</th>
<th>HEV IgM</th>
<th>P.value</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-25</td>
<td>32 (35.2%)</td>
<td>5 (56%)</td>
</tr>
<tr>
<td>26-35</td>
<td>46 (50.5%)</td>
<td>3 (33%)</td>
</tr>
<tr>
<td>36-45</td>
<td>13 (14.3%)</td>
<td>1 (11%)</td>
</tr>
<tr>
<td>Total = 91(100%)</td>
<td>9 (100%)</td>
<td>82 (100%)</td>
</tr>
</tbody>
</table>

P. value < 0.05 consider significant
**Table 2** Frequency of HEV IgM among the pregnant women (n=91) according to their Gestational age

<table>
<thead>
<tr>
<th>Gestational age</th>
<th>HEV IgM</th>
<th>P.value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (%)</td>
<td>Negative (%)</td>
</tr>
<tr>
<td>First Trimester</td>
<td>2 (22.2%)</td>
<td>21 (26%)</td>
</tr>
<tr>
<td>23 (25%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second Trimester</td>
<td>3 (33.3%)</td>
<td>28 (34%)</td>
</tr>
<tr>
<td>31 (34%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Third Trimester</td>
<td>4 (44.4%)</td>
<td>33 (40%)</td>
</tr>
<tr>
<td>37 (41%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total = 91(100%)</td>
<td>9 (100%)</td>
<td>82 (100%)</td>
</tr>
</tbody>
</table>

P. value < 0.05 consider significant
Table 3 Distribution of positive HEV IgM among pregnant women (n=91) according to their history of abortion

<table>
<thead>
<tr>
<th>Past history of Abortion</th>
<th>HEV IgM</th>
<th>P.value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (%)</td>
<td>Negative (%)</td>
</tr>
<tr>
<td></td>
<td>3 (33%)</td>
<td>22 (27%)</td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 (27.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>6 (67%)</td>
<td>60 (73%)</td>
</tr>
<tr>
<td>66 (72.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total = 91(100%)</td>
<td>9 (100%)</td>
<td>82 (100%)</td>
</tr>
</tbody>
</table>

P. value < 0.05 consider significant
Table 4 Distribution of positive HEV IgM among pregnant women (n=91) according to their educational status

<table>
<thead>
<tr>
<th>Educational Status</th>
<th>HEV IgM</th>
<th>P.value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (%)</td>
<td>Negative (%)</td>
</tr>
<tr>
<td></td>
<td>Positive (%)</td>
<td>Negative (%)</td>
</tr>
<tr>
<td>Educated</td>
<td>7 (78%)</td>
<td>59 (72%)</td>
</tr>
<tr>
<td>66 (72.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non Educated</td>
<td>2 (22%)</td>
<td>23 (28%)</td>
</tr>
<tr>
<td>25 (27.2%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total = 91(100%)</td>
<td>9 (100%)</td>
<td>82 (100%)</td>
</tr>
</tbody>
</table>

P. value < 0.05 consider significant
Table 5 Distribution of positive HEV IgM among pregnant women (n=91) according to their occupational status

<table>
<thead>
<tr>
<th>Occupational Status</th>
<th>HEV IgM</th>
<th>P.value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (%)</td>
<td>Negative (%)</td>
</tr>
<tr>
<td>Employed</td>
<td>2 (22%)</td>
<td>13 (16%)</td>
</tr>
<tr>
<td>15 (16.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Housewives</td>
<td>7 (78%)</td>
<td>69 (84%)</td>
</tr>
<tr>
<td>76 (83.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total = 91(100%)</td>
<td>9 (100%)</td>
<td>82 (100%)</td>
</tr>
</tbody>
</table>

P. value < 0.05 consider significant
CHAPTER FIVE

DISCUSSION
5.1 Discussion

Hepatitis E virus (HEV) cause epidemic, especially in developing countries where hygiene is poor and many affected pregnant women suffer from hepatitis (Pal et al., 2005).

IgM antibody to HEV in healthy subject has been used to measure the virus which is appears early during acute clinical illness but wanes over a few months, so it detect acute HEV infection (Boccia et al., 2006).

In the present study, the seroprevalence of anti-HEV IgM was (9.9 %) (9 out of 91) among pregnant women.

When compared with different previous studies it founds to be slightly lower than that reports from Africa which demonstrated a rate of 12% in Tunisia (Hannachi et al., 2011),14% in Gabon (Caron and Kazanji, 2008) and 28% in Ghana (Adjei et al.,2009), for anti-HEV IgM seroprevalence among pregnant women.

In Sudan, a fatality rate of 17.8% was found during an outbreak in Darfur, with a rate of 31.1% among pregnant women (Boccia et al., 2006).

Also another study in Khartoum State conducted by Alngashi, 2014 revealed higher rates of HEV infection (14.5%), however the present study result was higher than that which obtained by Walla, 2014 study also in Khartoum state, showed that (3.3%) of pregnant women were seropositive for anti-HEV IgM.

Most of studied population were belonged to (26-35 years) age range (46(50.5%)), however highest positivity observed among (15-25 years) age range (5(56%)) our result showed that HEV was decreased with age, these results found to be in agreement with other studies in Khartoum State, (Zuhal et al., 2014) found 45.9% seropositive HEV IgM in youngest age group , also (Adjei et al., 2009) showed similar result with (43%), while it disagreed with Stoszek et al., 2006 study in Egypt, who reported high rate of HEV in older age.

Consistent with these results, our findings revealed that most of studied pregnant women were in third trimester, also highest seropositivity (4(44.4%)) was observed among this group. This result is in agreement with Rasti et al., 2014 study in Ahfaz, India and Alngashi, 2014 study in Khartoum. Since women in the third trimester of pregnancy
associated with high levels of steroid hormones, these steroid hormones are immunosuppressive and mediate lymphocyte apoptosis that may promote viral replication. It also has a direct inhibition on hepatic cells, which may predispose to hepatic dysfunction when exposed to infectious Pathogens (Navaneethan et al., 2008).

Most of studied pregnant women had no past history of abortion (66(72.5%)), also highest seropositivity was observed among this group (6(67%)), our study revealed that there is no clear association between HEV and past history of abortion which similar with (Eltayeb et al., 2015) study in Wad Medani, Sudan which showed no significant difference between HEV and history of miscarriage.

Our results demonstrated that educational status had no significant difference in the occurrence of HEV infection, in which most of studied pregnant women were educated (66(72.5%)), also highest seropositivity was observed among this group (7(78%)), this may due to fact that educated women are more exposed to HEV infection since they are spending long time out houses.

Our result found to be in agreement with Walla, 2014 study in Khartoum State, while it disagree with another Turkish study (Oncu et al., 2006), which indicate that rate of HEV seropositivity was significantly higher in women with a lower education degree compared to women with a higher education degree.

According to occupational status, most of studied pregnant women were housewives (76(83.5%)), also highest seropositivity (7(78%)) was observed among this group, there is no association between occupational status and HEV infection in pregnant women according to study result. This indicates that housewives need awareness program about HEV infection, the route of transmission and important of eating clean and well cooked food.

The difference of the relation between our study and risk factors, from other studies may due to variation in study sample size, study area, awareness of HEV infection among different population, different of Food and water safety in different study area, and variation in sensitivity and specificity of the test performance.
5.2 Conclusion
This study found a high frequency of anti-HEV IgM among Sudanese pregnant women who attend in El Ribat University hospital in Khartoum. HEV IgM antibodies were detected in 9.9% (9 out of 91). The highest percentages were recorded in the third trimester of pregnancy. The serofrequency was highest among age group (15-25 years). There was insignificant statistical correlation between history of abortion, education, occupation and HEV seropositivity.

5.3 Recommendations
- Conduct further studies with advanced techniques to better understand the risk factors of HEV infection.
- Conduct awareness program about HEV infection, the route of transmission and it is affect on pregnant women and newborn.
- HEV screening must checked with routine investigation for pregnant women, which help in early detecting and controlling any possible HEV complication for mother and baby.
- Reinforce the importance of food safety and establish routine screening of HEV in food maker and all people work in food manufacture, this provide an important preventing agent to reduce the risk of HEV and other enteric infections for our community.
References


43. Khuroo MS (2010). Sero-epidemiology of a second epidemic of hepatitis E in a population that had recorded first epidemic 30 years before and has been under surveillance since then. *Hepatol Int.* 4 (2):494-499.


APPENDICES
Appendix (1)

Questionnaire

Sudan University of Science and Technology

College of Graduate Studies

Title: Serofrequency of HEV among pregnant women attending El Ribat University Hospital - Khartoum

Prepared by : Asma Saad Ibrahim Algadi
Supervisor : Dr Wafa Ibrahim Elhag

Name ……………………… Serial number……………………

Age : 15-25 ( ) 26-35 ( ) 36-45 ( )

Education : Educated ( ) Non Educated ( )

Job ………

Gestational age :

First trimester ( ) Second trimester ( ) Third trimester ( )

History of abortion : Yes ( ) No ( )

Specimen: Serum ( ) Other ( )

Method: ELISA IgM for HEV

Laboratory Findings:

Positive ( ) Negative ( )
Appendix (2)

Permission letter for specimen collection
Appendix (3)

Euroimmun Anti-HEV ELISA (IgM) kit
Appendix (4)

Assay principle

This is an ELISA assay for semi-quantitative determination for human antibodies of the IgM in serum or plasma. The assay is intended to be used in clinical laboratories for diagnosis and management of patients to infection with hepatitis E virus. A solid phase antibody capture ELISA assay in which polystyrene microwell strips are coated with recombinant antigens of hepatitis E virus. The patients’ serum samples added, and during the first incubation step, any IgM class antibodies will be captured in the well. After washing all other substances removed, the specific HEV IgM captured o is then detected by the addition of anti human IgM labeled with enzyme horseradish peroxidase (HRP-conjugate). During second incubation, the Anti human IgM-HRP conjugated will specifically react only with HEV IgM antibodies. After washing to remove the unbound HRP-conjugate, chromogen solutions are added into the wells. In presence of HEV IgM the colorless chromogens are hydrolyzed by the bound HRP-conjugate to a blue colored product. The blue color turns yellow after stopping the reaction with sulfuric acid. The amount of color intensity can be measured which proportional to the amount of antibody captured in the wells, wells negative for HEV IgM remain colorless.
Appendix (5)

Diluted samples
Appendix (6)

HEV IgM Microplate
Substrate Incubation
Appendix (7)

HEV IgM Microplate result