

بسم الله الرحمن الرحيم



Sudan University of Science and Technology

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**Seroprevalence of Hepatitis E Virus
among Blood Donors in Omdurman Locality**

الانتشار المصلي لفيروس الكبد الوبائي E لدى المتبرعين بالدم

- محلية أم درمان

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By:

Alaaeldeen Balal Ahmed Abdalgader

B.Sc in Medical Laboratory Science, Omdurman Islamic
University (Microbiology), 2012

Supervisor:

Dr. Yousif Fadlalla Hamedelnil

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بسم الله الرحمن الرحيم

قال تعالى :

يَا أَيُّهَا الَّذِينَ آمَنُوا إِذَا قِيلَ لَكُمْ تَفَسَّحُوا فِي الْمَجَالِسِ فَافْسَحُوا
يَفْسَحِ اللَّهُ لَكُمْ ۖ وَإِذَا قِيلَ انشُزُوا فَانْشُزُوا يَرَفَعِ اللَّهُ الَّذِينَ آمَنُوا
مِنْكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ ۚ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ

صدق الله العظيم

سورة المجادلة (الآية 11)

DeDication

To my family

To my teachers

To those who interested in microbiology in the world

Student who want to be pioneers microbiologist

Acknowledgement

Piously my gratitude and prayers to ALMIGHTY ALLAH for the mercy which followed me during the long path of this research.

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Abstract

This study was conducted to determine the seroprevalence of Hepatitis E Virus (HEV) infection among blood donors attending Blood Bank department at Omdurman Teaching Hospital during the period from April to July 2014.

A total of ninety subjects ($n = 90$) were included in this study. The blood donors ages range from 22 to 50 and all of them were males. From the study participants, serum samples were obtained and personal and clinical data were collected and tested for HEV IgM and IgG antibody using Enzyme Linked Immunosorbent assay (ELISA).

Out of the 90 blood donors tested, 26.7% and 7.8% were HEV IgG and IgM positive, respectively. Subjects were divided into two age groups (≤ 31 years and > 31 years) in order to evaluate the influence of age on HEV seropositivity. From the above finding this study revealed that there was significant difference ($P < 0.05$) between the two age groups on HEV IgG seropositivity.

Also this study concluded that, there was high percentage of hepatitis E virus (HEV) infection among blood donors, and there was no significant difference ($P > 0.05$) between the residence and HEV seropositivity. Since about 7.8% of blood donors are seropositive for HEV IgM, it would be very useful to screen the blood donors for HEV to avoid the transmission of HEV to the patients. The use of HEV vaccine is recommended to decrease the transmission of infection.

ملخص الأطروحة

أجريت هذه الدراسة لتحديد معدل الانتشار المصلي للإصابة بفيروس التهاب الكبد E لدى المتبرعين بالدم بينك الدم في مستشفى أم درمان التعليمي في الفترة من ابريل إلى يوليو 2014.

شملت هذه الدراسة 90 شخصا من المتبرعين بالدم , وتراوحت أعمار المشاركين في الدراسة من 22 إلى 50 سنة وجميعهم من الذكور. تم جمع عينات المصل من المشاركين بالدراسة وتم جمع المعلومات الشخصية والطبية وأخذت العينات وفحصت لمعرفة احتوائها على أجسام مضادة من النمط IgG و IgM لفيروس التهاب الكبد E باستخدام اختبار الإليزا.

من مجموع 90 شخصا من متبرعي الدم 26.7% كانت لديهم أجسام مضادة من النمط IgG و 7.8% كانت لديهم أجسام مضادة من النمط IgM. تم تقسيم أعمار المشاركين إلى مجموعتين (≤ 31 years and > 31 years) خلصت الدراسة إلي انه توجد فروق ذات دلالة إحصائية ($P < 0.05$) بين مجموعتي أعمار المشاركين في الدراسة ومعدل ايجابية HEV IgG.

كما خلصت هذه الدراسة إلى أن هناك نسبة عالية من عدوى فيروس التهاب الكبد E بين المتبرعين بالدم , أيضا كما خلصت على انه لا توجد فروق ذات دلالة إحصائية ($P > 0.05$) بين مكان الإقامة ومعدلات ايجابية HEV IgG/IgM , وحيث ان معدل ايجابية HEV IgM كان 7.8% فمن المهم جدا اختبار عينات المتبرعين بالدم لفيروس التهاب الكبد E وذلك للحد من انتقال الفيروس للمرضى. كما أوصت باستخدام لقاح فيروس الكبد E لتقليل انتقال العدوى.

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CHAPTER 0nE

INTRODUCTION

1. Introduction

1.1. Background

Hepatitis E virus (HEV) is a spherical, non-enveloped, single-stranded RNA virus that belongs to the new genus, *Hepevirus* (Okamoto, 2007).

HEV is the major cause of enterically transmitted non-A, non-B (NANB) hepatitis in many parts of the world (Balayan, 1997).

Hepatitis E is an acute disease with abrupt onset of nonspecific symptoms followed by jaundice, anorexia, malaise, nausea and vomiting (Teshale and Hu, 2011). It affects primarily young adults and is generally mild (Aggarwal and Krawczynski, 2000). Among all the responsible hepatitis viruses, HEV is associated with the largest number of fulminant hepatitis cases (3.0%) in the general population, and up to 20% in pregnant women (Bradley, 1990).

HEV is significant international public health problem and is estimated that 2.3 billion people are infected globally (WHO, 2010).

Epidemiological studies have shown that HEV infection is quite common in some areas of the world such as India, Africa, and Southeast Asia, where it can represent the main cause of acute hepatitis (Krawczynski *et al.*, 2001). Hepatitis E virus causes sporadic infection, but also large epidemics, usually transmitted through orofecal route due to contaminated food or water (Emerson and Purcell, 2003; Irshad, 1999).

HEV can be transmitted by blood transfusion and has recently been found in donated blood in a number of countries and the increased HEV incidence raises concern about the safety of blood and blood products (Dalton *et al.*, 2013).

1.2. Rationale

Hepatitis E virus is probably the most common cause of acute hepatitis and jaundice in the world (Purcell and Emerson, 2008).

Blood-borne transmission of HEV had been investigated as indirect evidence implicating HEV as a potential transfusion risk by many investigators worldwide. It has been reported that a substantial proportion of blood donors (1.5%) were positive for HEV RNA and viraemic blood donors are potentially able to cause transfusion-associated hepatitis E in areas of high endemicity (Arankalle and Chobe, 2000). several case reports from Europe and Japan have documented clinical hepatitis in patients after being transfused with blood products from an HEV infected donors (Boxall *et al.*, 2006; Matsubayashi *et al.*, 2011).

Patients who are immunosuppressed (such as solid organ transplant recipients, patients treated for malignancies or those infected with HIV), pregnant or have chronic liver disease which commonly receive blood transfusions, these patients are at risk of fulminant hepatitis or chronic rapidly progressive liver disease from an HEV infection (Hoofnagle *et al.*, 2012). Therefore; this study was conducted to identify the prevalence of HEV among blood donors in Omdurman Locality.

1.3. Objectives

1.3.1. General objective

To determine the seroprevalence of hepatitis E virus (HEV) among blood donors in Omdurman Locality.

1.3.2. Specific objectives

1.3.2.1. To determine the infected blood donors with hepatitis E virus in Omdurman Teaching Hospital.

1.3.2.2. To detect the presence of HEV IgM and IgG antibodies among blood donors by using Enzyme Linked Immunosorbent Assay (ELISA).

1.3.2.3. To evaluate whether the rate of seroprevalence of HEV IgM and IgG antibodies is associated with the sociodemographic characteristics.

CHAPTER TWO

LITERATURE REVIEW

2. Literature review

2.1. History

Hepatitis E Virus (HEV) was discovered after a large epidemic acute viral hepatitis which caused 29,000 cases that occurred in 1955-56 in New Delhi, India, following severe flooding that led to contamination of drinking water with sewage. The outbreak was initially thought to be caused by hepatitis A virus (HAV) because that was the only hepatitis virus recognized as waterborne at that time (Khuroo, 1980). Fifteen years later, sera from those infected in the Delhi epidemic and two subsequent outbreaks in India (Ahmedabad and Pune) were tested. None of these patient samples were determined to have evidence of HAV infection and only 1% had evidence of HBV infection. Therefore, it was determined that the outbreaks were caused by an unknown enteric non-A, non-B hepatitis agent (Wong *et al.*, 1980). This new agent was recognized in 1980 but the virus was not visualized until 1983 by Mikhail Balayan and colleagues in Moscow using immune electron microscopy of feces (Balayan *et al.*, 1983).

After the genome was cloned and sequenced in 1991, the virus became known as hepatitis E virus (Tam *et al.*, 1991).

2.2. Classification

Hepatitis E virus was tentatively assigned to the Caliciviridae family for some years on the basis of its particle structure and overall genome organization. However, detailed analysis of the viral genome showed that it had many features that were inconsistent with classification in any existing virus family (Koonin *et al.*, 1992). HEV has now been assigned to the new genus *Hepevirus*, family Hepeviridae (Emerson *et al.*, 2005).

2.3. Molecular virology

Virions of HEV are non-enveloped, icosahedral particles of around 32-34 nm in diameter (Bradley *et al.*, 1988).

Hepatitis E virus has a single-stranded RNA genome of positive polarity that contains three open reading frames (ORFs), organized as 5'-ORF1-ORF3-ORF2-3', with ORF3 and ORF2 largely overlapping (Emerson *et al.*, 2001). ORF1 encodes the nonstructural, enzymatic activities required for viral replication, and ORF2 encodes the structural, viral capsid that includes neutralizing epitopes. The function of ORF3 is unknown, but it appears to be necessary for cellular egress (Emerson *et al.*, 2010).

The genomic structure of HEV is unique and defines the Hepeviridae family, of which it was the first member to be identified (the genus *hepevirus*). The four genotypes of HEV that have been identified fall into two major groups. Genotypes 1 and 2 are human viruses that have been identified as causing epidemic hepatitis and are associated with water borne and fecal–oral transmission (Meng, 2011).

Genotypes 3 and 4 are swine viruses that are common in domestic and wild pigs and appear to infect humans as an accidental host, these viruses are thus zoonoses (Meng, 2011). There is cross-neutralization among the four HEV genotypes, indicating that they belong to a single serotype despite clinical and epidemiologic differences (Tang *et al.*, 2011). HEV grows poorly in vitro, but recently, several cell-culture systems have been developed for genotypes 3 and 4 (Takahashi *et al.*, 2012).

The virus appears to be relatively stable to environmental and chemical agents (Jothikumar *et al.*, 1993).

2.4. Routes of transimission

HEV is mainly an enterically transimitted virus (via the fecal-oral route) that causes waterborne epidemics in developing countries and sporadic cases in developed countries. There are four reported routes of transimission, namely waterborne, zoonotic (foodborne), bloodborne and perinatal (Mushahwar, 2008).

Hepatitis E is not commonly transmitted via person-to-person contact (in contrast to hepatitis A), so household transmission is therefore not a significant risk factor. Nosocomial infections with HEV are probably rare, but transfusion-associated HEV has been reported in developed countries including Japan, the United Kingdom and France, and Saudi Arabia (Matsubayashi *et al.*, 2004).

2.5. Risk groups

People who at risk of contracting HEV (WHO, 2001):

- Persons residing in areas where extended community outbreaks exist.
- International travelers to regions of the world where HEV is endemic.
- Refugees residing in overcrowded temporary camps following catastrophes, especially in Sudan, Somalia, Kenya and Ethiopia.
- Persons who have chronic liver disease.
- Possibly persons working with non-human primates, pigs, cows, sheep and goats.

2.6. Epidemiology

Apart from hepatitis A, hepatitis E is another important enteric infection causing large scale outbreaks in many parts of the world. Since the 1950, epidemics of enterically transmitted non-A, non-B hepatitis have been frequently documented in the Indian sub-continent. Large epidemics of hepatitis E have been reported in southeast and central Asia, northern and western Africa, and Mexico. In some highly endemic areas, HEV infection also accounts for more than 50% of acute sporadic hepatitis. Infection occurs more frequently in regions with hot climates. The highest prevalence of infection occurs in regions where low standards of sanitation promote the transmission of the virus. One of the largest waterborne hepatitis E outbreaks occurred in Kanpur city, India in 1991 where over 79,000 clinical cases were reported. The source of this outbreak was traced to fecal contamination of drinking water supplied from the river (Naik *et al.*, 1992).

In parts of Africa (Sudan, Chad, Uganda, Kenya and Somalia) a number of large hepatitis E outbreaks have occurred among persons living in refugee camps or internally displaced persons camps (Boccia *et al.*, 2006).

Persons living in such camps may not have adequate access to clean water and sanitary conditions. Furthermore, such populations may be vulnerable to infectious diseases because of crowded living and poor nutrition, leading to higher risk of exposure to infectious agents and poor immune response during infectious exposures. Available medical care services may not be optimal and thus mortality from serious complications of infection may be high. This may explain in part the observed high mortality during hepatitis E outbreaks in Africa (Teshale and Hu, 2011).

Outbreaks have been reported among migrant worker who move to cities and reside in crowded urban slums (Shidrawi *et al.*, 1994).

2.6.1. Geographic Distribution According to Genotypes

The genomes of many HEV strains from Asia, South and North America have been entirely or partially sequenced. The human HEV strains have at least four major groups (Purcell and Emerson, 2008).

Human genotype 1 has been the major cause of water-borne epidemics in Asia and north Africa and a significant cause of sporadic disease, while human genotype 2 has been recovered from epidemics in Mexico and central Africa. Hepatitis E caused by genotypes 3 and 4 more commonly occurs in developed countries and on average affects older people (Purcell and Emerson, 2008).

2.7. Pathogenesis

2.7.1. Incubation period

The incubation period from exposure to the onset of clinical disease is approximately 15 to 60 days, with a mean of 40 days (Teshale *et al.*, 2010).

2.7.2. Viral replication

Although some progress has been made in understanding the replication cycle of HEV with use of animal models, cell culture systems, and infectious cDNA clones, most aspects of viral replication in vivo can only be inferred. After ingestion of HEV, the virus may be absorbed directly through the gastrointestinal mucosa into the circulation to reach the liver or after one or more rounds of amplification in enterocytes. However, no evidence has been obtained for replication of HEV at this site, in contrast to HAV for which replication in enterocyte derived Caco₂ cells is well established (Blank *et al.*, 2000).

HEV is likely to interact with one or more specific receptors/coreceptors leading to penetration and uncoating of the virus, and the input viral RNA then serves as mRNA for PORF1 (Takahashi *et al.*, 2008).

The PORF1 polyprotein is then cleaved by viral (and perhaps cellular) proteases to yield the mature replicative proteins, but the viral protease activity has not been directly demonstrated. The viral RNA-dependent RNA polymerase (RDRP) then copies the input viral genome to yield negative-strand RNA, which in turn serves as a template for the transcription of further positive-strand RNA molecules, including new genomes and the subgenomic, bicistronic RNA encoding ORF2 and ORF3 proteins. Genomic RNA assembles together with PORF2, although the precise form involved in particle assembly (full length or truncated, glycosylated or nonglycosylated) is unclear. PORF3 may also associate with the virus particle during assembly, although it is dispensable for replication *in vitro*. The virus is then released from the hepatocyte via unknown mechanisms (Takahashi *et al.*, 2008). Most of the virus is likely to be excreted through the biliary system to complete the transmission cycle (Arankalle *et al.*, 1993).

2.8. Clinical features

Clinical features of HEV infection range from asymptomatic hepatitis to severe fulminant hepatitis, which can result in liver-related mortality. The asymptomatic patient typically clears the virus rapidly, while the symptomatic patient experiences clinical signs and symptoms including anorexia, hepatomegaly, myalgia, sometimes abdominal discomfort, fever, nausea, vomiting, general weakness, and jaundice lasting for 1 to 6 weeks after an incubation period of 2 to 6 weeks (Panda *et al.*, 2007; Meng, 2010).

HEV causes self-limited, acute hepatitis in immunocompetent hosts. However, persistent HEV infection accompanied by chronic hepatitis and liver cirrhosis has been documented in immunocompromised hosts, such as organ transplantation recipients (liver, kidney, or pancreas), lymphoma, leukemic patients and HIV infected patients (Pischke *et al.*, 2010; Dalton *et al.*, 2009).

Hepatitis E has been shown to produce a range of extra-hepatic manifestations, including arthritis, acute pancreatitis, thrombocytopenia, aplastic anaemia (Shah *et al.*, 2012).

Of most interest, 5% of cases present with a neurological illness. The spectrum of neurological injury is wide and includes Bell's palsy, encephalitis, brachial neuropathy, peripheral neuropathy and Guillain-Barre' syndrome (Kamar *et al.*, 2011).

The typical infection begins with an incubation period of 2 weeks to 2 months and a transient viraemia followed by viral shedding in the feces, disappearance of viraemia with onset of clinical signs, and regression of viral shedding with potential jaundice setting in around 2-3 weeks into the infection (Haagsma *et al.*, 2008). The severity of HEV infection is considered dose-dependent and host factors such as concurrent hepatic disease or alcohol overuse may also contribute to the disease course (Yugo and Meng, 2013).

2.9. Immune response

Typically, both IgG and IgM antibodies are detectable at the onset of disease, which allows serologic diagnosis of infection at the time of presentation of the patient. IgM declines to undetectable levels over a period of 2 to 6 months, and an approximately 10-fold decline in IgG levels is seen over this period. Levels then stabilize, but the duration of

protective immunity is unknown (Li *et al.*, 1994).

Immunoglobulin A (IgA) responses to HEV (as a correlate of mucosal immunity) have been detected in around 50% of patients (Chau *et al.*, 1993). These antibodies rapidly declined to undetectable levels, although IgA may persist somewhat longer than IgM (Tian *et al.*, 2006).

The role of IgA in immunity to HEV infection is unknown, but because passive immunization with IgG appears to be sufficient for protection, IgA is likely not essential (Tsarev *et al.*, 1994).

Cellular immune responses during HEV infection have not been extensively studied. In patients with HEV, T-cell responses (proliferation) to peptide libraries derived from the PORF2 and PORF3 proteins are observed with peptide pools derived from PORF2 but not PORF3 (Viswanathan, 1957).

2.10. Laboratory diagnosis

2.10.1. Virus isolation

Establishment of a practical cell culture system to allow the propagation of HEV *in vitro* is vital for virological characterization as well as for diagnosis and prevention of HEV infection. Several *in vitro* culture systems, such as human lung, kidney or liver (2BS, A549, Hep-G2) and macaques hepatocytes for HEV replication have been reported. Most of these, however, cannot provide authentic HEV particles or a high titre of viral particles and have poor reproducibility (Tam *et al.*, 1997; Worm *et al.*, 2002).

2.10.2. Immune electron microscopy (IEM)

IEM detects viral particles in clinical specimens (Balayan *et al.*, 1983). HEV particles are precipitated with the native antibody to HEV derived from acute or convalescent phase sera. Anti-HEV antibodies

concentrations can be determined semiquantitatively by rating the antibody coating. Although immune electron microscopy (IEM) is a superior technique for specificity, the sensitivity of the assay is insufficient for routine analysis. IEM is difficult to perform and most clinical specimens do not contain sufficient virus like particles (VLPs) to be detected (Yarborough, 1999). Other antigen detection methods have not been reported (Anderson and Shrestha, 2002).

2.10.3. Immune fluorescence microscopy (IFE)

A few specialized laboratories use this technique for the detection of antibodies. IFE detects antibodies that react against the HEV antigen semiquantitatively. Anti-HEV antibodies block the binding of fluorescein-conjugated anti-HEV IgG to HEV antigen in frozen liver tissue. The concentration of anti-HEV antibodies is estimated semiquantitatively. This method is laborious and expensive and thus not useful for routine diagnosis (Vasickova *et al.*, 2007).

2.10.4. Diagnostic assay formats

Based on the analysis of serum specimens collected during various stages of human HEV infection, which include the incubation period, acute and convalescent phases, a classic serological pattern of IgM and IgG anti-HEV appearances has been observed (Khudyakov and Kamili, 2011).

IgM anti-HEV appears during the early acute phase of illness and may be detected as early as 4 days after the onset of jaundice and lasts for up to five months (Khudyakov and Kamili, 2011).

The serological appearance of IgM anti-HEV is succeeded shortly by IgG anti-HEV, so that both seem to appear almost simultaneously in the acute phase of infection. IgG anti-HEV persists for a longer time and may be

detectable for 1-14 years (Khudyakov and Kamili, 2011). HEV RNA can be detected in serum and stool of infected patients during the acute phase of infection by RT-PCR using conventional and real time formats (Mushahwar, 2008).

2.10.5. Rapid diagnostic assays

In addition to standard enzyme immunoassays, other assays formats like rapid tests have also been evaluated for HEV serology.

The immunochromatographic methods for the detection of serological markers of infections are fast and simple and are frequently formatted into rapid diagnostic assays suitable for point of care testing. A rapid immunochromatographic assay ASSURE™ has been developed by Genelabs Diagnostics, Singapore and evaluated for the detection of IgM anti-HEV in serum specimens from patients with acute hepatitis E infection (Khudyakov and Kamili, 2011).

This test is an IgM-capture lateral-flow immunochromatographic assay. In this format, anti-HEV IgM is captured by anti-human IgM monoclonal antibody immobilized onto the membrane and detected using a colloidal gold-labeled HEV antibody attached to HEV antigen. Rapidity and simplicity to perform are major advantages of the ASSURE™ HEV IgM test. It was recently found to be highly sensitive (82%) and specific (100%) in detection of acute HEV genotype 3 infections (Khudyakov and Kamili, 2011).

2.10.6. Enzyme immunoassay (EIA)

2.10.6.1. Indirect ELISA

Indirect ELISA is one of the most popular diagnostic formats. In this format, HEV-specific immunoglobulins captured from serum specimens by HEV antigens attached to the solid-phase surface (usually wells of

microtiter plates) are detected using labeled species-specific antibody. The differential detection of IgG or IgM is achieved using , for example, antibody against gamma-or mu-chains of antibodies, correspondingly. There are numerous examples of application of this format to the detection of IgG anti-HEV in serum specimens using E. coli-expressed HEV antigens (Obriadina *et al.*, 2002) and virus-like particles expressed in insect cells (Khudyakov and Kamili, 2011).

2.10.6.2. Sandwich ELISA

The detection of anti-HEV in humans or swine requires assays designed specifically for specimens from human or animals. The host-independent detection of acute or past HEV infections has significant advantages for epidemiological investigations in the field. The format that allows for such host-independent detection is the double-antigen sandwich ELISA. In this format, antigen attached to solid phase is used to capture specific antibody from serum specimens. Detection of this antibody is achieved using the same antigen labeled with, for example, horseradish peroxidase (Khudyakov and Kamili, 2011).

2.10.7. Western blot-based assay

HEV recombinant proteins have been used in different formats of diagnostic assays. A Western blot-based assay for the detection of HEV IgG and IgM antibodies was developed using a recombinant polypeptide containing the C-terminal half of the ORF2-encoded protein (Khudyakov and Kamili, 2011). Another Western blot assay based on a different set of fusion proteins containing sequences from ORF2 and ORF3 of the HEV genotype 1 strain was used to examine temporal appearance of HEV antibodies in experimentally infected rhesus monkeys (Khudyakov and Kamili, 2011).

2.10.8. Molecular detection of HEV

Nucleic acid-based techniques, especially nested RT-PCR and real-time RT-PCR, have emerged rapidly as the method of first choice for sensitive and specific detection of RNA viruses (Vasickova *et al.*, 2007). However, nested RT-PCR is prone to contamination and virus quantification cannot be undertaken. To overcome these difficulties, rapid and sensitive real-time RT-PCR assays have been developed for the detection of HEV RNA in clinical samples (Ahn *et al.*, 2006; Enouf *et al.*, 2006). It should be noted that the choosing a suitable method for RNA extraction to ensure an adequate recovery of intact viral RNA and the elimination of inhibitory substances, is very important for the successful detection of viral genomes (Jothikumar *et al.*, 2006).

2.11. Treatment of HEV infection

There is no specific antiviral therapy for hepatitis E, and supportive care is the main therapy (Kamar *et al.*, 2010).

Most cases of acute HEV infection are self-limiting and require no treatment. However, patients with or without pre-existing chronic liver disease with acute severe HEV infection, have been treated successfully with ribavirin monotherapy (Gerolami *et al.*, 2011).

Pegylated interferon could induce sustained virological response in the post transplantation setting (Kamar *et al.*, 2010).

2.12. Prevention and control of hepatitis E virus infection

Two subunit vaccines have been developed against HEV infection and have been shown to be highly protective against clinical hepatitis E in clinical trials (Shrestha *et al.*, 2007). Currently only one of these vaccines has been licensed and that too only in China. Though several additional pieces of information are needed before these vaccines can be used for prevention of hepatitis E in general population. In the meanwhile, our knowledge about epidemiology of hepatitis E allows us to device and use

non-vaccine approaches for prevention of this disease. In high endemicity areas, these primarily include provision of safe drinking water, proper disposal of human feces, and education about personal hygiene. In addition, during outbreaks, boiling and chlorination of water should be useful. In contrast, in low endemicity areas with zoonotic transmission, sanitary handling and proper cooking of pig and deer meat may be important (Aggarwal, 2013).

2.13. Background studies in Sudan

In Sudan studies on patients with acute hepatitis during the floods of 1988 in Khartoum demonstrated that infection was mainly due HEV (58%) with low incidence of HAV infection (5.45%). Amongst children with acute hepatitis in Khartoum state concluded that HEV was also the commonest cause of acute clinical hepatitis among the pediatric population with HEV infection at 59%, HAV at 33.3%, and HBV at 2.6% (Mudawi, 2008).

The largest documented outbreak of HEV infection in displaced populations was reported from Mornay camp in Western Darfur in 2004, when, out of total population of 78.800 people, 2621 were infected with HEV, with an attack rate of 3.3% and an overall case-fatality rate of 1.7% (Mudawi, 2008).

Recent study was conducted in Sudan, have revealed a high frequency of anti- HEV IgG among Sudanese pregnant women in Khartoum (Al-Tayeb *et al.*, 2014).

CHAPTER THREE

MATERIALS AND METHODS

3. Materials and Methods

3.1. Study design

This study is a descriptive cross sectional study conducted to determine prevalence of HEV infection among blood donors in Omdurman Teaching Hospital.

3.2. Study area and population

Blood donors attending blood bank department at Omdurman Teaching Hospital.

3.3. Study period

This study was conducted during the period from April to July (2014).

3.4. Sampling technique

The study was based on non-probability convenience sampling technique during attendance of blood donors to Omdurman Teaching Hospital.

3.5. Sample size and sample collection

A total of ninety specimens (n=90) were collected from blood donors under aseptic technique in sterile plain containers and allowed to clot at room temperature. The sera were obtained by centrifugation (3000 rpm for 5 mins) and separated from the clot then transferred into new sterile labeled plain containers and stored at -20°C until processing.

3.6. Sample processing

All the blood specimens collected were tested for the presence of anti-HEV IgM and IgG antibodies using the commercially available ELISA kit.

3.7. Data collection

Personal and clinical data were obtained by direct interviewing questionnaire from each subject.

3.8. Data analysis

Collected data were analyzed by a computer system using statistical package for social sciences (SPSS-s) program using the Chi square test and crosstabulation. Statistical significant was set at P .values < 0.05.

3.9. Ethical consideration

Permission to carry out the study was taken from the College of Graduate Studies, Sudan University of Science & Technology. All subjects examined were informed for the purpose of the study before collection of the specimens and verbal consent was taken from them.

3.10. Laboratory work

All serum samples were analyzed for specific anti-HEV IgM and IgG using enzyme-linked immunosorbent assay (ELISA).

3.10.1. ELISA for detection of anti-HEV IgM

3.10.1.1. Principle

This kit is two-steps incubation, solid-phase antibody capture ELISA assay in which polystyrene microwell strips are pre-coated with antibodies directed to human immunoglobulin M proteins (anti- μ chain). The patient's serum sample is added, and during the first incubation step, any IgM-class antibodies will be captured in the wells. After washing out all the other substances of the sample and in particular IgG-class antibodies, the specific HEV IgM captured on the solid phase is detected by the addition of recombinant HEV ORF2 antigen conjugated to the enzyme horseradish peroxidase (HRP-conjugate). During the second incubation, the HRP-conjugated antigens will specifically react only with HEV IgM antibodies. After washing to remove the unbound HRP-conjugate, chromogen solutions are added into the wells. In the presence of (anti- μ) – (anti-HEV-IgM) – (HEV Ag-HRP) immunocomplex, the colorless chromogens are hydrolyzed by the bound HRP-conjugate to blue-colored product. The blue color turns yellow after stopping the

reaction with sulfuric acid. The amount of color intensity can be measured and is proportional to the amount of antibody captured in the wells, and to the amount of antibody in the sample respectively. Wells containing samples negative for IgM remain colorless (Wantai, China).

3.10.1.2. Procedure

Reagents preparation

The reagents and samples were allowed to reach room temperature (18-30°C) for 15-30 minutes. The stock wash buffer had been diluted 1 to 20 with deionized water.

Numbering the wells

The strips needed were set in strip holder and sufficient number of wells including one blank (A1), three negative control (B1, C1, D1) and two wells as positive control (E1, F1) were numbered.

Adding diluent

100 µl of specimen diluent were added into each well except the blank.

Adding samples

10 µl of samples, positive and negative controls were added into their respective wells except the blank.

Incubation of samples

The plate was covered with the plate cover and incubated for 30 minutes at 37° C.

Washing (1)

After the end of the incubation, the plate cover was removed and discarded. Each well was washed 5 times with diluted washing buffer. Each time the wells were allowed to soak for 30-60 seconds. After the washing cycle, the plate was turn down onto blotting paper to remove any remainders.

Adding HRP-Conjugate

100 µl of HRP-Conjugate reagent were added into each well except the blank.

Incubating HRP-Conjugate

The plate was covered with the plate cover and incubated for 30 minutes at 37° C.

Washing (2)

The plate cover was removed and discarded. Each well was washed 5 times with diluted washing buffer. Each time the wells were allowed to soak for 30-60 seconds. After the washing cycle, the plate was turn down onto blotting paper to remove any remainders.

Coloring

50 µl of Chromogen A (Urea peroxide solution) and 50 µl of Chromogen B (Tetramethyl benzidine dissolved in citric acid) were added into each well including blank. The plate was incubated at 37 °C for 15 minutes with avoiding light. Blue color was developed in positive control and HEV IgM positive sample wells.

Stopping Reaction

50 µl of Stop solution (diluted sulfuric acid) were added into each well and mixed gently. Intensive yellow color was developed in positive control and HEV IgM positive sample wells.

Measuring the absorbance

The absorbance was read at 450nm using ELISA reader.

3.10.1.3. Calculation of the results and quality control**Calculation of results**

The results were calculated by relating each specimen absorbance (A) value to the cut-off value (C.O) of the plate.

Cut-off value = $N_c + 0.26$ (N_c = the mean absorbance value for three negative controls).

Quality control (assay validation)

The A value of the blank well, which contains only Chromogen and Stop solution should be less than 0.08 at 450 nm.

The A value of the positive control must be more than or equal 0.800 at 450 nm.

The A value of the negative control must be less than 0.100 at 450 nm.

3.10.1.4. Interpretation of the results**Negative results**

Samples giving A value less than cut-off value are negative for this assay, which indicates that no HEV IgM antibodies have been detected with Wantai HEV-IgM ELISA kit, therefore there are no serological indications for current infection with HEV.

Positive results

Samples giving A value which is equal to, or greater than cut-off value are considered initially reactive, which indicates that IgM-class antibodies to hepatitis E virus have probably been detected using this HEV-IgM ELISA kit.

Borderline

Samples with A value to cut-off ratio between 0.9 and 1.1 are considered borderline and retesting of these specimens in duplicates is required to confirm the initial results.

3.10.2. ELISA for detection of anti-HEV IgG**3.10.2.1. Principle**

Bioelisa HEV IgG is an immunoenzymatic method in which the wells of a microplate are coated with three recombinant Hepatitis E antigens from the structural region of the Hepatitis E virus. Test specimens, diluted in diluents buffer, are incubated in these coated wells. HEV if present, will bind to the solid phase HEV antigens. The wells are thoroughly washed to remove unbound materials and a goat IgG anti-human IgG labeled with

peroxidase is added to the wells. This labeled antibody will bind to antigen-antibody complexes previously formed. Any excess unbound-labelled antibodies are removed by washing. After washing, an enzyme substrates solution containing a chromogen is added. This solution will develop a blue colour if the sample contains anti-HEV IgG. The blue colour changes to yellow after blocking the reaction with sulphuric acid. The intensity of the colour is, in general, proportional to the anti-HEV IgG content of the test sample (Biokit, Barcelona, Spain).

3.10.2. 2. Procedure

Reagents preparation

The reagents and samples were allowed to reach room temperature (20-25° C) and gently mixed. The stock wash buffer had been diluted 1 to 20 with deionized water and working conjugate was prepared.

Numbering the wells

The strips needed were set in strip holder and sufficient number of wells including one blank (A1), three negative control (B1, C1, D1) and two wells as positive control (E1, F1) were numbered.

Adding diluent

100 µl of specimen diluent were added into each well except the blank.

Adding samples

10 µl of samples, positive and negative controls were added into their respective wells except the blank.

Incubation of samples

The microplate was covered with the adhesive seal and incubated for 30 minutes at 37° C.

Washing (1)

After the end of the incubation, the adhesive seal was removed and discarded. Each well was washed 5 times with diluted washing buffer. Each time the wells were allowed to soak for at least 15 seconds. After

the last washing, the microplate was turn down onto absorbent tissue to remove any excess liquid from the wells.

Adding HRP-Conjugate

100 µl of diluted conjugate reagent (goat IgG anti-human IgG conjugated with peroxidase) were added into each well except the blank.

Incubating HRP-Conjugate

The plate was covered with an adhesive seal and incubated for 30 minutes at 37° C.

Washing (2)

The adhesive seal was removed and discarded. Each well was washed 5 times with diluted washing buffer. Each time the wells were allowed to soak for at least 15 seconds. After the last washing, the microplate was turn down onto absorbent tissue to remove any excess liquid from the wells.

Coloring

100 µl of substrate-TMB solution (Tetramethylbenzidine) were added into each well including blank. The plate was incubated for 15 minutes at room temperature (20-25° C).

Stopping Reaction

The reaction was stopped by adding 100 µl of stopping solution (Sulphuric acid).

Measuring the absorbance

The absorbance was read at 450nm using ELISA reader.

3.10.2.3. Calculation of the results and quality control

Calculation of the results

The results were calculated by dividing the sample absorbance by the cut-off value (C.O) of the plate.

Cut-off value = $N_c + 0.500$ (N_c = the mean absorbance value for three negative controls).

Quality control

Substrate blank : absorbance value must be less than or equal to 0.100.

Negative control: each individual absorbance value must be less than or equal to 0.100 after subtracting the blank.

Positive control: each individual absorbance value must be more than or equal 0.700 after subtracting the blank.

3.10.2.4. Interpretation of the results**Negative results**

Ratio absorbance/cut-off < 0.9

Positive results

Ratio absorbance/cut-off ≥ 1.0

Equivocal results

Ratio absorbance/cut-off $\geq 0.9 < 1.0$

CHAPTER FOUR

RESULTS

4. Results

4.1. Frequency of HEV IgG among blood donors

Out of the 90 blood donors tested, 24 subjects (26.7%) were HEV IgG positive, while 66 subjects (73.3%) were negative for HEV IgG (Figure 4.1).

4.2. Frequency of HEV IgM among blood donors

Out of the 90 blood donors tested, 7 subjects (7.8%) were HEV IgM positive, while 83 subjects (92.2%) were negative for HEV IgG (Figure 4.2).

4.3. Influence of age on HEV IgG and IgM seropositivity among blood donors

The mean age of subject was 31 years, subjects were divided into two age groups (≤ 31 years and > 31 years) in order to evaluate the effect of age on HEV seropositivity, there was significant difference ($P < 0.05$) between the two age groups on HEV IgG seropositivity whereas there was no significant difference ($P > 0.05$) between the two age groups on HEV IgM seropositivity (Table 4.1).

4.4. Influence of residence on HEV IgG and IgM seropositivity among blood donors

The results presented in table 4.2 demonstrate that there was no significant difference ($P > 0.05$) between the residence (urban or rural areas) and HEV IgG and IgM seropositivity.

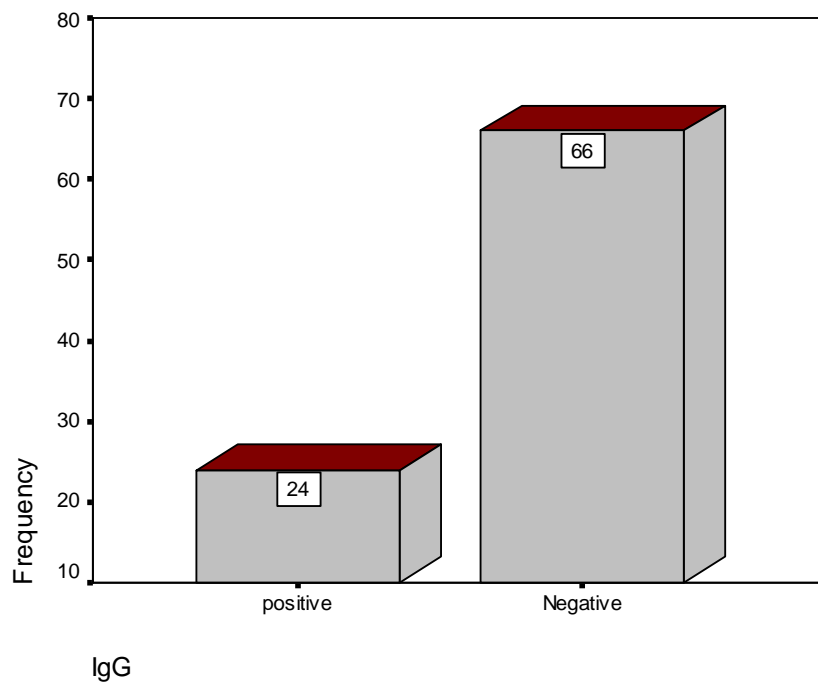


Fig. 4.1. Frequency of HEV IgG among blood donors

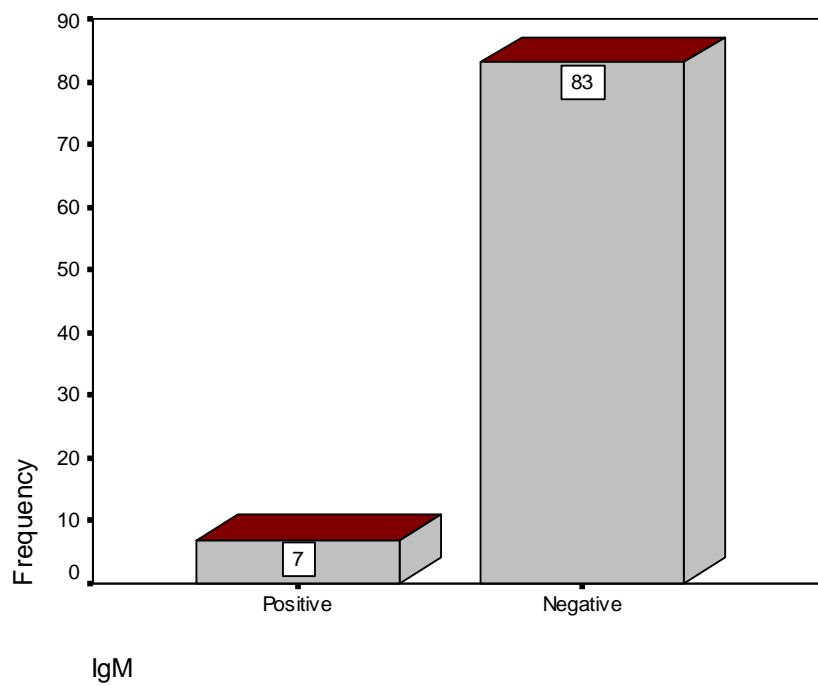


Fig. 4.2. Frequency of HEV IgM among blood donors

Table 4.1: Influence of age on IgG and IgM seropositivity among the blood donors

Serological marker (Anti-HEV antibodies)		Age groups (Years)			
		≤ 31 years		> 31 years	
		NO	%	NO	%
Anti HEV IgG	Positive	9	10.0	15	16.7
	Negative	40	44.4	26	28.9
Anti HEV IgM	Positive	2	2.2	5	5.6
	Negative	47	52.2	36	40.0

Influence of age on IgG seropositivity: *P*. value = 0.04.

Influence of age on IgM seropositivity: *P*. value = 0.15.

Table 4.1: Influence of residence on IgG and IgM seropositivity among the blood donors

Serological marker (Anti-HEV antibodies)		Residence			
		Urban		Rural	
		NO	%	NO	%
Anti HEV IgG	Positive	16	17.8	8	8.9
	Negative	53	58.9	13	14.4
Anti HEV IgM	Positive	4	4.4	3	3.3
	Negative	65	72.2	18	20.0

Influence of residence on IgG seropositivity: *P*. value = 0.17.

Influence of residence on IgM seropositivity: *P*. value = 0.20.

CHAPTER FIVE

DISCUSSION

5.1. Discussion

This study was conducted on 90 blood donors, and revealed that the seroprevalence among blood donors in blood bank of Omdurman Teaching Hospital was 26.7% and 7.8% for anti-HEV IgG and anti-HEV IgM, respectively.

The seroprevalence rate of anti-HEV IgM observed in this study was 7.8% which considerably higher than those findings previously reported by Johargy *et al* (2013) in Makkah, Saudi Arabia (4.3%), Ibrahim *et al* (2011) in Egypt (0.45%) and Guo *et al* (2010) in Hong Kong which have reported prevalence rates of 3.6%.

However, the prevalence rate of anti-HEV IgG among our blood donors was higher than those reported by Johargy *et al* (2013) in Makkah, Saudi Arabia (18.7%), Mansuy *et al* (2008) in South West France (16.6%), Assarehzadegan *et al* (2008) in Khuzestan Province, Southwest Iran (11.5%), Taremi *et al* (2007) in Tabriz, Islamic Republic of Iran (7.8%). Also our anti-HEV IgG rate was higher to that reported in Northern France (3.2%) (Boutrouille *et al.*, 2007), in Brazil (2.3%) (Bortoliero *et al.*, 2006). Also higher than recent studies which have revealed seroprevalence rates of 13.5%, 16.6% and 20.6% among blood donors in England, France and Denmark, respectively (Kaufmann *et al.*, 2011).

Seroprevalence in Sudan was lower than countries of the Eastern Mediterranean Region where reports of up to 52% seroprevalence for anti-HEV have been reported (Aminiafshar *et al.*, 2004).

The low or high rates observed by different investigators could be attributed to several factors including the size of study population, differences in the demographics of the population studied, incidence rate of the virus in different environments, the living conditions, and the

situation with regard to public health services and in the HEV antibody detection assays used.

In this study, the high rate of positive anti-HEV IgM among the blood donors raise the potential risk of HEV transmission by blood transfusion.

This study was not able to assess the sex association of anti-HEV due to low flow of female donors, therefore all subjects were male.

In the current study, seroprevalence of anti-HEV IgM and anti-HEV IgG increased with age, from 10% in subjects less than or equal 31 years to 16.7% in those more than 31 years for anti-HEV IgG and 2.2% in subjects less than or equal 31 years to 5.6% in those more than 31 years for anti-HEV IgM, that was in agreement with Johargy *et al* (2013), Kaufmann *et al* (2011), Taremi *et al* (2007) which stated that older donors tended to have higher HEV seroprevalence rates. Other studies have found older age to be a risk factor for anti-HEV positivity (Christensen *et al.*, 2008; Meng *et al.*, 2002). It is probable that this represents cumulative exposure over time.

In term of area of residence, there was no significant association between HEV IgG and IgM seropositivity and the residence, but the prevalence rate was more in urban as compared to that in rural subjects. This similar to Bortoliero *et al* (2006) but disagree with Mansuy *et al* (2011) in South West France which stated that the prevalence rate was more in rural as compared to that in the urban subjects. These results indicate that the populations with higher density may be at greater risk of hepatitis E.

5.2. Conclusion

This study demonstrates the high prevalence rate of HEV seropositivity among male blood donors at Omdurman Teaching Hospital, this will raise the potential risk of HEV infection by blood transfusion and may be source of outbreak. This underlines the importance of evaluating HEV screening for blood donors to avoid the transmission of HEV to the

patients. The prevalence of anti-HEV appears to increase with age and no significant association was found between the presence of anti-HEV IgG and IgM antibodies and rural or urban areas.

5.3. Recommendations

1. Enhance the screening of HEV among blood donors.
2. Blood and blood units screened positive for anti-HEV IgM is recommended to be discarded.
3. Isolation and characterization of HEV in Sudan is requested.
4. HEV vaccine is recommended to decrease the transmission of infection.
5. Further studies are warranted to identify additional risk factors involved in the epidemiology of the HEV infection.

RefeRences

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Appendices

بسم الله الرحمن الرحيم

Sudan University of Science & Technology

Faculty of Graduate studies

Faculty of Medical Laboratory Sciences (Microbiology)

Questionnaire

Seroprevalence of Hepatitis E Virus Infection among Blood Donors

1. Date.....
2. Name of donors.....
3. Sample number.....
4. Gender.....
5. Age.....
6. Residence
7. Signs and symptoms

Jaundice: Yes ☐ No ☐

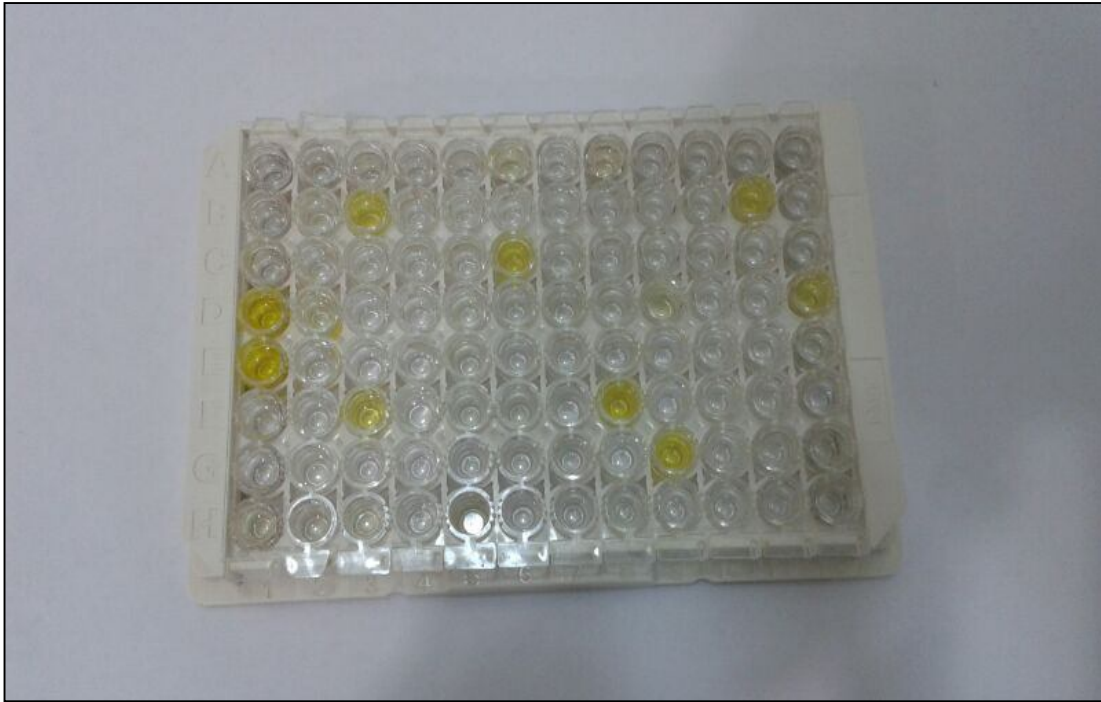
Previous history of jaundice: Yes ☐ No ☐

Result:

HEV IgM.....

HEV IgG.....

COLOR PLATES



ELISA microwell plate for HEV IgM.



ELISA microwell plate for HEV IgG.