

Chapter One

Introduction

1.1. Background

Hepatitis E virus (HEV) is an inflammatory liver disease (Levinson, 2012), HEV is a major cause of hepatitis transmitted by the fecal oral route and it is a common cause of waterborne epidemics of hepatitis in Asia, Africa, India & Mexico but is common in the United States (Brooks *et al.*, 2010). HEV is first documented in samples collected during the Delhi outbreak of 1955, when 29,000 cases of icteric hepatitis occurred after sewage contamination of the city's drinking water supply (Forbes *et al.*, 2007).

HEV was discovered in Asia by a Russian virologist who volunteered to drink stool filterates from a patient with an unidentified form of hepatitis (Purcell and Emerson, 2008).

A large outbreak of hepatitis E was reported in June 2004 in the internally displaced population camps of Darfur, in Western Sudan, and across the border in Chad, at least 5,000 HEV infections were recorded from June to December 2004 (Guthmann *et al.*, 2006). Pregnant women and patients with pre-existing chronic liver diseases are at a particular risk of fulminant hepatic failure upon HEV infection (Haaheim *et al.*, 2002).

The HEV particles are spherical, non-enveloped with a diameter of 32-34 nm. They belong to the caliciviridae family, although their relation to the other members of the family is relatively distant (Brain and Marc, 2010).

The capsid protein encoded by the open reading frame 2 (ORF2) gene of HEV is the only known structural protein on the virion. Pregnancy appears to be a potential risk factor for viral replication and leads to a low immune status of Indian/Asian pregnant women. Mortality rates

among pregnant women, especially those infected in the third trimester, have ranged between 5% and 25%, much higher than in men and nonpregnant women (Pischke *et al.*, 2011).

Hepatitis E in pregnancy is also associated with high rates of spontaneous abortion, intrauterine death, and preterm labour (Khuroo *et al.*, 1981).

1.2. Rationale

HEV is probably the most common cause of liver diseases (Levinson, 2012).

Extra-hepatic manifestations of HEV arthritis, pancreatitis and neurological complications such as Bell's palsy, peripheral neuropathy, ataxia, and mental confusion (Kamar, 2011).

The mortality rate may reach up to 20% in pregnant women with each passing trimester, making HEV infection the most severe hepatitis in pregnancy of all recognized hepatitis viruses (Khuroo and Kamili, 2003). Some virologist reported that observed pregnancy has been reported to result in significantly higher mortality in pregnant than in nonpregnant women.

Hepatitis caused by HEV is clinically indistinguishable from hepatitis A disease (Dalton *et al.*, 2009).

High mortality associated with an outbreak of hepatitis E among displaced persons in Darfur, Sudan (Boccia *et al.*, 2006).

1.3. Objective

1.3.1. General objective:

To investigate the seroprevalence of HEV infection among pregnant women in Khartoum Teaching hospital.

1.3.2. Specific objectives:

- 1.To detect IgG and IgM antibodies in pregnant women.
- 2.To detect association of infection with different trimester in pregnant women infected with HEV.
- 3.To see if there is any relation between the seroprevalence of the virus and age of the pregnant women.

Chapter Two

2. Literature review

2.1. Historical background

Hepatitis E was previously recognized as epidemic or enterically transmitted non-A,-B hepatitis (Shrestha, 1991).

The first well documented epidemic of water-borne hepatitis occurred in New Delhi in 1955-1956 (Viswanathan, 1957).

In the absence of serological tests for hepatitis it was then believed to be caused by hepatitis known at that time. When the stored sera from the epidemic were examined 25 years later, these were found negative for serological tests of acute HAV and hepatitis B virus (HBV) infection and the outbreak was labeled as epidemic non-A, non-B hepatitis (Wong *et al.*, 1980).

In 1980 Khuroo reported a waterborne outbreak of non-A, non-B Hepatitis in Kashmir valley (Khuroo, 1980). Balayan and colleagues in Moscow at 1983 isolated a spherical 27-30nm virus-like particles (VLP) from acute phase stool samples by immune-electron microscopy (IEM) (Balayan *et al.*, 1983). Virus like particles isolated from stool of Nepali patient with non-A, non-B hepatitis during 1981-1982 epidemic was transmitted to marmoset (Kane *et al.*, 1994). In 1990, Reyes *et al* succeeded in molecular cloning of a portion of the genome of this virus, and name the new agent hepatitis E virus (Reyes *et al.*, 1990).

2.2. HEV genome

Hepatitis E virus has a single – stranded positive –sense RNA genome of 7.2 kb. It consists of a short 5' non-translated region (27 to 35 nucleotides in length) followed by three partially overlapping forward open reading

frames (ORfs, from 5' end ORF1,ORF3,ORF2). The 3' nontranslated region is 65 to 74 nucleotides in length, terminated with a poly (A) end with 150 to 200 nucleotides in length (Kabrane *et al.*, 1999).

The HEV genome structure in all human and animal strains is comparable (Tam *et al.*, 1991).

2.3. Open reading frame 1(ORF1)

ORF1 begins at the 5' end of the viral genome following the 27 to 35 nucleotide long non-coding region. This gene consists of 5079 nucleotides and codes for a non-structural protein (polyprotein) with a length of 1693 amino acids. ORF1 polyprotein participates in the replication of viral particles and the modification of structural proteins (Worm *et al.*, 2002).

2.4. Open reading frame 2 (ORF2)

The ORF2 segment of the HEV genome comprises of 1980 nucleotides before a 65 nucleotide long polyadenylated terminal sequence at the 3' end of the ribonucleic chain. It is separated from ORF1 with nucleotides. This gene encodes the main structural (If not the only) virion capsid protein. It is a glycoprotein of 71-88 kDa (660 amino acids; saccharide component lies at the N end) with a potential signal region (It contains high arginine and lysine concentrations) for the endoplasmic reticulum. The protein is synthesized as a precursor, which is then, due to the signal sequence, changed into a mature protein and glycosylated at three possible sites. The presence of extra saccharide is more usual for surface proteins of enveloped viruses than for capsid proteins of non-enveloped viruses (Zafrullah *et al.*, 1997).

HEV capsid protein was used to form virus-like particles (VLPs) by means of baculovirus expression of the ORF2 gene inside cells (Yakamawa *et al.*, 1997). These particles have been characterized by means of cryo-electron microscopy and by three dimensional

reconstruction of the structure. ORF2 protein (between 112 to 607 amino acids) formed 30 homodimers in a form of icosahedra; smaller in size in comparison with fresh virions (Xing *et al.*, 1999). It should be emphasised that the size and modification of infectious viral particle proteins has not been determined yet. Phylogenetic affinity of HEV to the rubella virus (genus alphavirus), with characteristic glycoprotein protrusions on the envelope (Emerson and Purcell, 2003).

2.5. Open reading frame 3 (ORF3)

The last and smallest open reading frame, ORF3, is found between ORF1 and ORF2. ORF3 overlaps the ORF1 segment by one nucleotide near its 5' end and at the 3' end overlaps ORF2 by 328 nucleotides (Wang *et al.*, 2000). It should be noted that ORF3 does not overlap ORF1 in HEV-T1. The start codon is 28 nucleotides behind the ORF1 stop codon. Genotype IV forms a protein of 114 amino acids; the protein size in the other HEV types is 123 amino acids. The genotype group IV lacks the first 9 of 32 amino acids, which code for the hydrophobic domain associated with cytoskeleton binding. It is not known whether the ORF3 protein is a part of a virion, or whether it is a non-structural protein. The protein is non-glycosylated, but phosphorylated *in vivo* in serine-80, which is a residue, conserved in genotype groups I and III, but not in groups II and IV. Function of this protein remains obscure (Emerson and Purcell, 2003).

2.6. Genotypes and prevalence of human HEV strains

The HEV genome has only been completely mapped in a few HEV strains, while most of them have been sequenced only partly. Based on all available data, phylogenetic analysis and comparison of sequences have been performed: these have revealed that there are four major genotypes of HEV: I, II, III and IV. Homology of members of the same genotype is presumed not to be less than 81% (Schlauder and Mushahwar, 2001). The

phylogenetic analysis divided HEV genotype I into five subtypes, genotype II into 2 subtypes, whereas genotypes III and IV were divided into 10 and 7 subtypes, respectively (Hagedorn, 2006).

2.7. Classification and Phylogeny

At least four phylogenetically distinct genotypes have been defined, which distribute by geographic regions . Genotype 1 includes Asian and African HEV strains, genotype 2 includes the single Mexican HEV strain and few variants identified from endemic cases in African countries, genotype 3 includes human and swine HEV strains from industrialized countries and genotype 4 includes human and swine HEV strains from Asia, particularly China, Taiwan and Japan. The avian HEV was proposed to belong to a new genotype 5 (Haqshenas *et al.*, 2001), but this has not yet been confirmed.

A HEV genotype is dominant in a given geographic area, but not limited to it. For example, genotype 2 first identified in Mexico (Huang *et al.*, 1992) was later found on the African continent (Buissonet *et al.*, 2000). Recently, HEV genotype 1 was observed in Cuba in the Americas (Montalvo *et al.*, 2008). Swine HEV isolates belong to either genotype 3 or 4 (Hsieh *et al.*, 1999), but recently genotype 1 was detected in a pig in Cambodia (Caron *et al.*, 2006). Though there is inter- and intra-patient diversity of HEV, its relevance to viral pathogenesis is not clear (Grandadam *et al.*, 2004). All HEV genotypes show varying degrees of intra-genome diversity (Okamoto, 2007). Some recent reports indicate an effect of genotype on viral transmission and disease severity. Outbreaks due to HEV genotype 1 and 2 are the result of efficient human-to-human feco-oral transmission. HEV strains of genotype 3 and 4 are maintained among animal species and occasionally infect humans probably due to inefficient cross-species transmission. This is supported by the recovery of HEV isolate HE-JA4 from a patient who was infected after ingestion

of undercooked pig liver; the sequence was identical to the swine HEV isolate swJL145 (Okamoto, 2007). Genotype 4 was recently shown to cause more severe disease than genotype 3 (Mizuo *et al.*, 2005) and higher viral loads were observed for genotype 4 in a co-infected patient (Takahashi *et al.*, 2002).

2.8. HEV replication

A model for HEV replication and gene expression was proposed based on similarities and sequence homology to better characterized positive strand RNA viruses (Reyes *et al.*, 1993) (Fig 1).

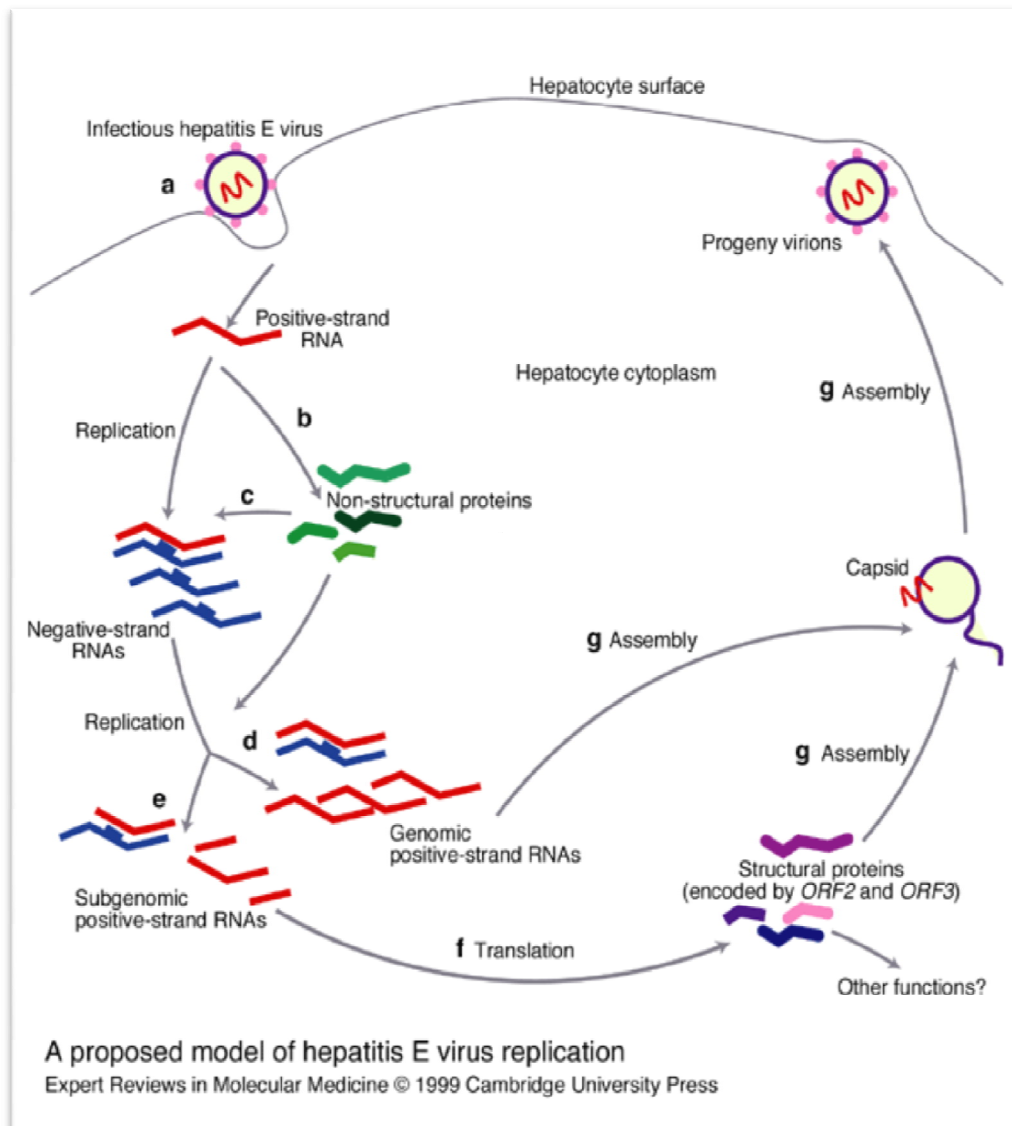


Figure 1. A proposed model of hepatitis E virus replication. This model is based on domain homologies between hepatitis E virus (HEV) and other positive-stranded RNA viruses. (a) Following attachment to an as-yet-uncharacterised receptor on the surface of hepatocytes, HEV is internalised and uncoated in the cytoplasm by unknown mechanisms. (b) The genomic protease, helicase and replicase activities. (c) The replicase so generated can use the positive-strand RNA as a template to synthesise the negative-strand replicative intermediates. Because of homology to alphaviral junction sequences (shown as a boxed region on the negative-

strand RNA), it is proposed that two classes of positive-strand RNA species, genomic (d) and subgenomic (e), are synthesised from the negative-strand RNA intermediates. (f) The subgenomic RNAs are translated into pORF2 viral structural proteins encoded by *ORF2* (and possibly pORF3 encoded by *ORF3*). (g) The structural protein subunits assemble into a capsid that includes the genomic positive-strand RNA to form progeny virions, which can infect other cells or be shed as infectious virus. The *ORF2*- and *ORF3*-encoded proteins might also provide other functions. Experimental proof for most of the steps in this model is awaited. Modified from Ref.(Jameel, 1999).

2.9. Transmission

Four modes of transmission of HEV infection have been reported : faecal-oral transmission, food-borne transmission, vertical transmission and Blood-borne transmission is rare but has been documented in some cases involving blood transfusion (Khuroo and Kamili, 2003; Halacet *al*, 2012) . The most common mode of transmission of HEV, also responsible for the majority of the HEV infection out breaks, is through the faecal-oral route, usually by ingested of contaminated water. Potential exists for food-borne transmission and some cases have been observed where consumption of raw or uncooked meat from wild boar and deer has led to HEV infection. Some cases of vertical (perinatal) transmission from mother-to-child have been documented, particularly in India, but this is considered to be of minor importance as a mode of transmission for HEV and more investigation is required. Person-to-person transmission and secondary household cases are uncommon, particularly in epidemic (poor hygienic) conditions. In non-endemic regions, where autochthonous cases have been observed, zoonotic transmission has been considered as the

likely mode of transmission, but more investigation is required (Halac *et al.*, 2012).

2.10. Pathogenicity

The disease caused by HEV is generally self-limiting with symptoms typical of acute viral hepatitis including, jaundice, malaise, anorexia, abdominal pain, nausea, fever, diarrhoea, discoloured stool and/or urine, and hepatomegaly (Tashale and Hu, 2011). An icteric hepatitis and cholestasis are also observed in some cases. Mortality rate due to infection by HEV have been reported to be high as 1% (Prinja *et al.*, 2008); however, the mortality rate may reach up to 20% in pregnant women with each passing trimester, making HEV infection the most severe hepatitis in pregnancy of all recognized hepatitis viruses. Analysis of serum specimens collected from volunteer blood donors shows that the prevalence of HEV varies from region-to-region but is higher in endemic countries/regions as compared to developed countries (Khuroo and Kamili, 2003). Hepatitis caused by HEV is clinically indistinguishable from hepatitis A disease (Dalton *et al.*, 2009).

2.11. Clinical finding

Clinical features of HEV infection range from asymptomatic hepatitis to severe, fulminant hepatitis, which can result in liver-related mortality. Typical symptoms include 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).

Hepatitis E superinfection with underlying stable chronic liver disease can present as acute hepatic decompensation. However, presence of immunoglobulin G (IgG) anti-HEV in patients with chronic liver disease did not differ from that of healthy blood donors. Moreover, previous exposure to HEV did not result in different outcomes among patients with chronic liver diseases (Hamid *et al.*, 2003).

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) and HIV infected patients (Pischke *et al.*, 2010; Dalton *et al.*, 2009).

Hepatitis E in pregnancy has been reported to result in significantly higher mortality than in nonpregnant women, especially in India or Pakistan, where genotype 1 and 2 HEV are prevalent. Parra *et al* (Patra *et al.*, 2007) reported that HEV infected icteric, pregnant women showed a significantly higher rate of fulminant hepatitis (55%), maternal mortality (41%), poorer fetal outcome (79%), and lower live birth (21%) than those of icteric, pregnant women with non HEV acute viral hepatitis (20%, 7%, 51% and 49% respectively). The mechanism of severe outcomes of HEV infection in pregnancy has not been elucidated; however, pregnancy induced suppression of T cell immunity and T helper 2 (Th2) skewed cytokine patterns as well as increased viral load may be related to poor outcomes (Navaneethan *et al.*, 2008). However, HEV infected pregnant women in the other endemic area of Egypt, where genotype 3 is prevalent, did not show different outcomes from those of non-pregnant women. Moreover, no difference in outcomes was observed between pregnant and nonpregnant animals in experimentally infected animals. Further study of the mechanism of pathogenesis during pregnancy is warranted (Myint *et al.*, 2000).

2.12. Immunity

There is evidence that immunity following HEV infection is not short lived. Second attack of hepatitis E in the same person have been documented only in a few, and occurred mostly after 5 to 10 years. Anti-HEV IgG was found to persist for 10 to 15 years after the infection in

some patients. Other investigators also detected long-lived protective antibody levels following acute infection with HEV (Myint *et al.*, 2000).

2.13. Epidemiology

Phylogenetic studies indicate at least four distinct genotype of HEV (1-4) based on geographical origin (Khuroo and Kamili, 2003; Halac *et al.*, 2012). Genotypes 1 and 2 are considered more pathogenic, restricted to humans, and are responsible for the large majority of cases and outbreaks in endemic regions.

Genotypes 3 and 4 are somewhat less pathogenic, infect humans, pigs, and other animal species, and are generally responsible for sporadic HEV infection cases within endemic and non-endemic regions. Outbreaks and sporadic cases of HEV have occurred over a large geographic area, most notable in regions with poor sanitation. There have been some cases of food-borne HEV infections, but the majority of confirmed cases have been associated with the consumption of water contaminated with faeces. The attack rate of HEV is highest in young adults between ages of 15-40. Males are more likely to develop clinical hepatitis when infected with HEV as compared to females.

In developed countries, HEV infection is generally reported from people who travel to HEV endemic or epidemic areas; however, some cases of locally-acquired (autochthonous) HEV infection have been observed in non-endemic countries including USA, Australia, France, Greece, New Zealand, Italy and UK. Documented epidemic outbreaks have occurred in Algeria, Ivory coast, Ghana, Chad, Ethiopia, Somalia, Namibia, India, Nepal, Pakistan, Burma, Myanmar, China, Vietnam, Indonesia, and Mexico (Kamar, 2011).

2.14. Diagnostic methods of HEV

2.14.1. Molecular detection of HEV

Nucleic acid-based techniques, especially nested RT-PCR and real-time-PCR, have emerged rapidly as the method of first choice for sensitive and specific detection of RNA viruses. This method is very useful in research for the characterisation of divergent HEV strains whose serological responses have not been detected by some assays, especially in countries where infection is not endemic (Kamar, 2011).

This molecular method consists of two or three steps (nested RT-PCR). The first step of RT-PCR, reverse transcription, uses specific primers, random hexamers or Oligo dT to rewrite viral RNA into cDNA. In the second or third step, PCR or nested PCR uses specific primers to amplify specific segments of viral RNA. Primer binding sites can be spread over the whole genome. 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 (Jothikumare*et al.*, 2006).

2.14.2. Immunologic diagnosis

2.14.2.1. Enzyme immunoassay (EIA)

EIA is a practical, highly sensitive and inexpensive diagnostic method for detection of anti-HEV antibodies. Antigenic domains have been found in all ORFs proteins of HEV (Khudyakov *et al.*, 1999):

- (i) 12 antigenic domains in ORF1 (particularly in the domain of the putative RNA-dependent RNA polymerase).
 - (ii) six antigenic domains in the ORF2 protein.
 - (iii) three antigenic domains within the ORF3 protein
- Recombinant proteins, originating from the ORF2 and ORF3 C-end domain or from a larger ORF2 segment and complete ORF3, are used for the detection of IgG and IgM anti-HEV. A wider range of antigens expressed from a larger part of ORF2 or “capsid-like” particles are more effective in the

detection of antibodies in the convalescent stage of the disease rather than rare antigens from the ORF2 and 3 C-ends or the whole of ORF3 (Krawczynski and Bradly,1989). Synthetic peptides may also be used as antigens; however, antibodies cannot be reliably detected in the convalescence stage due to a low sensitivity. These peptide antigens are usually used for the confirmation of the EIA result with recombinant proteins and for the exclusion of non-specific reactions. Their use might increase the reaction specificity and determine the genotype group of HEV in the acute stage of hepatitis (Worm *etal.*,2002).

2.14.2.2. Immune fluorescence microscopy (IFM)

A few specialised laboratories use this technique for the detection of antibodies. IFM 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 (Krawczynski and Bradly, 1989). This method is laborious and expensive and thus not useful for routine diagnosis.

2.14.2.3. Immune electron microscopy (IEM)

IEM detects VLPs in clinical specimens (Balayan *etal.*,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 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 VLPs to be detected (Yarbough,1999). Other antigen detection methods have not been reported (Anderson and Sherestha,2002).

2.14.3. Virus isolation

Establishment of a practical cell culture system to allow the propagation of HEV in vitro is vital for virological characterisation 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 VLPs and have poor reproducibility (Worm *et al.*, 2002). Currently there is no reliable cell culture system for HEV.

2.15. Treatment and vaccines for hepatitis E

There is no specific antiviral therapy for hepatitis E, and supportive care is the main therapy. However, specific anti viral treatment is required in severe or persistent hepatitis in immunocompromised host (Kamar *et al.*, 2010) reported that pegylated interferon could induce sustained virological response in the post transplantation setting.

To date, 2 type of HEV vaccine have been developed. The first vaccine produced by GlaxoSmithKline is a genotype 1 recombinant HEV protein vaccine prepared by a recombinant baculovirus system containing capsid antigen with aluminium hydroxide as an adjuvant; the vaccine efficacy was reported as 95.5% after three doses in a phase 2 clinical trial including 5,323 members of the Nepalese Army and the U.S. Army (Shresha *et al.*, 2007). The second HEV vaccine (HEV 239, Hecolin), which is a genotype 1 recombinant HEV capsid protein vaccine prepared by a recombinant *E. coli* system and adsorbed with aluminium hydroxide, was developed by Xiamen Innovax Biotech in China; the vaccine efficacy after 3 doses was reported as 100% in a phase 3 trial including 11,165 Chinese participants (Zhang *et al.*, 2009). Travelers from areas of low-endemicity to highly endemic areas should be protected from HEV infection using the above vaccine. Although universal vaccination of

children in highly endemic areas would be highly effective, specific program or strategies for HEV vaccination of children in highly endemic areas would be highly effective, specific programs or strategies for HEV vaccination should be developed.

2.16. Prevention of Hepatitis E

In the endemic areas HEV is a predominantly a waterborne infection, spread by sewage contamination of drinking water. Provision of safe drinking water in urban areas in developing countries is a challenging task. Efficient chlorination of the water at the treatment sites should be insured. Care should be taken to prevent contamination of drinking water supply system from sewage during construction of road and house.

3. Materials and Methods

3.1. Study design

This study is descriptive cross sectional study to investigate prevalence of HEV among pregnant women in Khartoum State.

3.2. Study area

This study was conducted in Khartoum Teaching Hospital.

3.3. Study population

Pregnant women attending Khartoum Teaching Hospital.

3.4. Study period

This study was conducted during the period from April to August 2014.

3.5. Sampling technique

The study was based on non-probability convenience sampling technique during attendance of pregnant women to Khartoum Teaching Hospital.

3.6. Sample size

A total of ninety blood samples (n=90) were collected from pregnant women.

3.7. Sample processing

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

3.8. Inclusion criteria

Pregnant women were included in this study.

3.9. Exclusion criteria

Non pregnant women were excluded from this study.

3.10. Data collection

Personal data were obtained by direct interviewing questionnaire from each subject (appendix).

3.11. 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 significance was set at $P\text{-values} < 0.05$.

3.12. 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.

3.13. Laboratory work

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

Separate serum from blood sample by 3000 round per Min for 10 Min.

3.13.1. ELISA for detection of anti-HEV IgM

3.13.1.1. 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/plasma 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).

3.13.1.2. 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

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

Adding samples

Ten 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

Hundred μ l 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

Fifty μ l of Chromogen A and 50 μ l 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

Fifty μ l of Stop solution 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.

3.13.1.3. 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.

3.13.1.4. 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.

3.13.2. ELISA for detection of anti-HEV IgG**3.13.2.1. 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).

3.13.2. 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

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

Adding samples

Ten 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 turned down onto absorbent tissue to remove any excess liquid from the wells.

Adding HRP-Conjugate

Hundred μ l of diluted conjugate reagent 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 turned down onto absorbent tissue to remove any excess liquid from the wells.

Coloring

Hundred μ l of substrate-TMB solution 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 μ l of stopping solution.

Measuring the absorbance

The absorbance was read at 450nm using ELISA reader.

3.13.2.3. 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.

3.13.2.4. 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 $\geq 0.9 < 1.0$

4. Results

4.1. Seroprevalence of HEV among pregnant women

Figure 4.1 demonstrates that out of the 90 pregnant women, 13 (14.5%) were positive for HEV IgM. Figure 4.2 also explains 41(45.6%) out of 90 samples were positive for HEV IgG.

4.2. Duration of pregnancy among positive pregnant women

Table 4.2 displays that duration of pregnancy in first trimester 7(7.7%) were positive for IgG and 2(2.3%) for IgM, in second trimester 15(16.6%) were positive for IgG and 5(5.6%) for IgM and third trimester 19(21.1%) were positive for IgG and 6(6.6%) for IgM.

4.3. Distribution of age groups among positive pregnant women

Table 4.3 displays that distribution according to age it was found that 19(22%) were positive for IgG and 7(7.7%) for IgM among age group (15-25years), 14(17%) were positive for IgG and 4(4.4%) for IgM among age group (26-35) and 8(10%) were positive for IgG and 2(2.2%) for IgM among age group (36-45).

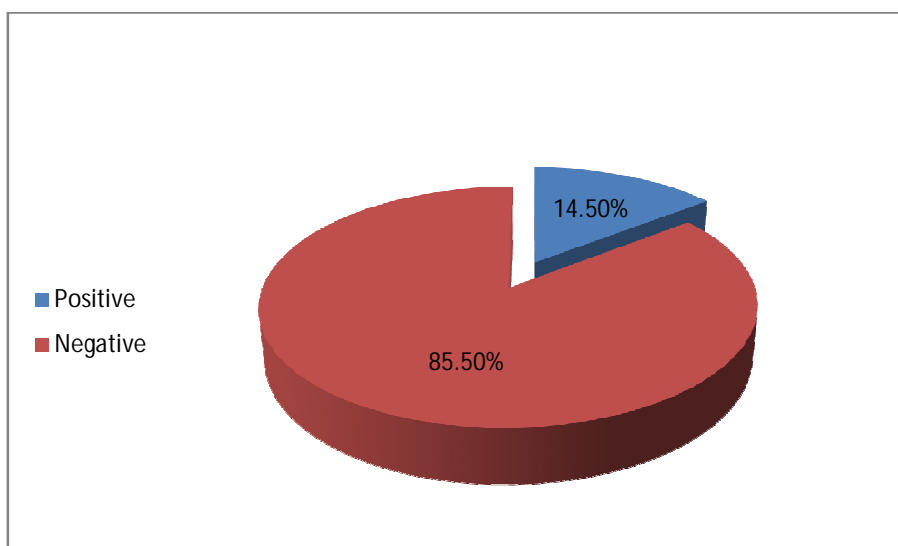


Figure 4.1. Frequency of HEV IgM among pregnant women

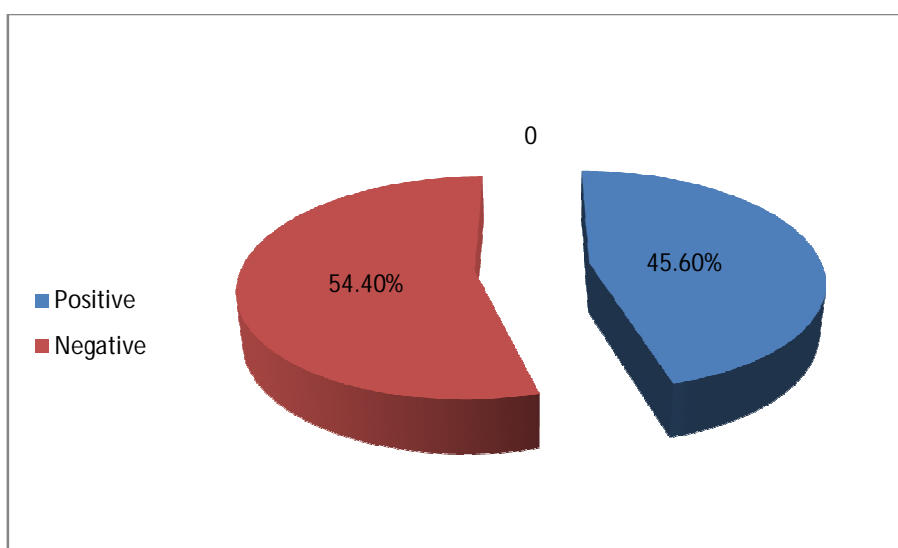


Figure 4.2. Frequency of HEV IgG among pregnant women

Table 4.2: Frequency of IgG and IgM among positive pregnant women related to duration

Daration	IgG				IgM			
	Positive		Negative		Positive		Negative	
	No	%	No	%	No	%	No	%
1 st trimester	7	7.7	14	15.5	2	2.2	22	24.4
2 nd trimester	15	16.6	18	20	5	5.6	29	32.2
3 rd trimester	19	21.1	17	18.9	6	6.7	26	28.9
Total	41	45.6	49	54.4	13	14.5	77	85.5

Table 4.3:Frequency of IgG and IgM among positive pregnant women related to age

Age group	IgG				IgM			
	Positive		Negative		Positive		Negative	
	No	%	No	%	No	%	No	%
15-25	19	21.1	22	24.5	7	7.7	44	48.8
26-35	14	15.5	17	18.9	4	4.5	21	23.3
36-45	8	8.8	10	11.1	2	2.3	12	13.3
Total	41	45.5	49	54.5	13	14.5	77	85.5

Discussion

The interaction of Hepatitis E and pregnancy is fascinating and has provided new insights into the pathophysiology and understanding of the immunology and host susceptibility factors and their interaction to produce the disease processes. The severe liver injury due to HEV infection during pregnancy may be related to several possible factors, such as differences in immune and hormonal factors occurring during pregnancy, the genetic and environmental factors with its occurrence in certain developing countries (Navaneethan *et al.*, 2008).

This study showed very high overall frequency rates for IgG (41.1%) of HEV antibody among pregnant women. The overall frequency of HEV among pregnant women attending Khartoum Hospitals was higher than that found in Darfur, Western-Sudan (31.1%) and lower than in Egypt (84.3%) and India (60%) (Boccia *et al.*, 2006; Stoszek *et al.*, 2006; Patraet *et al.*, 2007).

Significant relation also could be seen between trimester of pregnancy and HEV antibody positivity demonstrating that highest rate of anti HEV was obtained in second trimester, this agreed with Tabarraei *et al* (2011) and Al-Tayeb *et al* (2014). Also highest rate was obtained in third trimester, this is also agreed with Al-Tayeb *et al* (2014) and Adjeiet *al* (2009), this may be due to low immune status of the pregnant women that increases with trimester. The prevalence was found to be decreased with age, this was agreement with Al-Tayeb *et al* (2014) and disagreement with Adjeiet *al* (2009).

Recently HEV screening program has been recommended as part of the routine in several countries. The antenatal screening program also should be performed to minimize prenatal HEV transmission to take further precaution to protect fetus's life (Weber and Taylor, 1996).

Conclusion

The present study concluded that 45.6% for IgG and 14.5% for IgM were detected among pregnant women in Khartoum State. This study reveals that HEV infection was more in second and third trimester than first trimester among pregnant women. The prevalence of the virus decreases with the increase in age.

Recommendations

1. Education of pregnant women to raise their awareness of attention to good hygiene to reduce the chances of infection.
2. Increase awareness of HEV infection amongst women during the period of pregnancy.
3. Attention of the increase of the virus during the productive age of the women.
4. The use of advanced techniques for diagnosis of virus like PCR.

References

1. **Adjei AA, Tettey Y, Aviyase JT, Adu-Gyamfi C, Obed S, Mingle J, Ayeh-Kumi PF and Adiku TK.** (2009). Hepatitis E virus infection is highly prevalent among pregnant women in Accra, Ghana. *Virology Journal*. 6: 108-114.
2. **Al-Tayeb ZA, Nafi M and Yassin ME.** (2014). Frequency of Hepatitis E Virus among Pregnant Women Attending Khartoum Hospitals. *American Journal of Research Communication*. 1-7.
3. **Anderson DA and Shrestha IL.** (2002). Clinical Virology, Hepatitis E virus. 2nd ed. ASM Press, American Society for Microbiology, Washington, DC, USA.
4. **Balayan MS, Andzhaparidze AG, Savinskaya SS, Ketiladze ES, Braginsky DM., Savinov AP. and Poleschuk VF.** (1983). Evidence for virus in non-A/non-B hepatitis transmitted via the faecal-oral route. *Interovirology*. 20: 23–31.
5. **Boccia D, Guthman JP, Klovstad H, Hamid N, Tatay M, Ciglenecki I, Nizou JY, Nicand E and Guerin PJ.** (2006). High mortality associated with an outbreak of hepatitis E among displaced persons in Darfur, Sudan. *Clin Infect Dis*. 42:1679-1684.
6. **Brain WJ and Marc HV.** (2010). Hepatitis E virus. In: Desk Encyclopedia of Human and medical virology 11th edition Elsevier USA PP:195-198.
7. **Brooks GF, caaroll KC Butel Js , Morse SA and Mietzer TA.** (2010). Hepatitis viruses, In jawetz, Menliick & Adel berg's Medical Microbiology ,25th edition ,MC grow hill medical ,new York:PP471-476.
8. **Buisson Y, Grandadam M, Nicand E, Cheval P, van Cuyck-Gandre H, Innis B, Rehel P and Coursaget P et al.** (2000).

- Identification of a novel hepatitis E virus in Nigeria; *J Gen Virol.* 81: 903–909
9. **Caron M, Enouf V, Than S C, Dellamonica L, Buisson Y and Nicand E** (2006). Identification of genotype 1 hepatitis E virus in samples from swine in Cambodia; *J Clin Microbiol.* 44: 3440–3442
 10. **Dalton HR, Bendall RP, Keane FE, Tedder RS and Ijaz S.** (2009). Persistent carriage of hepatitis E virus in patients with HIV infection. *N Engl J Med.* 361:1025-1027.
 11. **Emerson SU and Purcell RH.** (2003). Hepatitis E virus. *Reviews in Medical Virology.* 13:145–154.
 12. **Forbes BA,shm Df and wessfeld AS.** (2007). Laboratory Mthods in basic virology.In Bailey & Scott' diagnostic microbiology .12 th edition, Mosbyelsevier china. PP718-715.
 13. **Grandadam M, Tebbal S, Caron M, Siriwardana M, Larouze B, Koeck J L, Buisson Y, Enouf V and Nicand E.** (2004). *Evidence for hepatitis E virus quasispecies*; J. Gen. Virol. 85 :3189–3194.
 14. **Guthmann JP, klovstad H, Boccia D, Hamid n, Pinoges L, Nizou J.Y, Tatay M, Diaz F, Moren A, Grais R.F, Ciglenecki I, Nicand E and Guerin PJ.** (2006). A large outbreak of hepatitis E among adisplaced population in Darfur, sudan ,2004: The role of water treatment methods.*Clin Infect Dis.* 421685-1691.
 15. **Haaheim LR ,Pattison JR and Whitley RJ.** (2002). Hepatitis E virus .In :Apractical guide to clinical virology,second edition ,JoHN WILEY & SONS , LTD , England. PP 125-198.
 16. **Hagedorn CH.** (2006). Phylogenetic analysis of global hepatitis E virus sequences: genetic diversity, subtypes and zoonosis. *Review of Medical Virology.* 16, 5–36.

- 17. Halac U, Beland K, Lapierre P, Patey N, Ward P, Brassard J, et al.** (2012). Cirrhosis due to Chronic Hepatitis E infection in a Child Post-Bone Marrow Transplant. *J Pediatr.* 160(5):871-4.
- 18. Hamid SS, Atiq M, Shehzad F, et al.** (2003). Hepatitis E virus super-infection in patients with chronic liver disease. *Hepatology.* 36:474-478.
- 19. Haqshenas G, Shivaprasad H L, Woolcock P R, Read D H and Meng X J.** (2001). Genetic identification and characterization of a novel virus related to human hepatitis E virus from chickens with hepatitis-splenomegaly syndrome in the United States; *J Gen Virol.* 82 :2449–2462
- 20. Hsieh SY, Meng XJ, Wu YH, Liu S T, Tam AW, Lin DY and Liaw Y F.** (1999). Identity of a novel swine hepatitis E virus in Taiwan forming a monophyletic group with Taiwan isolates of human hepatitis E virus; *J. Clin Microbiol.* 37: 3828–3834.
- 21. Huang CC, Nguyen D, Fernandez J, Yun KY, Fry KE, Bradley D W, Tam AW and Reyes GR.** (1992). Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV); *Virology.* 19:1550–558
- 22. Jameel S.** (1999). Molecular biology and pathogenesis of hepatitis E virus; *Expert Rev. Mol. Med.* 1–16.
- 23. Jothikumar N, Cromeans TL, Robertson BH, Meng XJ and Hill VR.** (2006) broadly reactive one-step realtime RT-PCR assay for rapid and sensitive detection of hepatitis E virus. *Journal of Virological Method.* 131: 65–71.
- 24. Kabrane-Lazizi Y, Meng XJ, Purcell RH and Emerson SU.** (1999). Evidence that the genomic RNA of hepatitis E virus is capped. *Journal of Virology.* 73, 8848–8850.

25. **Kamar N, Rostaing L, Abravanegl F, et al** (2010). Pegylated interferonalpha for treating chronic hepatitis E virus infection after liver transplantation. *Clin Infect Dis*. 5:30-33.
26. **Kamar N.** (2011). Hepatitis e virus infection in Iranian Kidney-transplant patients. *Hepat Mon*.11(11): 927-8.
27. **Kane MA, Bradley DW, Shrestha SM, Maynard JE, Cook EH Mishra RP and Joshi DD.** (1994). Epidemic non-A, non-B hepatitis in Nepal: Recovery of a possible etiologic agent and transmission studies in marmosets. *Journal of the American medical Association*. 152:3140-3145.
28. **Khudyakov YE, Lopareva EN, Jue DL, Crews TK, Thyagarajan SP and Fields HA.** (1999). Antigenic domains of the open reading frame 2-encoded protein of hepatitis E virus. *Journal of Clinical Microbiology*. 37: 2863–2871.
29. **Khuroo MS .** (1980). Study of an epidemic non-A, non-B hepatitis virus distinct from post-transfusion non-A, non-B type. *American Journal of Medicine*. 68:818-824.
30. **Khuroo MS and Kamili S.** (2003). Aetiology, clinical course and outcome of sporadic acute viral hepatitis in pregnancy. *J viral Hepat*. 10(1): 61-9.
31. **Khuroo MS, Teli MR and Skidmore S.** (1981). Sofi MA, Khuroo MI. Incidence and severity of viral hepatitis in pregnancy. *Am J Med*. 70: 252-255.
32. **Krawczynski K and Bradley DW.** (1989). Enterically-transmitted non-A, non-B hepatitis: identification of virus-associated antigen in experimentally infected cynomolgus macaques. *Journal of Infectious Diseases*.

- 33. Levinson W.** (2012). Hepatitis viruses. In: Review of Medical Microbiology and Immunology , 12th edition , MC Grow Hill medical , New York, pp 324 – 332 .
- 34. Mizuo H, Yazaki Y, Sugawara K, Tsuda F, Takahashi M, Nishizawa T and Okamoto H.** (2005). Possible risk factors for the transmission of hepatitis E virus and for the severe form of hepatitis E acquired locally in Hokkaido, Japan; *J Med Virol.* 76 :341–349
- 35. Montalvo VM, Rodriguez LL, Chandra V, Bello CM, Sariego FS, Gutierrez MA and Jameel S.** (2008). Phylogenetic analysis of hepatitis E virus isolates from Cuba shows the first presence of genotype 1 in the Americas; *Emerg infect Dis.*
- 36. Myint K, Shretha M, Vaughn D, Innis B, Kuscher R, Seriwatana J, Endy T and Scott R.** (2000). Hepatitis E antibody Kinetics in Nepalese patients Antiviral Therapy; 5(suppl).
- 37. Navaneethan U, Al Mohajer M and Shata MT.** (2008). Hepatitis E and pregnancy: understanding the pathogenesis. *Liver Int.* 28:1190-1199.
- 38. Okamoto H.** (2007). Genetic variability and evolution of hepatitis E virus; *Virus Res.* 127:216–228.
- 39. Panda SK, Thakral D and Rehman S.** (2007). Hepatitis E virus. *Rev Med Virol.* 17:151-180.
- 40. Patra S, Kumar A, Trivedi SS, Puri M and Sarin KS.** (2007). Maternal and fetal outcomes in pregnant women with acute hepatitis E virus infection. *Ann Intern Med.* 147:28-33.
- 41. Pischke S, Heim A and Bremer B.** (2011). Hepatitis E: an emerging infectious disease in germany?. *Z gastroenterol* . 49:1255-1257

42. **Pischke S, Suneetha PV, baechlein C, et al.** (2010). Hepatitis E virus infection as a cause of graft hepatitis in liver transplant recipients. *Liver Transpl.* 16:47-82.
43. **Prinja S, Kumar S, Reddy GM, Ratho RK, Kumar R.** (2008). Investigation of viral hepatitis E outbreak in a town in Haryana. *J Commun Dis.* 40(4):249-54.
44. **Purcell RH and Emerson SU.** (2008). hepatitis E: an emerging awareness of an old disease. *J hepatol.* 424-503.
45. **Rayes GR, Purdy MA, Kim JP, et al ,** (1990). Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. *Science.* 247:1335-1339
46. **Reyes GR, Huang CC, Tam AW and Purdy MA.** (1993). Molecular organization and replication of hepatitis E virus (HEV); *Arch Virol Suppl.* 7:15–25.
47. **Schlauder GG and Mushahwar IK.** (2001). The genetic heterogeneity of hepatitis E virus. *Journal of Medical Virology.* 65, 282–292.
48. **Shrestha MP, Scott RM, Joshi Dm, et al.** (2007). Safety and efficacy of a recombinant hepatitis E vaccine. *N Engl j Med.* 356:895-903.
49. **Shrestha SM.** (1991). Enteric non-B hepatitis in Nepal: clinical and epidemiological observations. In ShiKata T, Purcell RH, Uchida T editors- *Viral Hepatitis C, D and E*, Amsterdam, Elsevier. 265-275.
50. **Stoszek SK, Abdel-Hamid M, Saleh Doa'a, Kafrawy SE, Narooz S, Hawash Y, Shebl FM, Daly ME, Said A, Purcell RH and Strickland T.** (2006). High prevalence of hepatitis E antibodies in pregnant Egyptian women. *Trans Roy Soc Trop Med Hyg.* 100:95-101.

51. **Tabarraei A, Moradi A, Rodgari D, Javid N and Bakhshandeh N.** (2011). Anti-Hepatitis E Virus seroprevalence in pregnant women, in Gorgan, Iran, North East of Caspian Sea. 3: 162-164.
52. **Takahashi M, Nishizawa T, Yoshikawa A, Sato S, Isoda N, Ido K, Sugano K and Okamoto H.** (2002). Identification of two distinct genotypes of hepatitis E virus in a Japanese patient with acute hepatitis who had not travelled abroad; J. Gen. Virol. 83 : 1931–1940.
53. **Tam AW, Smith MM, Guerra ME, Huang CC, Bradley DW, Fry K.E and Reyes GR.** (1991). Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. *Virology*. 185: 120–131.
54. **Tashale EH and Hu DJ.** (2011). Hepatitis E: Epidemiology and prevention. *World J Hepatol*. 3(12):285-91.
55. **Viswanathan R.** (1957). Epidemiology: Infectious hepatitis in New Delhi 1955-56. *Indian Journal of Medical Research*. 45:1-29.
56. **Wang YC, Zhang H, Ling R, Li H and Harrison TJ.** (2000). The complete sequence of hepatitis E virus genotype 4 reveals an alternative strategy for translation of open reading frames 2 and 3. *Journal of General Virology*. 81: 1675–1686.
57. **Weber JN and Taylor GP.** (1996). Antenatal screening is important. *BMJ*. 312: 706.
58. **Wong DC, Purcell RH, sreenivasan MA, Prasad SR and Pavri KN.** (1980). Epidemic and endemic hepatitis in india- evidence for a non-A, non-B, hepatitis virus aetiology. *Lancet*. 876-879.
59. **Worm HC, Van der Poel WH and Brandstatter G.** (2002). Hepatitis E: an overview. *Microbes and Infection*. 4: 657–666.
60. **Xing L, Kato K, Li T, Takeda N, Miyamura T, Hammar L and Cheng RH.** (1999). Recombinant hepatitis E capsid protein self-

assembles into dual-domain T=1 particle presenting native virus. *Virology*. 265: 35–45.

- 61. Yakamawa Y, Suzuki K, Tatsumi M, Razak MA, Uchida T, Takeda N and Miyamura T.** (1997). Expression and self-assembly of empty virus- like particles of hepatitis E virus. *Journal of Virology*. 71: 7207–7213.
- 62. Yarbough PO.** (1999). Hepatitis E virus. *Intervirology*. 42: 179–184.
- 63. Zafrullah M, Ozdener MH., Panda SK and Jameel S.** (1997). The ORF3 protein of hepatitis E is phosphoprotein that associates with the cytoskeleton. *Journal of Virology*. 71: 9045–9053.
- 64. Zhang J, Liu CB , Li RC, et al .** (2009). Randomized-controlled, phase II clinical trial of a bacterially expressed recombinant hepatitis E vaccine. *Vaccine*. 27:1869-1847.