SEROPREVALENCE OF SUBCLINICAL HEPATITIS E IN PREGNANCY

Dissertation submitted to

THE TAMIL NADU DR.M.G.R MEDICAL UNIVERSITY

In partial fulfilments of the regulation

for the award of the degree

M.S. OBSTETRICS AND GYNECOLOGY

BRANCH II

GOVT. STANLEY MEDICAL COLLEGE & HOSPITAL

THE TAMIL NADU DR. M.G.R MEDICAL UNIVERSITY

CHENNAI, INDIA

SEPTEMBER 2015

CERTIFICATE

This is to certify that this dissertation entitled **"SEROPREVALENCE OF SUBCLINICAL HEPATITIS E IN PREGNANCY"** is the bonafide original work done by **Dr.VINITRA.D,** Post graduate, under my overall supervision and guidance in the department of obstetrics & Gynaecology, Stanley Medical College and Hospital, Chennai, in partial fulfilment of the regulations of The Tamil Nadu Dr. M.G.R. Medical University for the award of **M.S. OBSTETRICS & GYNECOLOGY.**

Dr.Venkateshwaran.M.D.,
Prof & HOD,
Department of Medical Gastroenterology
Govt. Stanley Medical College
Chennai-600 001.

Dr.N.Thamizhselvi., MD.DGO
Professor
Department of Obstetrics & Gynaecology
RSRM Hospital,
Govt. Stanley Medical College,
Chennai-600 001.

Dr. ISSAC CHRISTIAN MOSES
DEAN,
Government Stanley Medical College,
RSRM Hospital,
Chennai-600 001.

Dr.Vasanthamani, MD. DGO
Prof. & H.O.D,
Department of Obstetrics &
Gynaecology, RSRM Hospital,
Government Stanley Medical College,
Chennai-600 001.

DECLARATION

I, solemnly declare that this dissertation**" SEROPREVALENCE OF SUBCLINICAL HEPATITIS E IN PREGNANCY"** is the bonafide work done by me at the Department of Obstetrics & Gynaecology, Government Stanley Medical College Hospital, Chennai, under the guidance and supervision of **Prof. Dr.N.Thamizhselvi. MD.DGO.,** Professor of Department of Obstetrics & Gynaecology, Government Stanley Medical College, Chennai-600 001.

This dissertation is submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the University regulations for the award of degree of M.S Obstetrics & Gynaecology examinations to be held in April 2016.

Place: Chennai. Signature of the candidate

Date : **(Dr. VINITRA.D)**

ACKNOWLEDGEMENT

First and foremost, I thank the **ALMIGHTY** for blessing and giving me the health and ability for carrying out this work successfully.

I sincerely acknowledge and wish to express my thanks to Professor **Dr.N.Thamizhselvi. MD. DGO, Department of obstetrics & gynaecology,** Stanley Medical College and Hospital, Chennai, for giving me permission to commence this dissertation and for her able guidance, constant encouragement and support during the course of the study.

I would like to thank **Dr.Vasanthamani. MD.DGO**, professor and HOD, Department of Obstetrics and Gynaecology, RSRM, Government Stanley medical college and hospital, Chennai, for granting me permission for this study and for her valuable suggestions and guidance.

I am extremely thankful to **Dr. VENKATESHWARAN M.D.,** Professor and Head of the Department of Medical Gastroenterology, for his guidance and support all times and for sponsoring the kit needed for this study.

I express my warmest respects and profound gratitude to **Dr. R.SELVI, M.D.,** The Professor and Head of The Department of Microbiology, Government Stanley Medical College, Chennai, to utilize the facilities of this Institution for the study.

I extend my sincere thanks to **Assistant Professor Dr.Ponnammal, M.D., and PG Dr.Sherin M.D.,** Department of Microbiology for their valuable help, constant support and guidance throughout my study.

I express my special thanks to mycolleague and my Friend **Dr.Thoorika** for her immense help, constant encouragement and unconditional support throughout the study.

I will always cherish the company of my junior PG's and my CRRI`s for their support and help during sample collection.

I gratefully acknowledges the help rendered by lab technicians during sample processing at Department of microbiology, Government Stanley Medical College and Hospital, Chennai.

I am indebted to the pregnant mothers from whom blood samples were collected for the study.

I would be failing in my duties, if I don't express my deep gratitude to my family members especially my husband **Dr.Vignesh Mantharam.,** MD. DM for their constant love, understanding, encouragement, support throughout my life.

CONTENTS

INTRODUCTION

INTRODUCTION

Hepatitis is the condition characterized by the presence of inflammatory cells in the liver tissue. It is caused by different etiological agents and is more commonly used to refer to a group of viral infections that affects liver, such as hepatitis A, B, C, D and E. Hepatitis A and E are transmitted through feco-oral route, hepatitis B and C are transmitted through infected blood and body fluids and hepatitis D occur exclusively in persons infected with hepatitis B virus.

All water borne epidemics of viral hepatitis was thought to be caused by hepatitis A virus. In Delhi there was a suspected outbreak of hepatitis A between 1955 and 1956. Serum samples from those patients were collected and preserved. In 1983 a specific and sensitive assay was applied to these preserved samples and they were found to be negative for specific antibodies against hepatitis A virus[4]. Thus a new clinical entity came into life with signs and symptoms similar to other forms of viral hepatitis.

It was designated as hepatitis E because of its enteric transmission, epidemic and endemic nature that capture the hepatitis E virus[1]. Later it was known as Enterically Transmitted Non A Non B (ENANB) hepatitis or Epidemic Non A Non B[5].

Hepatitis E virus (HEV) causes large outbreaks in endemic areas like India, Central Asia, parts of Africa and Mexico[1]. It is the most important cause of epidemic and sporadic viral borne hepatitis in countries with suboptimal sanitary conditions affecting susceptible population like young aged adults and pregnant women.

Prevalence of Hepatitis E in developing countries ranges from 7.2% to 24.5% when compared to <1% in developed countries[25,32].

Hepatitis E virus causes an acute short lived, self-limiting viral hepatitis typically lasting for 1-4 weeks. Itaffects both sexes targeting age group between 15 and 40.It exhibits a wide clinical spectrum, fluctuating from asymptomatic infection to fulminant hepatitis. Most cases of pregnancy will not affect the severity of hepatitis unless it is found to be hepatitis E which tends to worsen during the period of pregnancy. It causes fulminant hepatic failure (FHF) in pregnant women particularly those in 2^{nd} and 3^{rd} trimester of pregnancy. There is a high rate of occurrence of disseminated intravascular coagulation (DIC) associated with hepatitis E infection in pregnancy[27].Attack rate among 1^{st}, 2^{nd} and 3^{rd} trimester were 8.8%, 19.4% and 18.6% respectively. Whereas among non-pregnant women and men it is only 2.1% and 2.8%[2].

37% of acute viral hepatitis during pregnancy is caused by hepatitis E and 81% of them go in for fulminant hepatic failure more commonly during 3rdtrimester of pregnancy[20,22]. General mortality rate in hepatitis E is 1 to 2 percentage when compared with hepatitis A which is 0.4 percentage[1].

The case fatality rate among pregnant mothers is 25% [4,22,25] and has been reported to be very high with maximum severity in the third trimester of pregnancy i.e. 44.4%[2] .In fact, the major cause of mortality in epidemics is due to the high rate of fulminant hepatic failure in pregnant women[27].Besides the high maternal mortality rate, the other dangers of fulminant hepatic failure such as fetal malformation, abortion, still birth and neonatal death are noted among pregnant mothers with hepatitis E infection[4,25].

High rates of vertical transmission of hepatitis E were documented between 33.3% and 50% leading to high fetal mortality[25]. Studies have reported that 78.9% of babies had evidence of vertically transmitted HEV infection[30]. This data points to a relationship between severity of HEV infection in the pregnant women and the fetus.

Most of the cases of hepatitis E remain asymptomatic. The treatment of acute infection is supportive management. In spite of the

explosive nature of hepatitis E during pregnancy, there is no established treatment available for it[31]. Therapeutic termination of pregnancy as approved in disorders like HELLP syndrome have not been completely explored in hepatitis E infection[19]. The diagnosis of hepatitis E infection in individual patients remains challenging. It cannot be clinically distinguished from other forms of acute viral hepatitis. The routine laboratory diagnosis of hepatitis E is based either on serologic tests or nucleic acid amplification techniques[27].

Trials of HEV vaccines are presently continuing in many countries. This consists of a candidate vaccine which is very effective and accepted in China, showing efficacy of >90%. On January 23,2012 this vaccine has been licensed by State Food and Drug Administration of China for its production and sale. Perhaps it is a major milestone on the road towards protecting susceptible women in a disease endemic country like India.

Hepatitis E is accountable for approximately 9.8% of pregnancy associated deaths. In southern Asia, as many as 10,500 maternal deaths per year could be prevented by using the existing vaccine[24]. With the availability of an efficacious vaccine, we must consider prudent implementation of such an intervention, where appropriate, to avoid a significant proportion of preventable deaths in developing countries.

As there is very less data documented with respect to sero-prevalence of hepatitis E in pregnant mothers in India, this study was done to assess the sero-prevalence of subclinical hepatitis E viral infection in asymptomatic pregnant mothers attending routine antenatal check up in a tertiary care hospital in Chennai. Moreover, it helps in considering vaccination for the pregnant mothers and development of existing vaccine.

Since hepatitis E infection is a significant health problem world, there is a need for more public health involvement by provision of clean drinking water, health education of public and easy availability of approved serological assay for early detection of infection.

AIMS

AND

OBJECTIVES

AIMS AND OBJECTIVE

1) To study the seroprevalence of Hepatitis E virus infection in asymptomatic pregnant mothers attending routine antenatal check up in a tertiary care hospital in Chennai.

2) To emphasize the importance of screening, diagnosis and management of HEV infection in asymptomatic pregnant women

3) To study the implications of HEV vaccination if available in the future to all sero-negative antenatal mothers thereby decreasing morbidity and mortality associated with HEV infection

REVIEW

OF

LITERATURE

REVIEW OF LITERATURE

HEPATITIS E VIRUS

THE DISCOVERY

The clinical features of viral hepatitis wasrecognized by Hippocrates in 460-375 BC. In the year 1965, Blumberg et al discovered Australia antigen which consequently led to the detection of hepatitis B virus. In 1973, Feinstone et al demonstrated hepatitis A virus by immune electron microscopy (IEM) in stool extracts of patients with acute hepatitis A virus infection. Later, Choo et al identified hepatitis C virus from post-transfusion patients, and developed serological test for its diagnosis.

Till 1980 all water borne epidemic viral hepatitis disease were thought to be caused by hepatitis A. During the 1955-56 epidemic, about 29,300 patients developed symptoms of jaundice in Delhi which was typical of hepatitis A disease by Indian Council of Medical Research[13] and concluded that, the disease caused by a hepatitis virus, lasting for less than 6 weeks spread through contaminated water and was of non-A, non-B. They also concluded that the disease typically affected the young adults with maximum severity in pregnant women.

The experimental evidence of hepatitis E was first reported by Balayan et al[5] in the year 1983.He successfully demonstrated the fecal-oral transmission of hepatitis. A volunteer who was already infected with hepatitis A infection in the past was given a suspension inoculated with feces specimen collected from the patient from Uzbekistan[5]. On 36th day, the volunteer developed clinical feature of severe hepatitis and the fecal sample showed27 to 30 nm sized virus like particles in a immune electron microscopy (IME)[5].

Balayan observed that the volunteer did not develop antibodies to either HAV or HBV but to virus like particles which he detected from his faeces[5, 6]. The study members also demonstrated a similar transmission of HEV in cynomolgus monkey with the volunteer's faecal sample and the monkeys responded similarly[5].

This type of non-A non-B hepatitis was designated as enterically transmitted nonA nonB (ENANB) hepatitis or epidemic nonAnonB. Later it was found to be the major hidden cause for epidemics in many developing countries and also cause sporadic cases of hepatitis.

In those days Immune Electron microscopy and transmission to primates were the only means of studying HEV.

CLASSIFICATION

HEV is an RNA virus of the ***Hepeviridae*** family, genus ***Hepevirus***[5] as classified by Emerson et al, 2006[5].

HEPATITIS E VIRUS

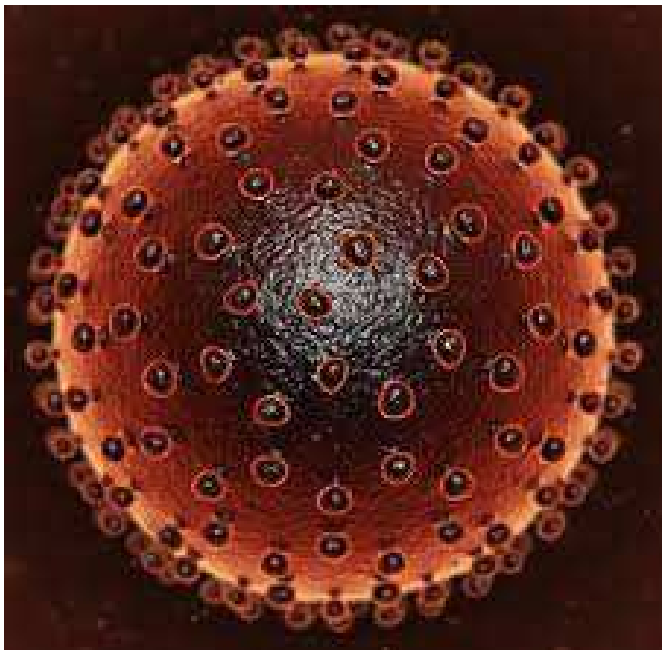


Fig 3.1 Hepatitis E structure

Hepatitis E viruses are small, 27-34 nm in diameter, symmetrical icosahedral, non-enveloped, with non-segmented positive-sense, single stranded, polyadenylated RNA (at its 3' terminus) comprising of a 7.5 kb genome[4, 7].

GENOTYPE AND PREVALENCE OF HEPATITIS E HUMAN STRAIN

Genotyping became prevalent during the mid-1990's[22]. Based on sequence analysis done from the hepatitis E strains from different parts of the world, HEV were known to have 4strains i.e., I, II, III, IV[5]. Upto 9 genotypes have been dicovered[6].

Further phylogenetic analysis showed multiple subtypes in each genotype. HEV I into 5 subtypes, II into 2 subtypes, III into 10 and IV into 7 subtypes[7].

Genotype I – Asia(India, Pakistan), North Africa

Genotype II – Mexico, South Africa

Genotype III - North and South America, Europe, Asia

Genotype IV – Asia (China, Taiwan, Vietnam, Japan)

Genotype I and II cause hepatitis outbreaks especially in developing countries and Genotype I is the most common subtype in India.

Genotype I contains human hepatitis Estrains obtained from endemic regions. Limited sequence strains appears to belong to genotype

II. Genotype III and IV have been identified from human and swine sources[6].

MOLECULAR CHARACTERIZATION OF THE VIRUS

Most of the molecular aspects of hepatitis Ewere gained by in vitro expression of recombinant proteins due to the lack of efficient cell culture system.

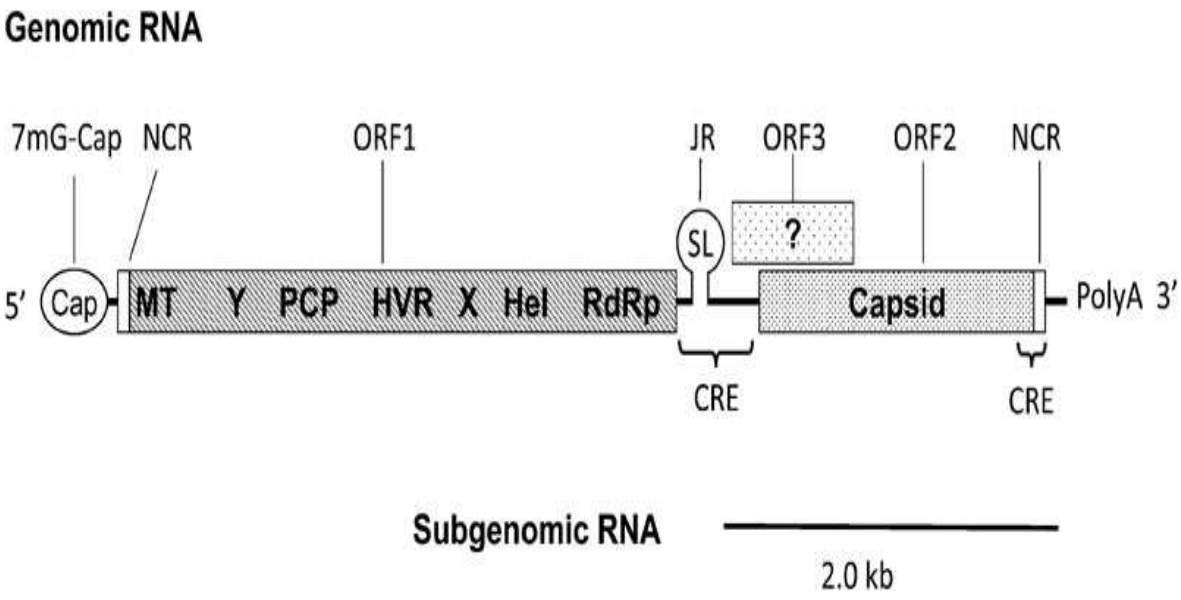


Fig 3.2: RNA of HEV virus

OPEN READING FRAME (ORF 1)

ORF1 begins at the 5' terminus of the hepatitis E. It contains 5,073 to 5,124 nucleotides long codes for nonstructural proteins (polyproteins with length of 1693 amino acid) that are involved in replication of viral genome and viral protein processing[4, 5,7].

OPEN READING FRAME 2 (ORF2)

ORF 2 begins at 37nt 3' of the termination coding frame of ORF1. It has 1977 to 1,980 nucleotides [5] and encodes for the main structural viral capsid proteins. In addition to the fresh virions, ORF2 forms 30 small sized icosahedral homodimers (size and modification are not yet determined)[5,6,7].

Hepatitis Evirus envelope protein form the Virus like Protein (VLP). These particles have been characterized with the help of 3-D reconstruction of the structure and Cryo-electron microscopy.

OPEN READING FRAME 3 (ORF3)

ORF3 is the smallest and last open reading frame with 366 to 369 nucleotides encodes and encodes for pORF protein (123 amino acid) expressed in an intracellular manner, shown to be associated with liver cell cytoskeleton in HIV replication[4, 6].

Other aspects such as assembly and release of viral progeny, some viral protein synthesis, HEV genomic transcription remains unclear.

KEY ANTIGENS[5]

HEV antigens are reactive in immune electron microscopy, enzyme linked immunosorbent assay (ELISA), western blot and

immunofluorescent microscopy. ORF3 is more heterogeneous, whereas ORF2 is highly conserved relatively and hence the ORF2 sequence based serological test are broadly reactive and tests containing ORF3 are more strain specific.

Expressed or synthetic peptides of ORF2 and ORF3 are used in diagnostic tests like ELISA and Western blot. Truncated proteins expressed from ORF2 in insect cells are better than proteins expressed in Escherichia coli for detecting antibodies against hepatitis E virus, though not satisfactorily sensitive for sero-epidemiological studies[5].

ORF1 incorporated test are not useful for routine diagnostic purposes, however, important for distinguishing infection-induced and vaccine induced immune response when non replicating hepatitis E vaccines are available commercially[5].

REPLICATION OF HEPATITIS E VIRUS

Little information is known about the stages of replication because hepatitis E virus is not closely related to any other well characterized virus and reports from conventional cell culture are insufficient about its strategy of replication[5, 6].

However, recently, replication of hepatitis E virus have been documented in primary cell cultures of hepatocytes obtained from cynomologus monkeys already infected in vivo. The process of attachment, entry and uncoating of hepatitis E virus is not determined because of limited availability and absence of characterized and permissive cell culture[5, 6].

It is assumed that hepatitis E virus attaches to the receptors on hepatocytes especially in the biliary tract and intestine. From the newly uncoated virus particle, 7.2-kb genome is released. The nonstructural proteins recognizes the capped 5'RNA after presumably translated via cellular mechanisms[5,6] and the required enzymes are provided for the synthesis of both positive and negative strand RNA.

Cellular proteases are found to cleave ORF1 that is sequenced with papain like protease motif, but its functions are not yet clear.

3' end of the viral genome shown to bind with RNA dependent RNA polymerase and ORF 2 binds to the first 76 nucleotide of the 5' region[6].

Positive sense full length genomic RNA and 2 bicistronic subgenomic mRNA of 3.7 and 2.0kb are transcribed from replicative full length negative strand RNA[5,6].

However, the importance of these subgenomic RNAs in translation of ORF2 and ORF3 protein are not determined. Nothing is known about how these processes are regulated[6].

HEPATITIS E VIRUS TRANSMISSION

Hepatitis E virus is classified as the transmitter of waterborne and foodborne diseases[11]. Most common route of transmission is feco-oral. Most of the epidemic serologically confirmed for hepatitis Evirus have been found to be linked with fecal contamination of drinking water. Some sporadic hepatitis have been associated with consumption of undercooked pork or raw/uncooked shellfish[7]. Consumption of undercooked or raw meat of infected pork, beef, wild boar meat and offal are significantly associated with hepatitis Einfection[12].

The spread of hepatitis E virus infection from cases to contact by person to person contact and fomites may play a role, but this is not always common[4]. This may be due to the low amount of intact hepatitis E particles present in a patient's stool[16].Transmission of hepatitis Evirus infection through person to person contact between family members is only one to two percentage compared to fifteen percentage in hepatitis A[7].

Transmission via exposure to infected blood has been reported in endemic regions. Post transfusion hepatitis E infection have also been documented [28]. Trans-placental transmission of hepatitis E virus in third trimester of pregnancy is associated high perinatal mortality of the newborns[14].There appears to be relatively higher risk among infants born to mothers with hepatitis E infection upto 33.3% of cases[20]. In a study of 469 pregnant women with hepatitis E infection, found to have vertical transmission up to 100%[19].

Though hepatitis E virus RNA is detected in the colostrum milk, no evidence suggest the spread of infection to offspring[1]. There is no evidence for HEV transmission through sexual route[4]. Hepatitis E infection has also been documented in 3 health care workers who had treated fulminant hepatic failure due to hepatitis E in South Africa[4]. Travelling history to HEV endemic region pose a high risk factor in a number of cases.

Improper treatment of drinking water and substandard sanitation is the major cause for hepatitis E virus outbreaks.

PATHOGENESIS AND PATHOLOGY

PRIMARY HOST TARGET CELLS FOR HEV-HEPATOCYTES[4,5,6,28]

The pathogenesis of Hepatitis E virus infection is not completely understood so far due to the lack of competent cell culture system. Most of the little knowledge about the pathogenesis of hepatitis E virus was obtained from the studies on non-human primates[28]. However, from the clinical picture and its serological events exhibited in some of the typical cases of hepatitis E disease, certain speculative conclusion have been arrived at about its pathogenesis. Ingestion of contaminated food with infected patient's faeces is the route of primary entry of HEV into the host[5].

It is presumed that hepatitis E virus replication takes place in the intestinal tract. It reaches the hepatocytes and starts replicating within the cytoplasm, followed by a phase of viremia with high concentration of the virus found in bile and is shed in the feces. Transient viraemia occurs for 3-4 weeks. Anti HEV IgM peaks with the symptomatic period and persist for 3 to 6 months and after that they become undetectable.

Anti HEV IgG appears 3 to 4 weeks post inection and have been shown to persist for 2 to 13 years. During convalescent period IgG

disappears within 6 to 12 months period[1].HEV RNA are detected in the biliary duct, on the luminal aspect of the epithelial cell surfaceusing in situ hybridisation[7].Hepatitis E virus antigens are expressed in the hepatocyte cytoplasm, faeces and bile. Antigens appears one week post infection indicating viral replication. HEV can be detected in the stool a week prior to the clinical symptoms.

It has been established that the liver injury is caused by the immune response produced by the host to the invading hepatitis Eand does not depend directly on the replication of the virus in the liver tissue. Viraemia and faecal shedding is followed by the onset of alanine transaminase (ALT) elevation. Observable histopathological changes in the liver usually correspond to the detectable levels of antibodies to hepatitis Ein the serum and decreased levels of hepatitis Evirus antigens in the hepatocytes of the liver.

During epidemic outbreaks of hepatitis Edisease, examination of the infected patient's liver shows either a characteristic cholestatic pattern with glandular modification of the parenchymal cells or a pattern similar to the other forms of acute viral hepatitis which includes acidophilic bodies formation and ballooning degeneration of the hepatocytes or confluent hepatocyte necrosis in the liver [4,6].

PREGNANCY AND HEV

Pregnant women, particularly those in 2nd and 3rd trimester of pregnancy are more frequently affected during hepatitis E outbreaks. They have a worse outcome than other form of viral hepatitis.

It was proposed that severe fetal infections and fetal death may produce toxins that overload circulation which causes severe maternal disease[27]. During pregnancy there is increased sensitivity to hepatitis E virus mediated endotoxins[1] called Schwartzman-like phenomenon. Hepatitis E virus infection are fatal as they precipitate pregnancy associated eclampsia with disseminated intravascular coagulation due to hepatic and renal failure [28].

Following are the two possible host factors that favor fulminant hepatic failure to occur in hepatitis E infected pregnant women:

- Immunological changes
- Hormonal changes during pregnancy

IMMUNOLOGICAL CHANGES DURING PREGNANCY[28]

During the early weeks of gestation up to 20thweeks, T cells are significantly reduced. It is a protective modulation of cell mediated immunity (CMI) to sustain the highly antigenic fetus and the placenta

during pregnancy. Placenta are resistant to cell mediated damage as they do not express MHC.

They express a unique HLA-G molecule which inactivates Natural Killer cells by binding to its receptors CD16 and CD56. It also produces indoleamine 2, 3 dioxygenase enzyme, inactivate and deplete the aminoacid tryptophan, which supports T cell function and hence suppresses CMI. Increase in cytokines such as TGF-β, IL-4 and IL-10 inhibits cell mediated immunity.

HORMONAL FACTORS IN PREGNANCY

Increased incidence of fulminant hepatic failure may be associated with hormonal changes. As the period of gestation progresses the hormones like estrogen, progesterone and human chorionic gonadotropin also increases. Estrogen causes shrinkage of thymus and deplete the number of CD4 and CD8 cells. Progesterone blocks T cell development i.e. they inhibit Th1 cells and promote Th2 cells by producing involution of thymus more effectively than estrogen. It was also shown that there is increased level of T helper2 cytokines assuming this may cause liver injury and have a role in increased severity of HEV infection in pregnancy[25].

PROBABLE MECHANISM OF FULMINANT HEPATIC IN PREGNANCY[19]

In addition to the above mentioned hormones, high levels of steroid hormones are also appreciated during pregnancy. Through NF-κB steroids mediate lymphocyte apoptosis and this physiological down regulation helps to retain the fetus. In studies it was shown that the activity of p65 component of NF-κB was decreased in peripheral blood mononuclear cells as well as liver biopsy of pregnant women who died of fulminant hepatic failure. It was established that absence of p65 is responsible for liver damage in fulminant hepatic failure associated in hepatitis E infected mothers.

CLINICAL MANIFESTATION

Incubation period ranges from 2 to 8 wks for the clinical manifestation of the disease. Wide spectrum of clinical manifestation have been observed, from self-limiting, subclinical, acute hepatitis to fulminant hepatitis in case of pregnant mothers but never proceeds to chronicity.

It is most commonly seen in the age group of 15 to 45. It is mostly asymptomatic in children. 1% mortality in the general population is due to fulminant hepatic failure.

SIGNS AND SYMPTOMS

PRE-ICTERIC OR FIRST PHASE

Influenza like symptoms

Abdominal pain

Tenderness

Nausea

Vomiting

ICTERIC OR SECOND PHASE

Jaundice

Dark urine

Viremia

Elevated liver enzymes

Antibody sero conversion

Clearing of the viruses

First phase or pre-icteric phase lasts for 1 to 10 days followed by second or icteric phase persist for 15 to 40 days.

OUTCOME OF HEV

Typically hepatitis E virus infection is self-limiting without progression to the chronic illness. However hepatitis E infection is more

severe than hepatitis A. It causes fulminant hepatic failure in pregnant women and can be fatal.

Fulminant form of hepatitis E infection occurs more frequently in the third trimester of pregnancy with a mortality rate of 25%.

However, on the basis of clinical presentation hepatitis E cannot be differentiated from other forms of viral hepatitis. In fact, not all hepatitis E infections are clinically apparent.

Increased frequency of abortion, neonatal deaths and still births are noted among pregnant mothers with hepatitis Einfection[4]

Other complications are prolonged cholestatic hepatitis, acute HEV super infection in patients with cirrhosis, co-infection with other hepatotropic viruses and autochthonous hepatitis E virus in developed countries

SEROEPIDEMIOLOGY

Hepatitis E cause waterborne epidemic disease with peak clinical attack rate in young adults and high rate of fulminant hepatitis in pregnancy. The earliest recorded outbreak of HEV occurred in the year 1955 in Delhi, India, following heavy flooding of the river Yamuna. Thereafter, many number of outbreaks and sporadic cases have been

documented in a wide variety of developing and tropical regions, together with India, Nepal, Pakistan, Myanmar, Indonesia, China, the central Asian region of the former Soviet Union, Ethiopia, Egypt, Algeria, Jordan, the Ivory Coast, Sudan, Chad, Somalia, Ethiopia, and Mexico[4,5].

HEV epidemiology in hyper endemic regions

Epidemics of hepatitis E are known to occur frequently In hyper endemic regions, typically separated by a few years. The outbreaks are regularly large, and affect several 100 to several 1000 persons. They are often related to drinking of water contaminated with infected person's feces. Their time-course varies from unimodal (few weeks) to prolonged, multipeaked (last for over a year) epidemics. The multipeaked represent continuous water contamination. The outbreaks normally follow heavy rainfall and floods. It create conditions that favor contamination of drinking water with human excreta, or during summer months, probably due to reduced water flow in rivers cause increased concentration of fecal pollutants. In Southeast Asia, repeated outbreaks have been shown to be related with disposal of human excreta into rivers, and people tend to use the same water for cooking, drinking, and personal hygiene. They favor constant fecal contamination of water.

Occurrences of hepatitis E have happened in underdeveloped urban areas with leaky water pipes passing through soil that is contaminated with sewage. During periods of erratic water supply, negative pressure forms in pipes of no flow which allow inward suction of contaminants into the pipes. Few outbreaks are associated with food-borne transmission due to a relatively extended incubation period (up to 10 wks)Genotype 1 of hepatitis E virus is the predominant cause of sporadic hepatitis and epidemics in hyperendemic regions.

Sporadically occurring hepatitis in many of the regions have been serologically confirmed as hepatitis E virus infection. In fact, hepatitis E virus is one of the most common etiology of sporadic hepatitis in the endemic regions.

A salient feature of hepatitis E is that it has age specific clinical attack rate with its peak among young adults ranging from 15 to 40 years of age in developing countries[1].Survey of sera collected from a hepatitis E endemic area of India over a period of ten years documented that most hepatitis A infection occurred before five years of age, whereas, hepatitis E virus infection occurred after sixteen years of age. However, all age groups are affected with male preponderance and mostly adults develop clinical evidence of hepatitis than children during epidemics[2,3].

In most hepatitis E endemic areas, antibodies have been identified in 5% of children in the age group less than ten years when compared to 10% to 40% in young adults >25 years of age[4]. A recent study from India have reported >60% of children <5 years of age have antibodies against hepatitis Evirus[4].

By Nargis et al, seroprevalence of anti-hepatitis E IgG in New Delhi, India is 33.67% in asymptomatic healthy mothers[2]. Whereas in Spain and Turkey prevalence it is 0.6-2 and 12.6 percent respectively[2].Arankalle et al states a seroprevalence of23.62% of IgG in the general population is [2]. By Khuroo et al, it is 5% in asymptomatic healthy children[2].

By Radhakrishnan et al, among 361 cases of acute viral hepatitis 17.3%(66) were positive for IgM[29]. By Singh et al 40% of pregnant women were positive for IgM anti HEV antibody[29]. By Bista et al, out of 19 jaundiced pregnant women 16 (84.2%) were positive for anti HEV IgM antibody[29].

Relatively high seroprevalence rate were reported in developed countries like Japan (4%-7%) and U.S (15%- 20% in blood donors)[28]. The urban sewage samples from non-endemic areas like Spain, Greece, France, Sweden, and U.S tested positive for hepatitis E virus infection[28],

signifying that the healthy population of non-endemic areas may also be exposed to other types of animal HEV strains that do not develop any clinical manifestations.

The development of a sensitive serological assay has permitted the complete analysis of HEV seroprevalence and its distribution worldwide. Contrary to the expectations, the prevalence of anti HEV IgG is much lower in endemic regions and higher in non-endemic region.

Molecular methodology to the epidemiology of hepatitis E virus hold some promise. The moderately low level of hepatitis Ein blood and feces restricts the practicality of hybridization without amplification. RT-PCR has been useful for checking the outcomes of serological test and for assessing the duration of infectivity of the cases. Limitation to molecular epidemiological studies are due to fairly short duration of faecal shedding and viremia. Its main significance is identification of different types of hepatitis E virus genotypes and their association to specific epidemics and environmental locations. Furthermore, RT-PCR has been used to identify hepatitis E in sewage and contaminated water.

RISK GROUP IN THE POPULATION[7,16]

1. People living in areas where community outbreaks of hepatitis E infection occurs

2. People living in overcrowded refugee camps following disastrous events
3. People travelling to hepatitis Eendemic areas
4. People with chronic liver disease
5. People handling pigs, cows, sheep, goat and non-human primates which may be infected with hepatitis E virus.
6. Travelers to endemic areas

DIAGNOSIS

Hepatitis E should be should be suspected in endemic areas during outbreaks of water-borne hepatitis in people living in sub optimal sanitary conditions. In developed nations hepatitis E should be suspected in patients presenting with signs and symptoms of hepatitis with history recently returned from endemic areas. Similar to the other forms of viral hepatitis, serological tests play an important role in establishing a definitive diagnosis of hepatitis E.

Specific test for anti HEV IgM and IgG antibodies are available. IgM is the acute phase marker and IgG determines the exposure to HEV. Detection of IgM is up to 90% during acute infection if the suspected patient’s serum samples are collected between 1 and 4 weeks after the onset of symptoms. During the first 4 weeks of infection after the onset

on disease, IgM reaches peak titer of 1:1000 to 1:10000 and disappears within 3 months in 50% of the patients[5]. Between 2 and 4 weeks, IgG peaks in titer of 1:1000 to 1:100000 and thereafter diminishes rapidly relatively[5]. According to Clayson et al., in the infected people IgG antibodies against HEV persist for more than 14 years in 47% of the patients[7].

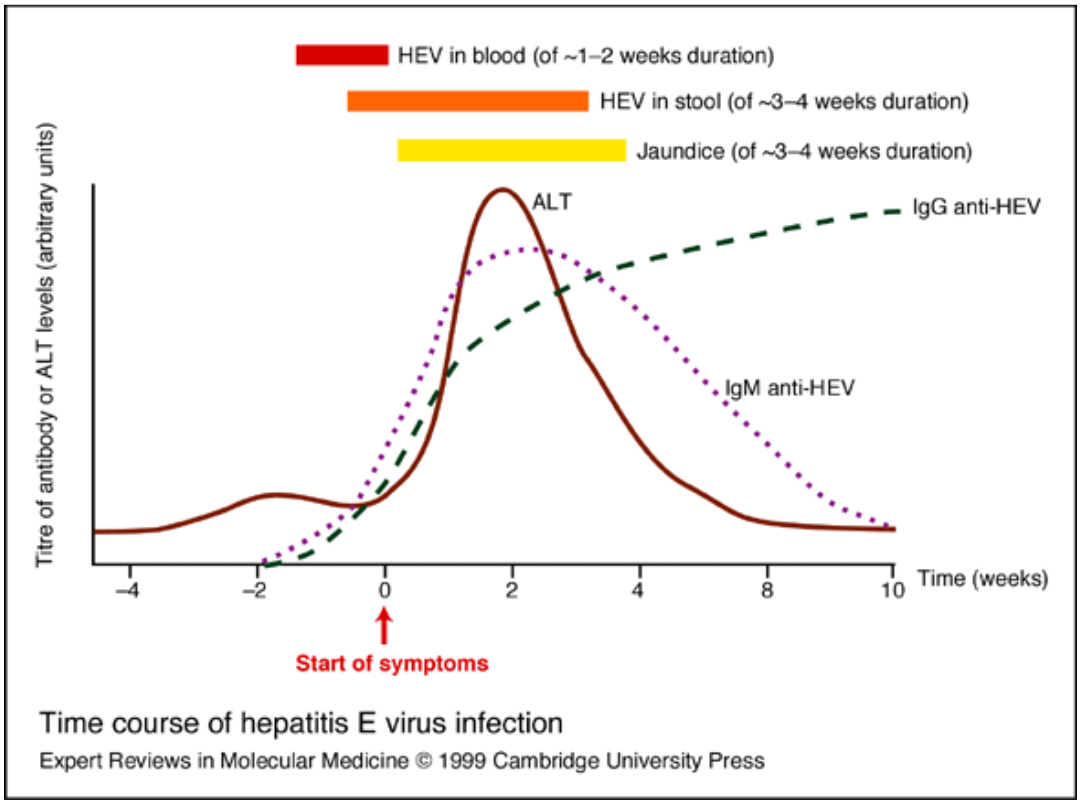


Fig 3.3: Diagnosis of HEV infection

IMMUNOLOGICAL DIAGNOSIS

ENZYME IMMUNO ASSAY (EIA)[7]

IMMUNOFLUORESCENCE MICROSCOPY (IFE)

Enzyme immune assay is a highly sensitive, inexpensive and a practical method for detection of antibodies against hepatitis E virus.

IgM- acute phase marker

IgG- measures exposure to hepatitis E

Specific antibodies can be detected in either blood or serum. Following are the antigenic domains found in the ORF proteins

1. ORF1 – 12 antigenic domains
2. ORF2 – 6 antigenic domains
3. ORF3 – 3 antigenic domains

Complete ORF3 and a large segment of ORF2 or ORF3 C end domain and recombinant proteins originating from the ORF2 are used for detection of IgM and IgG antibodies of HEV. In the convalescent stage of the disease course, ‘capsid-like’ or large ORF2 particles are more effective in detection of anti HEV.

Synthetic peptide antigens are used to confirm enzyme immune assay results and to exclude nonspecific reactions. Their use increase the

specificity of the reaction and helps to determine the genotype ofhepatitis E. Disadvantage of this peptide antigens is that it has low sensitivity and not reliable in detection of antibodies in the convalescent period[7].

4 short recombinant proteins derived from 3' end of ORF-2 with 42 amino acid and ORF-3 with 33 amino acid of Burmese (genotype –I) and Mexican (genotype –II) are used by two Genelab EIA. 2 recombinant proteins obtained from the complete ORF3 with 123 amino acid of the Burmese genotype I strain is used by Abbott –EIA[7]. Sensitivity, specificity, positive predictive value and negative predictive values of all three combination of EIA's are 100%, 99.5%, 75% and 100% respectively[7].

According to Mast et al, 12 different enzyme immune assay showed a concordance from 41 to 94 percent in blood donors and 0 to 89% among reactive sera with mean 68 and 32 percent respectively[7].

During acute HEV infection inflammation of liver or damage to liver tissues can be assessed by elevated liver enzymes like glutamyl transpeptidase (GGT) alanine aminotransferase (ALT)

IMMUNOFLUORESCENCE MICROSCOPY (IFE)

This is a semi-quantitative test which measures the concentration of anti HEV antibodies against hepatitis E virus. Fluorescein–conjugated anti-HEV IgG to hepatitis E antigen in the liver tissue is blocked by anti HEV antibodies.

IFE is expensive and laborious hence not useful for routine diagnosis.

MOLECULAR DETECTION OF HEV

IMMUNE ELECTRON MICROSCOPY

NUCLEIC ACID AMPLIFICATION TEST

VIRAL ISOLATION

IMMUNE ELECTRON MICROSCOPY (IEM)[17]

Balayan et al., in 1983 used IEM to detect virus like particles in the clinical specimens. Antibodies to HEV derived from acute phase or convalescent phase sera, precipitate the HEV particles. By rating the antibody coating, concentration of the anti HEV antibody can be determined semi-quantitatively.

For routine analysis, immune electron microscope (IEM) is not suitable as its sensitivity and specificity of the assay is insufficient.

Moreover clinical samples contains less number of virus like particles (VLPs) to be detected by IEM.

Molecular methods in diagnosis of hepatitis E have replaced Immune electron microscopy as it is positive in only 10% of cases[5].

NUCLEIC ACID AMPLIFICATION TEST

They can be used to detect HEV RNA either in serum/blood or stool. These tests are of importance during the window period as the specific antibodies to HEV are not mounted to the detectable levels, hence the serological test are found to be negative[3].

Nested RT PCR and real time RT PCR are nucleic acid based techniques is the method of choice for the specific detection of HEV RNA in blood and faeces during the acute phase of infection. This method is very advantageous in detecting divergent hepatitis Evirus strains in non-endemic countries where some assay fail to detect serological response to hepatitis E. But the sensitivity of the test depends on a proper match between the hepatitis E virus strain and the PCR primers.

Conventional RT-PCR detects viral RNA, not only from sera and faeces in humans but also from clinical samples like bone marrow,

plasma, serum, salivary gland, mesenteric lymph nodes, inguinal lymph nodes, kidney, urine, liver and bile of domestic pigs and other animals like wild boar, sika deer, mongoose, chickens and also from contaminated water[7].

VIRAL ISOLATION[17]

For viral characterization and diagnosis, establishment of a practical cell culture that allow the multiplication of hepatitis Ein vitro is very critical. The use of human lung diploid cell culture system (2BS, PLC/PRF/5, A549,HepG2) and primary cell culture system from non-human primates like chimpanzees, African green monkeys, cynomolgus macaques and tamarins have been reported[7,17].

But these cell culture medium cannot be used for virological and biophysical studies of hepatitis E because they do not provide high-tire of HEV in the culture medium and have reproducibility.

Toshinori Tanaka et al used Faecal suspension with 2.0×10^{-7} of HEV copies ml^{-1} were used successfully in developing a cell culture system with an HEV titre of up to 10^8 in PLC/PRF/5 cells [17].

TREATMENT

Treatment of hepatitis E virus is supportive and involves bed rest, adequate hydration and electrolyte repletion. Almost all patients are able to clear the virus spontaneously. There is no specific therapy available in altering the course of acute hepatitis E infection. Antivirals have not been effectively established for the treatment of hepatitis E infection. In acute severe form of infection, patients are treated with ribavirin for 21 days. Pilot studies in cell culture suggest that interferon alpha and ribavirin may inhibit hepatitis E virus[1].Though ribavirin is contraindicated in pregnancy for its teratogenicity, the risk of untreated hepatitis E infected mother and fetus are essentially high.

PREVENTION OF HEPATITIS E VIRUS

Following are the most important means of prevention

- Good personal hygiene
- High quality public water supplies
- Proper disposal of sanitary waste and
- General food safety

Recommendation[16]:

1. Disease surveillance and outbreak detection

a. Enhance hepatitis Ediagnosis and reporting in all acute hepatitis cases particularly those that test negative for hepatitis A and hepatitis B virus to enable epidemiological investigation and out breaks

b. Use of molecular methods to determine the different genotype prevalence in an area.

2. Food and water safety

a. Increased awareness among food handlers

b. Food surveillance programme for hepatitis E

3. Public health education

a. Sea foods like shell fish, pork and pig offal should be cooked completely before consumption

b. To take appropriate measures to prevent hepatitis Einfection when travelling to an endemic areas

4. Local study

To monitor hepatitis Eprevalence and epidemiological changes

SWINE HEV

For the first time in the year 1997 by Meng et al, HEV from the clinical samples of pigs in USA was identified and demonstrated. Subsequently, other countries from all over the world with high production of pork, hepatitis E virus strains have been detected. These hepatitis E genotypes showed their association with genotype III and genotype IV. Swine HEV strain is almost homologous with the human hepatitis E from the same geographic location(Hsieh et al, Pina et al, Wang et al, Huang et al, Yazaki et al)[7,].The swine hepatitis E virus shared more than 97% amino acid identity with human hepatitis E genotype III[16]. Isolates of hepatitis E virus strains from Thailand and Mexico have been classified as genotype III where human isolates of hepatitis E has been categorized as genotype I and II. In India human HEV strains are designated as genotype I and swine as genotype IV[7].

AVIAN HEV

In U.S., Haqshenas et al chicken isolated a novel HEV strain, the avian HEV from the bile sample of chicken suffering from Hepatitis-splenomegaly syndrome [7]. Comparison of avian HEV and BSLV(big liver and spleen disease virus) revealed resemblance of about 80%. Sequenced genome segment of isolates of avian HEV showed 50 to 60

percent similarity with human and swine HEV strains[7]. Variation in the position of ORF was observed, ORF 3 does not shown overlapping with ORF-1 unlike classical hepatitis E viral genome. According to Wang et al, and Haqshenas et al, it is not clear that whether isolates of avian HEV is a different genotype V of hepatitis E or it is another member of Herpevirus genus[7]. Later, phylogenetic analysis and sequence comparison of avian HEV discovered that it is most divergent of the hepatitis E virus strains and had 50% sequence identity[28]. Newly, another new avirulent strain of avian HEV was isolated from seemingly healthy chickens. It is assumed that the 2 avian HEV strains fit into presumed genotype V[28].

ANIMAL EXPERIMENTS

Several species of monkey and chimpanzees are susceptible to hepatitis E virus infection. Most of our understanding of the pathogenesis of hepatitis E infection is derived from the reliable non- human experimental animal models like rhesus, cynomolgus macaques, owl monkeys and tamarins.

Incubation period was 21 days after intraven

ous inoculation of Hepatitis E virus into cynamolgus macaques. During the initial highly replicative cycle, expression of HEV Ag appears approximately 7^{th} day post infection[4,6]. During 2^{nd} or 3^{rd} week after inoculation, HEV Ag has been detected in faeces, bile and hepatocyte cytoplasm due to excretion of HEV into bile.

Antigenic expression of hepatitis E covers approximately 70%to90% of the hepatocytes and the quantity of viral antigen declines with the onset of raise in liver enzymes and there was faecal excretion of virus and viremia in the blood. Detectable antibodies to hepatitis E virus appears just before the elevation of ALT(alanine aminotransferase) and coincides with resolution of fecal virus shedding, viraemia in the blood and reduction in the viral antigen in the liver and correspond to the presence of histopathogical changes in the liver. In the last decade, a growing trend of hepatitis E notification has been observed. From 2001 to 2010, the yearly notification of hepatitis E infection in the past decade has ranged from 26 to 117.In fact, hepatitis E turn out to be the most common cause of viral hepatitis reported in 2010. Hepatitis E accounts for 44.3% of all viral hepatitis cases, followed by 27.7% of viral hepatitis B and 24.2% of viral hepatitis A

India is endemic for hepatitis E infection and it is the most common cause of viral hepatitis in pregnancy with high mortality during

3rd trimester following fulminant hepatic failure which may be associated with the hormonal changes during the course of pregnancy[22].Hepatitis E virus gives a large global burden of sporadic and epidemic hepatitis.

HEV IN INDIA:

Table: 3.1 Studies on HEV in India

Study	Location	Population	No. of patients	Prevalence of antibodies	Comments
Naik et al, 1992	Kanpur, UP	Outbreak Population	79000	-	Municipal water was the source of contamination
Nayak et al 1989	South India	Outbreak population (NP women and adult men)	526	81.6%	0.06% CFR
Malathi et al 1995-96	Madras	Children	127	15.7%	-
Madan et al, 1998	North India	Sporadic	50	64.7	-
Radhakrishnan et al 2000	South India	Patients attending hospital	381	17.3	-
Jaiswal, 2001		Central India	273 (127P and 146NP)	57.5(P) 46 (NP)	Highest mortality in pregnant women in 3rd trimester (56%)
Beniwal, 2003	North India	3rd trimester women	97	47.4%	Mortality rate 18%
Singh et al, 2003	North India	Pregnant women	60	37%	72% in 3rd trimester, 64% mortality in pregnancy
Khuroo 2003	Kashmir	ALF patients	180	44%	95.8% pregnant women had ALF
Mishra 2003	Bangalore	Hospital patients	569	18%	CFR 3.75%

Naik et al (2002) published the data regarding a large outbreak of hepatitis E occurred in Kanpur, Uttar Pradesh during 1991. The outbreak was related to contamination of the city's municipal water supply system. Based on a sample survey, it was estimated that nearly 79,000 persons were affected. The attack rate was the highest in young adults, and was higher among males. The number of deaths recorded was 48; of this 13 were among pregnant women.

Nayak et al I n 1989 studied 169 pregnant women, 70 non-pregnant women and 287 adult men with hepatitis and found that NANB hepatitis accounted for 81.6%, 48.6% and 57.1% of cases in the three groups, respectively.

Between February 1995 to January 1996, children with acute viral hepatitis (n=127) were studied in Madras in southern part of India. HEV was the sole infectious cause in 15.7% cases; in addition, 13.4% of cases had HAV and HEV co infection, and 0.8% had HAV, HBV and HEV coinfection.

In another study from northern India by [Madan, 1998], role of HEV infection in causation of sporadic AVH (n=34) and FHF (n=16) was studied. Evidence of HE [Madan, 1998] infection was found in22

(64.7%) and 8 (50%) cases, respectively. It was the sole cause in 15/34 (44%) cases with AVH and 7/16 (43.7%) cases with FHF.

Prevalence of HEV infection was studied in 381 patients with acute hepatitis attending a hospital in southern India. HEV IgM was detected in 66 (17.3%) patients. Most of those with hepatitis E were older children and adults; only 5.5% of these were children < 12 years of age [Radhakrishnan, 2000].

A study conducted in central part of India comprising 273 women with viral hepatitis (age 18-23 years) included 127 pregnant (AVH 83, FHF 44) and 146 non-pregnant (AVH 129, FHF 17) women. Of the pregnant women, 73 (57.5%) had HEV infection;of these, 42 had FHF. Among non-pregnant women, 67 (46% had HEV infection; of these, 2 had FHF. HEV-infected women with FHF during third trimester of pregnancy had the highest mortality rate (56%) [Jaiswal, 2001].

In a study on 97 consecutive pregnant women in third trimester with acute viral hepatitis (AVH) or FHF, HEV was the leading cause in 47.4% of cases, including 36.2% of those with uncomplicated AVH and 75% of those with FHF .Twenty-four women died, including 18 with HEV infection [Beniwal, 2003].

Of 60 pregnant women with acute hepatitis tested during the years 1997 and 1998 in Delhi at AIIMS, 22 (37%) had detectable IgM anti-HEV. Of the latter, 16 (72%) were in third trimester of pregnancy. Fourteen of the 22 (64%) HEV-infected pregnant women had FHF and all died [Singh, 2003].

During April 1989 to April 1996, 180 persons [69 male, 111 female; age: mean ± SD [31.1 ± 14.7 years] with acute liver failure in Kashmir were studied. HEV was the cause in 79 (43.9%) patients. The study included 49 pregnant women; of them, 47 (95.8%) had HEV infection [Khuroo, 2003].

In another study done in bangalore by Mishra et al, 569 patients with hepatitis admitted to a hospital during1997-2000 were evaluated for IgM anti-HEV. Of these, 107 (72 male, 35 female) tested positive. The case-fatality rate was 3.75% (4/107). The highest hepatitis E rate was in the age group of 21-40 years.

In a study conducted in Delhi by Patra et al among 220 pregnant women with acute viral hepatitis, 60% of cases were due to Hep.E. There was higher rates of occurrence of FHF and of death in Women with hepatitis E than those in patients with hepatitis due to other causes.

MATERIALS

AND

METHODS

MATERIALS AND METHODS

TYPE OF STUDY:

CROSS- SECTIONAL STUDY

STUDY PLACE:

This study was done in the Department of Obstetrics and Gynecology, RSRM, Stanley Medical College and Hospital in association with Department of Microbiology, and Medical Gastroenterology, Stanley Medical College and Hospital, Chennai.

PERIOD OF STUDY:

May 2015 – Sept 2015

SAMPLE SIZE:

200

Inclusion criteria

All the asymptomatic pregnant mothers age > 18 yrs who were attending the antenatal OPD and who were willing to provide written informed consent were recruited for the study.

Exclusion criteria:

- Alcohol intake
- Chronic drug intake
- Past history of jaundice
- Chronic medical comorbidities

Those who are not willing to give consent to participate in the study.

PATIENT SELECTION:

The study was conducted among the asymptomatic pregnant mothers attending routine antenatal checkup in theDepartment of Obstetrics and Gynecology, RSRM at Government Stanley Medical College and Hospital, Chennai for a period of 5 months from MAY 2015 to Sept 2015.

Population group were from in and around Chennai.

The study was explained in detail to the pregnant mothers in their local language and informed consent were obtained.

All asymptomatic antenatal mothers Age >18 yrswho were willing to give informed consent irrespective of their gestational age were enrolled in the study.

No samples were repeated from the same patients.

200 asymptomatic pregnant mothers were selectedfor this study.

Blood samples were collected and serum was separated from them to detect the presence of IgM and IgG antibodies to HEV and to detect viral RNA by PCR.

Ethical consideration:

Ethical and research clearance was obtained from the ethical committee Stanley Medical College and Hospital. Permission to conduct the study was sought from the respective hospital authorities. Informed consent was obtained in the local language of the patientbefore enrolment into the study.

Statistical analysis:

The collected data was analyzed with Stata 12 version. To describe about the data descriptive statistics frequency analysis, percentage analysis, cross tabulation were used for categorical variables and the mean and S.D were used. To find the significance in categorical data Chi-

Square test was used. In all the above statistical tools the probability value .05 was considered as significant level.

Data collection:

Details were obtained directly from the patients with the help of a questionnaire which dealt with information regarding socio demographic data such as age, residential address, educational status, profession, socioeconomic status, source of drinking water and type of toilet facility to analyze various factors that contribute for the prevalence of HEV infection in pregnant mothers in the particular geographic area.

Sample collection:

Under aseptic precaution around 5 ml of blood sample was collected from each patients by venipuncture at the cubital fossa, by using 23G needle. Blood was dispensed into a sterile test tube without anticoagulant. Samples were transported to the Microbiology lab. Each blood sample was allowed to clot and retract. Serum sample was separated from the clot as early as possible by centrifugation at 2500 rpm for five minutes to avoid hemolysis of the red blood cells. Samples were duplicated and safely stored into 2 mL cryovials containing 50µl of EDTA at -80°C deep freezer until tested.

These set of serum samples were used for doing indirect ELISA (Enzyme Linked Immunosorbent Assay) to detect both IgG and IgM antibodies against Hepatitis E virus.

ELISA (Enzyme Linked Immunosorbant Assay)

ELISA was done with a commercial kit (Composed of 12 antigenic regions derived from ORF2 and ORF 3)

1. DSI-EIA-ANTI-HEV-IgM-KIT
2. DSI-EIA-ANTI-HEV-IgG-KIT

This test was intended for the screening of serum IgM and IgG antibodies against HEV. The test was performed according to the instructions provided in the kit literature.

PRINCIPLE OF THE TEST:

The microtitre test wells were coated with HEV antigens. Diluted patients serum was into their designated wells. If specific antibodies to the antigen are present in the patient serum, they get bind to each other in the wells during the incubation period. When the test wells are washed unbound antibodies are removed. Microtitre plate was tapped over tissue paper to get rid of the excess washing solution or bubbles in the plate formed during the washing procedure.

To this microtitre plate, freshly prepared enzyme conjugate was added to all the plates including PC and NC. If the antigen antibody complex had formed in the well during the first incubation, enzyme conjugate will specifically binds to it. Again second set of washing was repeated and to this a chromogen substrate was added. The peroxidase enzyme present in substrate will catalyse the reaction that consumes peroxide and turns the chromogen from clear to blue. Addition of stop solution end the reaction and turns the blue colour to a bright yellow colour. The reaction can be read with an ELISA reader.

MATERIALS AND EQUIPMENTS REQUIRED

1. Purified water
2. Pipettes to measure and dispense 10, 50, 90, 100
3. Pipette tips
4. Incubator at 37.0±1.0 C
5. Automated microplate washer
6. Microplate reader equipped with 450nm
7. Disposable gloves

REAGENTS

1. 96 microtitre wells coated with HEV antigens
2. Wash buffer

3. Sample diluent
4. Enzyme conjugate and diluent
5. TMB substrate and substrate buffer
6. Stop solution
7. Positive control (inactivated)
8. Negative control(inactivated)

HANDLING OF SPECIMENS

Plasma stored at -80°C was thawed for few minutes at 40°C to avoid fibrin precipitation.

AUTOMATIC MICROPLATE WASHER:

Washer was set up appropriately i.e. 380-400µl working washing solution into each wells and soak time at least 40 seconds and aspirate.

PREPARATION OF THE REAGENTS

HEV –Ag COATED STRIPS

The foil bag was opened and the strips were removed and placed in the microtitre plate. The strips were numbered and the wells were assigned with the respective samples with the help of a data sheet. Before initiating the assay all the strips were washed 2 times with the automatic microplate washer, as instructed in the manual.

PREPARATION OF WASH BUFFFER

To 50ml of washing buffer (concentrated 25 fold), 1200mlof distilled water (1:25 ratio), was added and mixed thoroughly according to the manual.

Note: the prepared working solution is stable for 14 days at 18-24°C or for 28 days when used in GLP condition.

PREPARATION OF THE WORKING CONJUGATE

Working conjugate was prepared just before the test procedure. To 0.65ml of conjugate (concentrated 21 fold), 13ml of conjugate diluent (1:20 ratio)was added and thoroughly mixed until diluted avoiding foaming. Intensive mixing was avoided.

Note: it can be stored for 12 hours in the dark at 18-24°C

PREPARATION OF THE SUBSTRATE MIXTURE

The substrate mixture was prepared just before use. To 0.65ml of TMB (concentrated 21 fold), 13ml of substrate buffer (1:20 ratio) was added and mixed thoroughly until diluted.

Note: substrate mixture is stable for 10 hours when stored in a dark place at 18-24°C in a clean vial

DS-EIA-ANTI-M ASSAY PROCEDURE:

ACCORDING TO THE MANUFACTURERS INSTRUCTIONS

BEFORE ASSAY

The coated strips was washed with working washing solution 2 times before the procedure. 380-400µ of working washing solution was added into each well during washing. Soak time at least 40 sec was kept. Automated microplate washer was used after appropriate set up. The microtitre plate was tapped over tissue paper and was made sure that no fluid or bubbles are left inside the wells as they may adversely affect the assay precision. Data sheet are made ready for the controls and for the samples.

1. 100µl of PC into one well and NC in 2 wells are added to their designated wells.
2. 90µl of sample diluent and 10µl of test serum are added in rest of the wells.
3. The plate was covered by a lid protective film.
4. The plate was incubate for 30 minutes at 37±1°C in the incubator.
5. The contents was removed by washing the plate for 4 times as described above.

6. 100µl of freshly prepared working solution of conjugate was then added into each wells.
7. Step 4 & 5 was repeated.
8. 100µl of freshly prepared substrate mixture was added into each wells.
9. The plate was kept in a dark place for 20 minutes at 18-24°C
10. 150µl of stopping reagent are added to all the reacting wells.
11. Optical density at 450/620 nm using microplate reader was used to read the result immediately after adding stop solution.

For the assay to be valid:

PC > 0.6 and NC < 0.2

CUT – OFF = AVERAGE OF NC + 0.200(coefficient – defined by manufacturer during statistical processing for each lot)

INTERPRETATION OF RESULTS

Sample is NEGATIVE if OD < CUT – OFF

Sample is POSITIVE if OD ≥ CUT - OFF

PREPARATION OF THE REAGENTS FOR IgG ANTI HEV

HEV –Ag COATED STRIPS

The foil bag was opened and the strips were removed and placed in the microtitre plate. The strips were numbered and the wells were assigned with the respective samples with the help of a data sheet. Before initiating the assay all the strips were washed 2 times with the automatic microplate washer, as instructed in the manual.

PREPARATION OF WASH BUFFFER

To 50ml of washing buffer(concentrated 25 fold), 1200ml of distilled water (1:25 ratio), was added and mixed thoroughly according to the manual.

Note: the prepared working solution is stable for 14 days at 18-24°C or for 28 days when used in GLP condition.

PREPARATION OF THE WORKING CONJUGATE

Working conjugate was prepared just before the test procedure. To 0.65ml of conjugate (concentrated 21 fold), 13ml of conjugate diluent (1:20 ratio) was added and thoroughly mixed until diluted avoiding foaming. Intensive mixing was avoided.

Note: it can be stored for 12 hours in the dark at 18-24°C

PREPARATION OF THE SUBSTRATE MIXTURE

The substrate mixture was prepared just before use. To 0.65ml of TMB (concentrated 21 fold), 13ml of substrate buffer (1:20 ratio) was added and mixed thoroughly until diluted.

Note: substrate mixture is stable for 10 hours when stored in a dark place at 18-24°C in a clean vial

DS-EIA-ANTI-G ASSAY PROCEDURE:

ACCORDING TO THE MANUFACTURERS INSTRUCTIONS

BEFORE ASSAY

The coated strips was washed with working washing solution 2 times before the procedure. 380-400µl of working washing solution was added into each well during washing. Soak time at least 40 sec was kept. Automated microplate washer was used after appropriate set up. The microtitre plate was tapped over tissue paper and was made sure that no fluid or bubbles are left inside the wells as they may adversely affect the assay precision. Data sheet are made ready for the controls and for the samples.

1. 100µl of PC and NC in duplicates are added to their designated wells.
2. 90µl of sample diluent and 10µl of test serum are added in rest of the wells.
3. The plate was covered by a lid protective film.
4. The plate was incubate for 30 minutes at 37±1°C in the incubator.
5. The contentswas removed by washing the plate for 4 times as described above.
6. 100µl of freshly prepared working solution of conjugate was then added into each wells.
7. Step 4 & 5 was repeated.
8. 100µl of freshly prepared substrate mixture was added into each wells.
9. The plate was kept in a dark place for 20 minutes at 18-24°C.
10. 50µl of stopping reagent are added to all the reacting wells.
11. Optical density at 450/620 nm using microplate reader was used to read the result immediately after adding stop solution.

For the assay to be valid:

PC > 0.6 and NC < 0.2

CUT – OFF = AVERAGE OF NC + 0.200(coefficient – defined by manufacturer during statistical processing for each lot)

INTERPRETATION OF RESULTS

Sample is NEGATIVE if OD < CUT – OFF

Sample is POSITIVE if OD ≥ CUT – OFF

OBSERVATION

AND

RESULTS

OBSERVATION AND RESULTS

Two hundred asymptomatic pregnant women attending routine antenatal clinic in a tertiary care hospital, were included in the study. Blood samples were collected and serum was separated by centrifuging at 2500 rpm. All the serum samples were stored to detect IgM, IgG antibodies against hepatitis E virus from May 2015 to September 2015. Our observations are as follows:

The mean age group of the pregnant women was 24.12 ± 3.64. Among the 200 women 82.2 % fell under 21-30 years of age as described in Fig 5.1

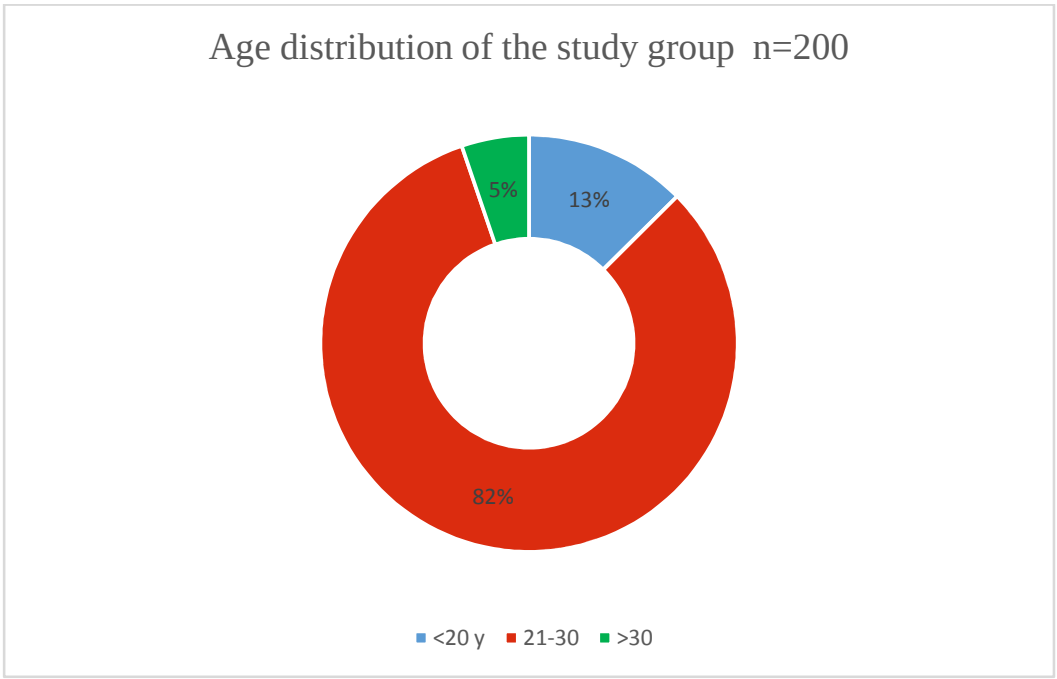


Fig 5.1: Age distribution of the study group

Among the women whom we studied, majority(49%) fell in the second or third trimester (39%) as in fig 5.2

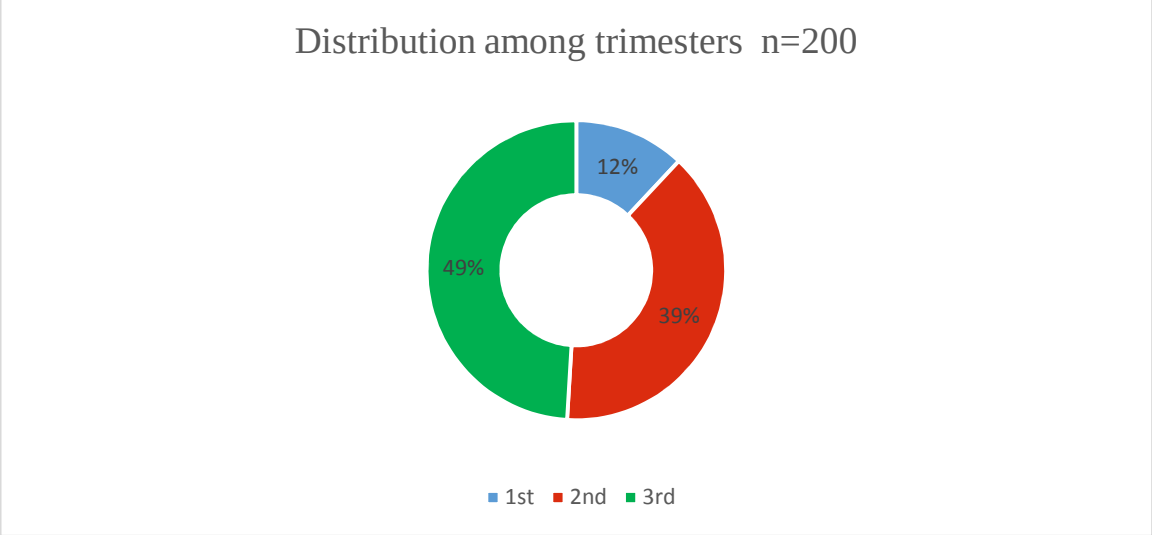


Fig 5.2: Distribution of the study women in different trimesters

There was equal representation primi and multigravida as depicted by fig 5.3

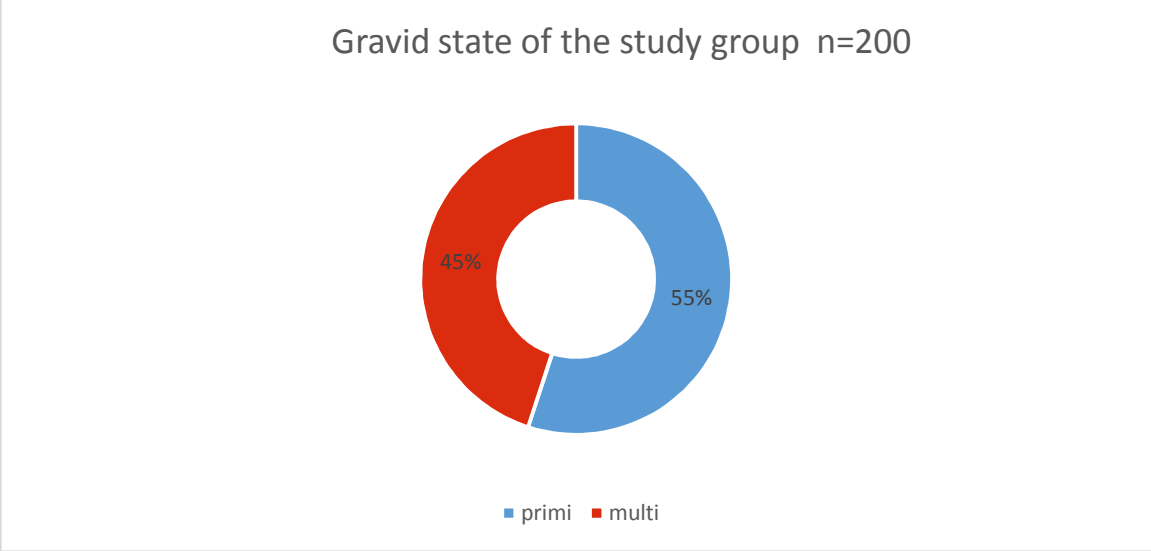


Fig 5.3: Gravid status of the study group

Majority of the members of the study group have completed higher secondary education as shown in fig 5.4

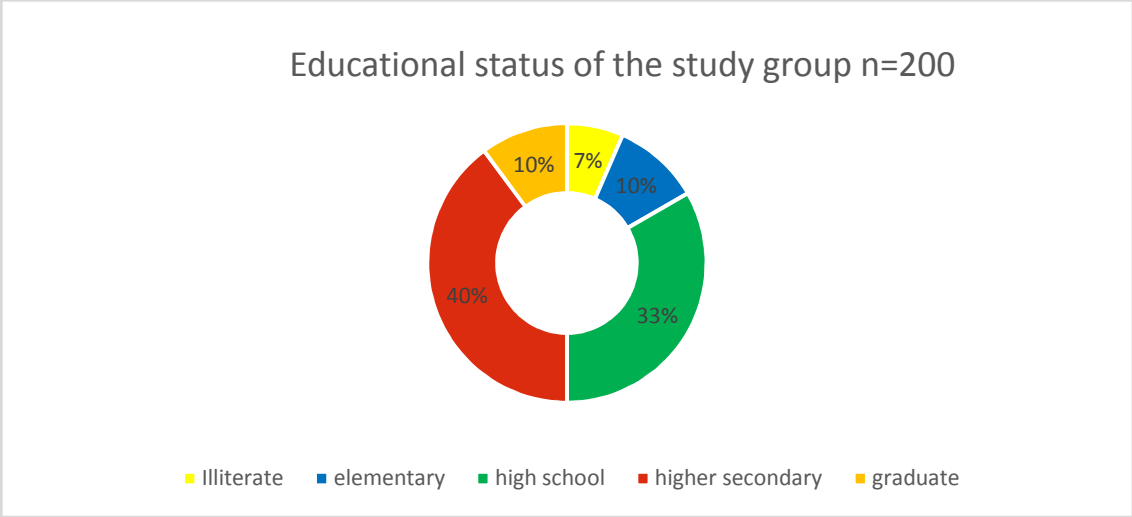


Fig 5.4: Educational status of the study group

Majority of the pregnant women were from upper lower(57%) followed by lower middle (29%) as explained by fig 5.5

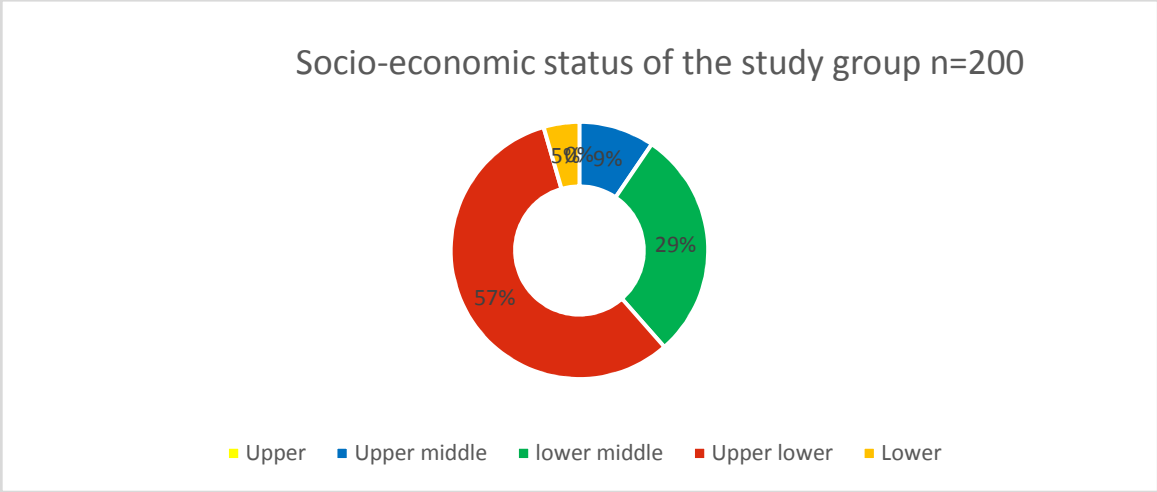


Fig 5.5: Socio-economic status of the study group

Majority of the population has been using Metro water (61.5%) as the source of drinking water, the rest using borewell and can water as shown in fig 5.6 and 24.5% have been boiling the water before use.

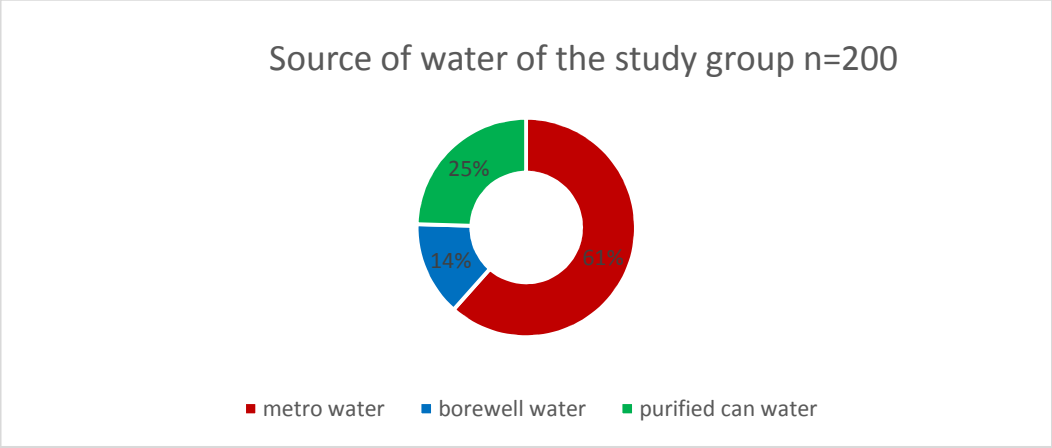


Fig 5.6: Source of water of the study group

24.5% of the pregnant women did not have adequate drainage facilities at home as shown in fig 5.7

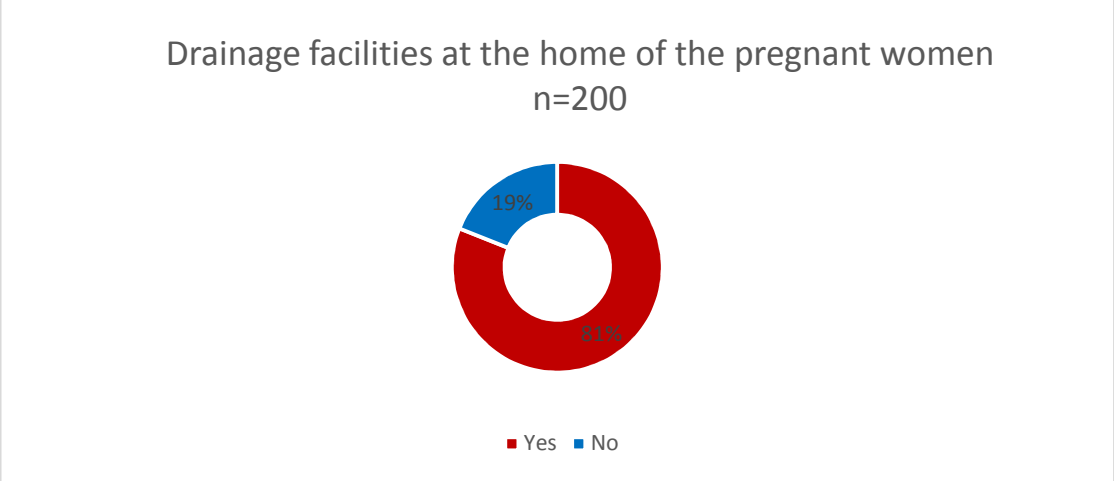


Fig: Drainage facilities at home of the study group

About 79.5 % responded positively when questioned about the habit of had washing before food and after using toilet and 45.5 % of women consumed non vegetarian food as depicted by fig 5.8, 5.9 respectively.

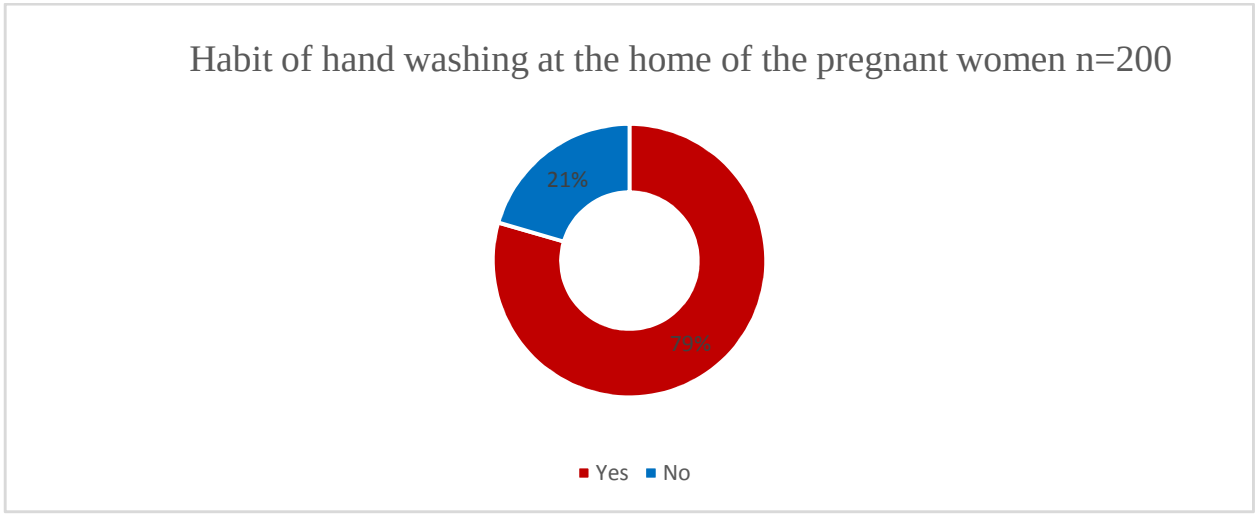


Fig 5.8 Habit of hand washing at the home of the pregnant women

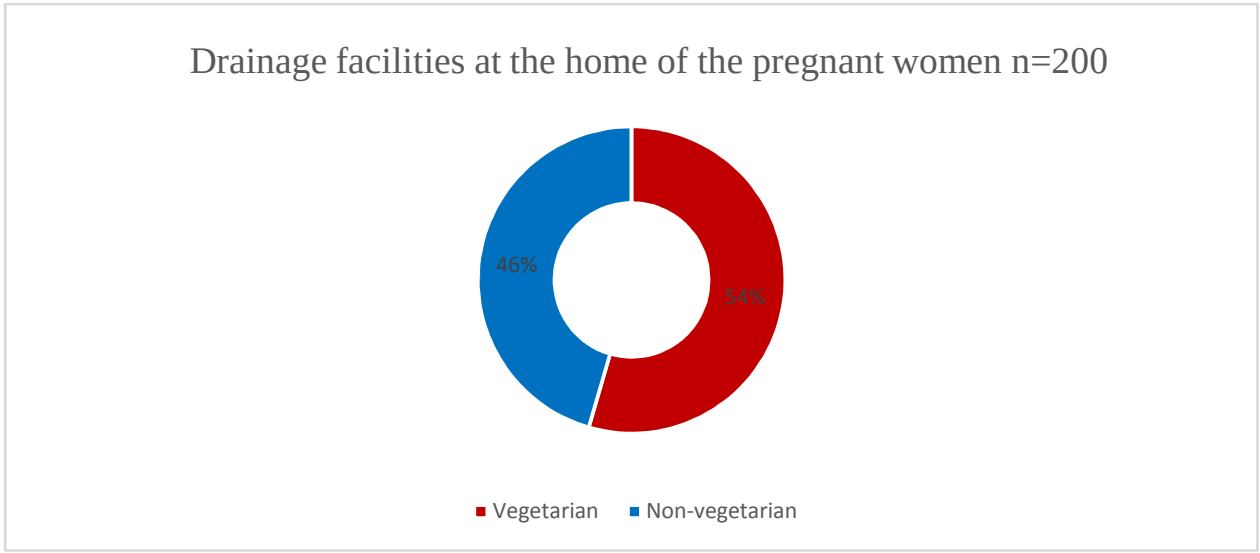


Fig 5.9: Drainage facilities at the home of the pregnant women

The results of Ig M and Ig G ELISA were analyzed with respect to the possible risk factors if HEV disease.

ELISA for Ig M was positive in 9 patients all within the age group of 21-30 y of age. Of the 9 positive women, 4 were primigravidae and 5 were multi-gravidae. These patients were equally distributed in all the three trimesters. 7 of these patients were consuming non vegetarian food which reached statistical significance ($p=0.04$) and 8 patients who tested positive to Ig M ELISA did not wash their hands before food and after using toilet ($p<0.05$). 6 patients were using metro water for drinking ($p=0.94$) and 7 of them did not boil the water before drinking ($p<0.05$). While we analyzed the sanitary facilities and Ig M positivity, we found that seven of the 9 positive patients did not have adequate drainage facilities at home ($p < 0.05$). Majority of the patients who turned positive were illiterates ($p<0.05$) and 5 were belonging to the upper lower class of modified Kuppusamy socio economic status scale 2015($p<0.05$) and the results are depicted in fig 5.10-5.13

ELISA for Ig G was positive in 13 patients and majority within the age group of 21-30 y of age. Of the 9 positive women, 7 were primigravidae and 6 were multi-gravidae. Majority of the sero positivity occurred in 3rd trimester ($p=0.05$). 9 of these patients were consuming non vegetarian food which reached statistical significance ($p=0.07$) and 9

patients who tested positive to Ig G ELISA did not wash their hands before food and after using toilet ($p<0.05$). 8 patients were using metro water for drinking ($p < 0.05$) and 10 of them did not boil the water before drinking ($p<0.05$). While we analyzed the sanitary facilities and Ig M positivity, we found that six of the 13 positive patients did not have adequate drainage facilities at home ($p =0.06$). Educational status did not differ within the positive population ($p=0.90$) and 6 were belonging to the upper lower class of modified Kuppusamy socio economic status scale 2015($p<0.05$) and the results are depicted in fig 5.14-5.16

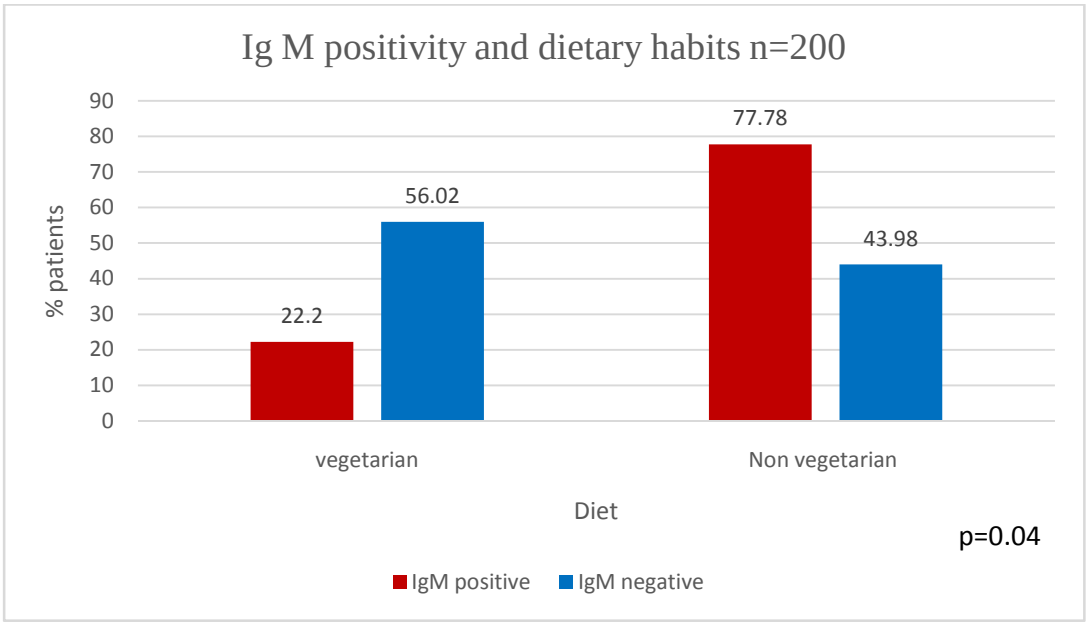


Fig 5.10: Ig M seropositivity and dietary habits

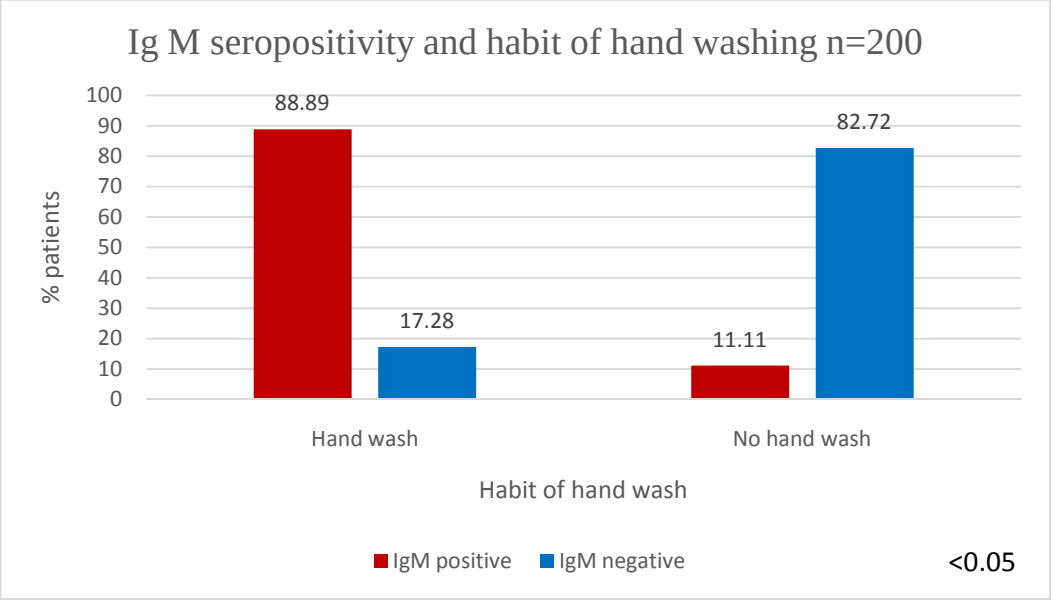


Fig 5.11: Ig M sero-positivity and habit of hand washing

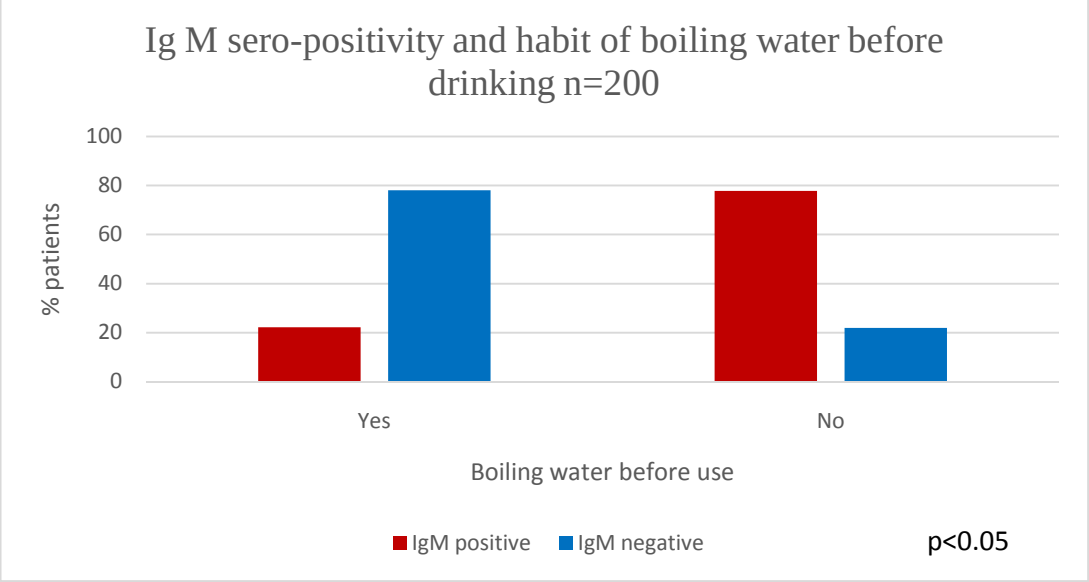


Fig 5.12: IgM sero-positivity and habit of boiling water before drinking

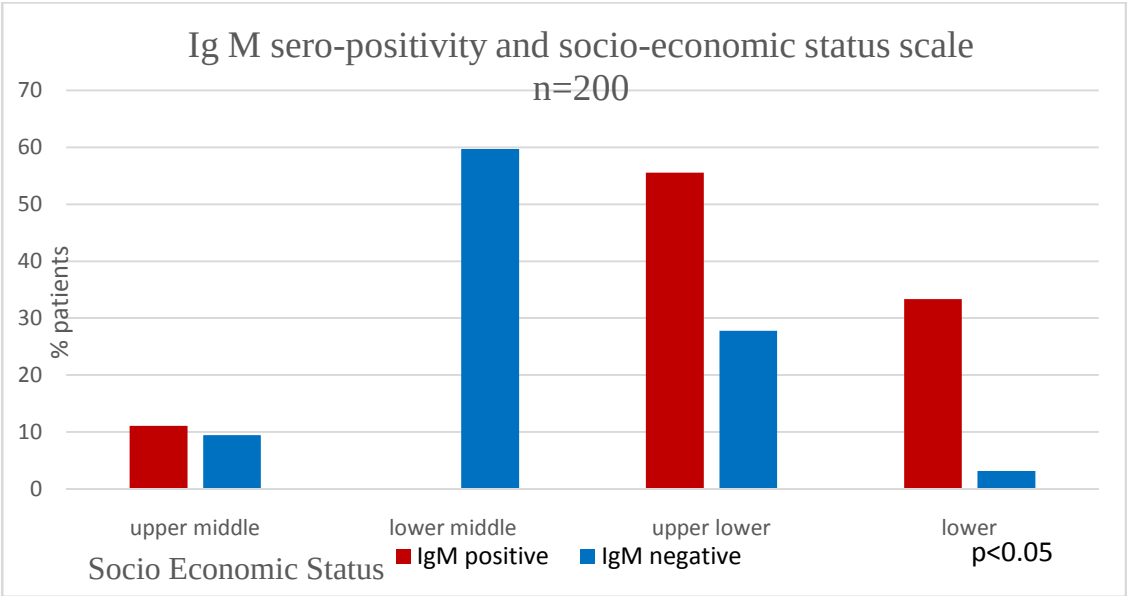


Fig: 5.13: Ig M sero-positivity and 2015 modified

Kuppusamy's socio-economic status scale.

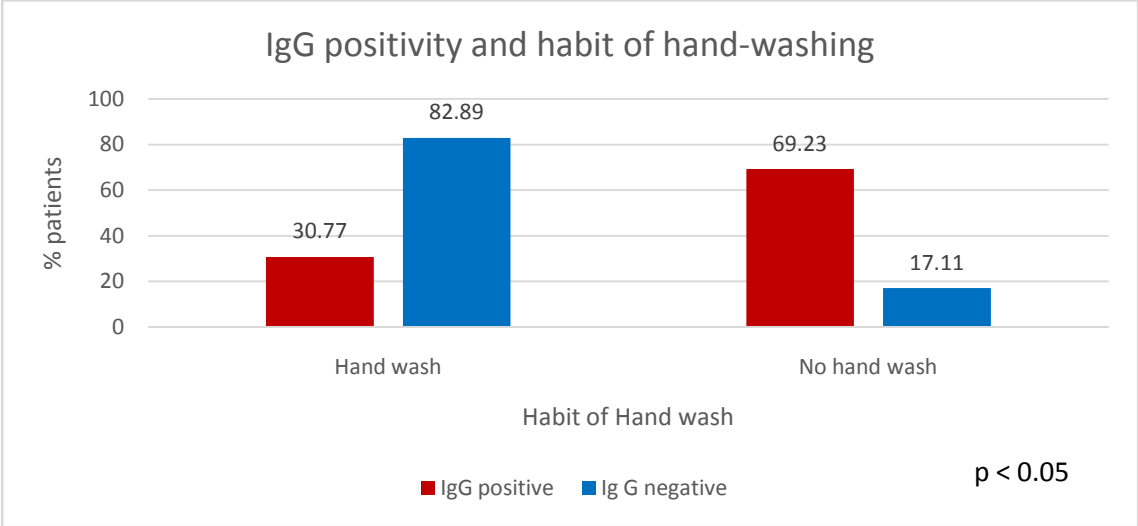


Fig 5.14: Ig G sero-positivity and habit of hand washing

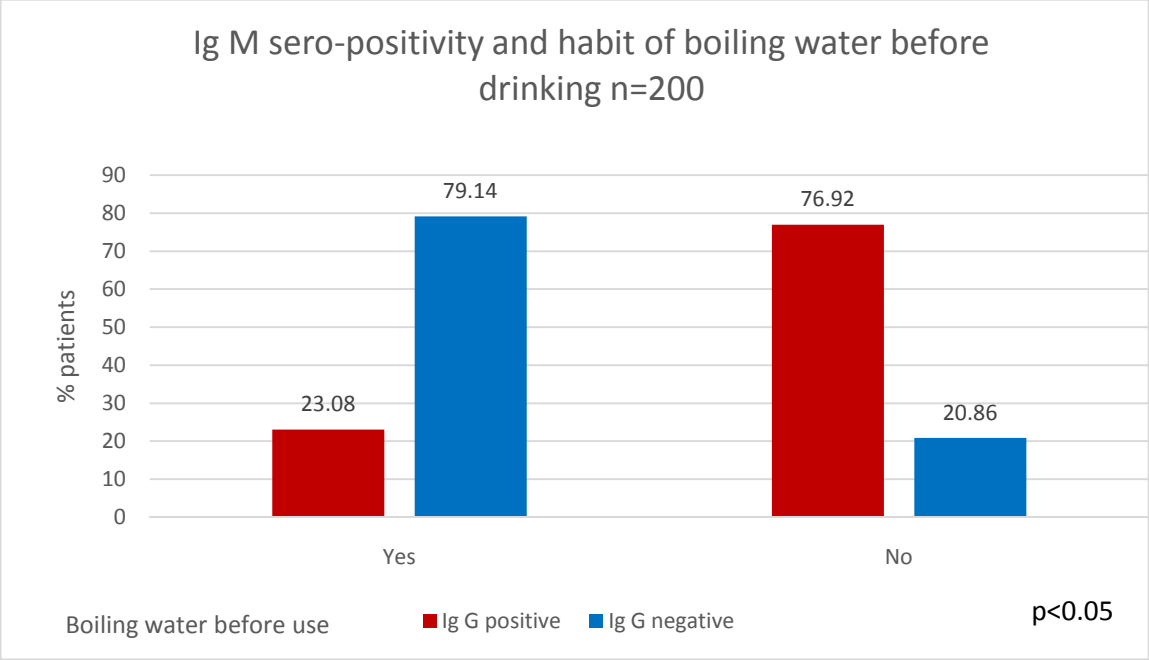


Fig 5.15: Ig M sero-positivity and habit of boiling water before drinking

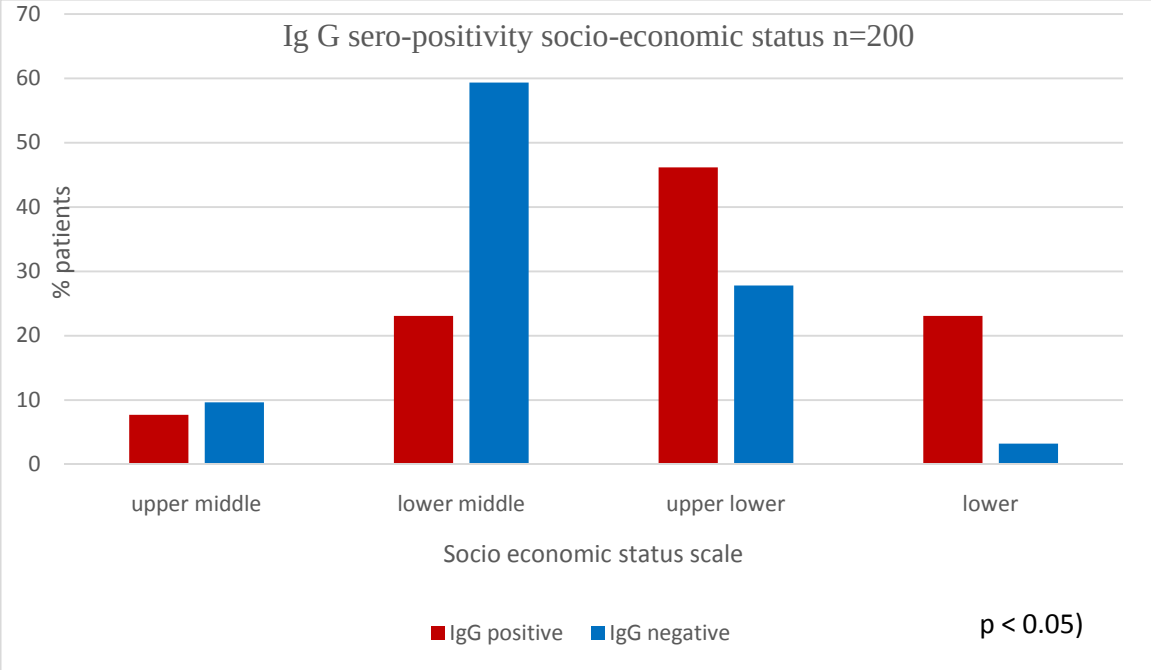


Fig 5.16: Ig G sero-positivity and 2015 modified Kuppusamy's socio-economic status scale

DISCUSSION

DISCUSSION

Enzyme Linked Immunosorbent Assay (ELISA) is the serological test available for antibody detection of both anti HEV IgM and IgG of hepatitis E virus infection. **It is highly sensitive, inexpensive and a practical method for detection of HEV antibodies.**

The population included in this study were asymptomatic pregnant women who comes for routine antenatal checkup in a tertiary care hospital. All patients were from different areas in and around Chennai.

Sera of all the enrolled patients were analyzed for the presence of anti-HEV IgM and IgG antibody against hepatitis E virus by a commercially available Enzyme Linked Immunosorbent Assay (***DSI - EIA - ANTI - HEV - M; DSI - EIA - ANTI - HEV - G***)

ANALYSIS OF HEV SEROPOSITIVITY

We found totally **4.5% (9/200)** IgM and **6.5% (13/200)** IgGsero prevalence in asymptomatic pregnant women. Concerning the Sero prevalence of hepatitis E virus infection in asymptomatic pregnant women, results of this study were similar to 5.62% sero prevalence of IgG a report given by Daniel et al (2004) at Vellore from 600 samples including blood donors, antenatal mothers and from pre-operative

patience. And also coincides with other studies from south India with 8% anti HEV seropositivity[2].

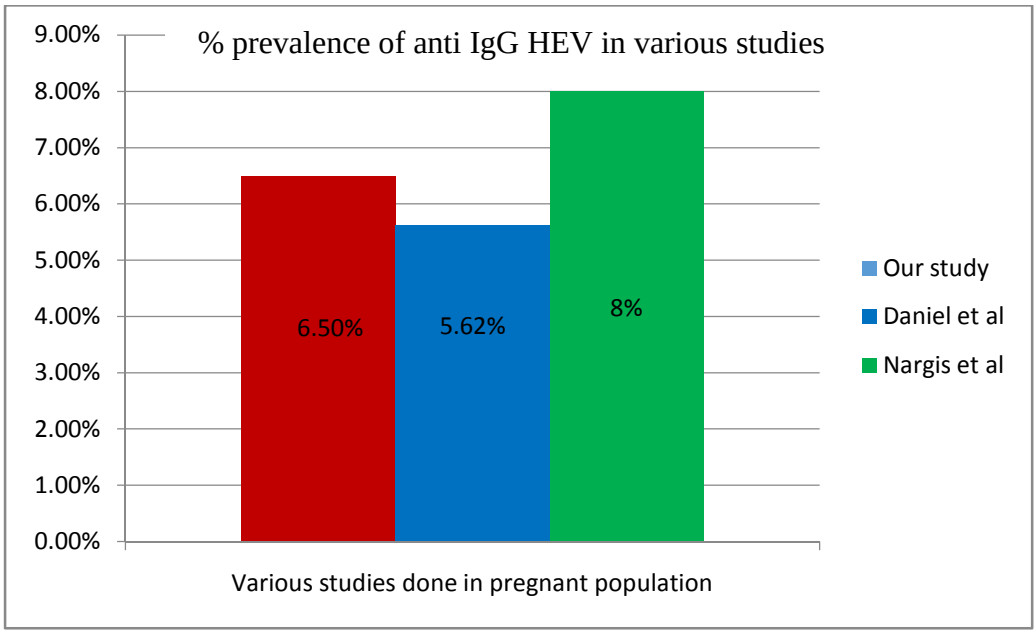


Fig 6.1: % prevalence of anti IgG HEV in various studies

And other studies from Pune and Lucknow documented a much higher prevalence in adult population (*40%-50%*). But the seroprevalence of this study is also comparable to the 3.6% seroprevalence of hepatitis E in pregnant women in Madrid, Spain and 6.6% seroprevalence of IgG antibodies to HEV reported in pregnant women infected with HIV in Gabon and central Africa[55].

Table 6.1 % prevalence of anti IgG HEV in various studies in India

Author	Year	Place	Sample size	Prevalence	Population
Khuroo et al	1994	Kashmir	40	5%	Asymptomatic healthy children
Arankalle et al	1995	Pune	1602	23.62%	Healthy general population
Aggarwaal et al	1997	Lucknow	95	59.25%	Healthy general population
Das et al	2000	New Delhi	500	35.6%	General population sporadic AVH
Mathur et al	2001	New Delhi	2070	26.25%	Children with mild non-hepatic illness
Mohanavalli et al	2003	Chennai	185	9.5%	Healthy children
Daniel et al	2004	Vellore	600	5.62%	Blood donors and pregnant women
Nargis et al	2009	New delhi	300	33.67	Asymptomatic pregnant women
Our study	2015	Chennai	200	6.5%	Asymptomatic pregnant women

AGE DISTRIBUTION

The study group included in asymptomatic pregnant mothers wereaged 18-35 years with the mean age of ***24.12***years. According to the study carried out by Shams et al, out of 65 pregnant women, the mean age

was 25 years[60]. Nargis Begum et al mean age of the 300 asymptomatic primigravida were ***21.92 ± 2.66*** years (range 18-35)[2].

All cases of IgM HEV and majority of Ig G sero-prevalence were reported in the age between 21 and 30 years and this might be attributed to the predominant population group that has been studied.

Table 6.2 HEV and Mean Age

Study	Population studied	Mean age	Comments
Nargis et al	Asymtomatic pregnant women	21.92 ± 2.66	Both falls within the age of 21-30 years
Our study	Asymtomatic pregnant women	24.12 ± 3.64	

GRAVIDA

Among 200 asymptomatic pregnant patients, ***55%*** were primigravida, 45% of them were multigravida. Compared to primigravida, 2nd and multi gravida were less in number. Most of them were in their second and third trimester of pregnancy. According to Nargis Begum et al mean period of gestation was 19.06 ±2.25 wk with a range of 16-24 wk[2].

Seropositivity of pregnant women of different gravidawere equally distributed in different gravida. This result was in contrast to the study done by Cosme et al, where seropositivity of hepatitis E were in

association with number of pregnancy though the reason was not yet clear[55].

EDUCATIONAL STATUS

Out of 200 asymptomatic pregnant women ***7%*** of them were illiterates Majority of them have studied up to higher secondary school. Diploma holders among asymptomatic pregnant women was only 10**%.**

Though IgG positivity was common in illiterates, IgGsero-prevalence did not differ much by the difference in educational status. This study results were similar according to Cosme et al observation, in which he states that there was no positive association with the education status and HEV seropositivity[55]. But the results were contrary to the study done by Sekan et al, according to which education seems to be the only risk factor for HEV seroprevalence[18,58].

Table 6.3 HEV and Educational Status

Study	Population	Percentage of illiterates with positive IgG	Comments
Nargis et al	Asymptomatic Pregnant women	80.6	Lower Ig G positives in illiterates than the above study But did not decrease with increasing level of education (p=0.90)
Our study	Asymptomatic Pregnant women	44.4	

SOCIOECONOMIC STATUS

According to the Kuppusamy's criteria, about 5*%, 57%, 29%* and 9% were in the lower, upper lower, lower middle and upper middle class of socioeconomic status respectively. Majority of them were in the upper lower class. Clubbing patients with upper lower and lower, there were 33% of women from lower socio-economic status and sero-positivity of IgG and Ig M were significantly more positive than in the higher groups ($p<0.05$). The results are similar to the study conducted by Nargis et al.

No seropositive cases were seen in the lower group of socioeconomic status. And this cannot be always true because out of 200 pregnant women only 5% of them were in the lower level of socioeconomic status in the study population. According to Nargis et al socioeconomic status appeared to be the risk factor for IgG sero prevalence.

Study	**Population**	**Percentage of women with lower socio-economic status & positive IgG**	**Comments**
Nargis et al	Asymptomatic Pregnant women	48.5	Similar increase in positivity with declining socio-economic status
Our study	Asymptomatic Pregnant women	33	

FOOD HABIT AND USE OF BOILED WATER

Out of 200 asymptomatic pregnant women ***54%*** of the pregnant women were following non-vegetarian diet whereas only ***46%*** of them were vegetarian.

24.5% of the pregnant women were using boiled water whereas majority ie., **75.5%** of them used water without boiling. These results were in contrast to Cosmo Alvarado et al where the above risk factors were not associated with sero-positivity.

Majority of sero-positive patients were consuming non-vegetarian diet which shows that the asymptomatic pregnant women following Non-vegetarian dietary habit were predominantly associated with HEV seroprevalence. This high prevalence may be due to consumption of undercooked meat. According to Rakesh et al HEV-RNA has been detected from domestic swine in their faeces and also HEV antibodies have been detected in the sera of cattle, sheep, pigs and rodents[4].

Most of the HEV positive pregnant women i.e., were not using boiled water for drinking. This shows, it may be related to HEV positivity as boiling can inactivate HEV[5].

But from the above result, no positive association was found between source of drinking water and use of boiled water with HEV positivity. Similar observation was documented by Cosmeet al[55] and also agrees with results found in other studies[18].

SUMMARY

SUMMARY

Asymptomatic pregnant women attending routine antenatal check up in a tertiary care hospital were enrolled and the observational study was conducted between “May 2015 and September 2015”. Total of 200 pregnant mothers were selected and evaluated by a questionnaire which dealt with information regarding socio demographic data such as age, residential address, educational status, profession, socioeconomic status, source of drinking water and type of toilet facility. Serum samples were collected and serological test was done using ELISA to detect anti-HEV IgM and IgG antibodies and were done according to the manufacturer’s instruction.

In the present study, out of 200 asymptomatic pregnant women majority of them were seen in the age group of 21-30 (mean age 24.15). Most of them were in the 2^{nd} and 3^{rd} trimester of pregnancy. Primigravidae outnumbered multi gravida. Educational status of most of the pregnant women was till higher secondary school. Graduates and illiterates were almost in equal percentage. Most of the pregnant mothers belong to upper lower class.

In this study we found totally **4.5%** of IgM and **6.5%** of IgG sero prevalence in asymptomatic pregnant women. Most of the positive cases

were in the age group 21-30. Sero prevalence was highly associated with women not having a habit of hand washing, not having adequate drainage facilities, who are consuming non vegetarian diet, poor socio-economic status. Analysis of anti HEV sero prevalence with age, gravida, educational level, however revealed no significant association. Our study was in contrast to the results of Cosme et al with respect to hand washing, educational level, socio-economic status, consumption of untreated water and undercooked meat. Our study had similarities with respect to age, socio-economic status and in contrast with respect to educational status, non-vegetarian diet, hand washing, boiling of water as studied by Nargiset al[2].

CONCLUSION

CONCLUSION

1) In India many factors are favorable for the transmission of the disease like low socioeconomic status, poor hygiene and low level of education.
2) The seroepidemiology of hepatitis E virus infection in pregnant women is largely unknown in different states in India.
3) Thus it is desirable to determine the magnitude of hepatitis E virus seroprevalence in pregnant women so as to consider HEV as one of the public health problem and also for the ideal planning of preventive measures against hepatitis E infectionin our area.
4) From the present study we conclude that there is very low prevalence of anti HEV IgM and IgG antibodies among asymptomatic pregnant women when compared with study results of north India.
5) Further studies with more numbers and improvement in the molecular testing will be an important research protocol.
6) Role of HEV vaccination is currently questionable in India at present may throw better insights into the HEV epidemiology.
7) Improved sanitation, boiling water for drinking, safe washing practices will go a long way in the eradication of this subclinical infection which can be deadly during the later trimester of pregnancy.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. The leading cause of acute viral hepatitis in the world.
2. Seroprevalence of subclinical HEV infection in pregnant women from north India: A hospital based study-Nargis Begum, Salam Gyaneshwori Devi, Syed A. Husain- Received September 2, 2008.
3. Min Liu1, Yili Chen1, Zhengyu Shen2 and Hongxu-Comparative clinical study on diagnostic detection of hepatitis E virus between nested polymerase chain reaction (PCR) and serological tests August, 2013 African journal of Microbiology Research.
4. Hepatitis E an over view and recent advances in clinical and laboratory research Journal of Gastroenterology and Hepatology
5. Fields text book of virology
6. Topley and Wilson text book of microbiology
7. P. Vasickova, I. Psikal, P. Kralik, F. Widen, Z. Hubalek, I. Pavlik-Hepatitis E virus: a review. VeterinarniMedicina, 52, 2007 (9): 365–384
8. T. P. E. Williams, C. Kasorndorkbua, P. G. Halbur, -Evidence of Extra hepatic Sites of Replication of the Hepatitis EVirus in a Swine Model Journal Of Clinical Microbiology Sept. 2001, p. 3040–3046.

9. Malcolm Banks, Richard Bendall, Sylvia Grierson- Emerging Infectious Diseases • www.cdc.gov/eid • Vol. 10, No. 5, May 2004
10. Nicole PAVIO1, Xiang-Jin MENG, Christophe- Zoonotic hepatitis E: animal reservoirs and emerging risks
11. Nicholas John Ashbolt - Microbial contamination of drinking water and disease outcomes in developing regions. Toxicology 198 (2004) 229-238
12. Robert H. Purcell and Suzanne U. Emerson- Hidden Danger: The Raw Facts about Hepatitis E Virus
13. MS Khuroo MD Riyadh, Saudi Arabia- DISCOVERY OF HEPATITIS E VIRUS - THE UNTOLD STORY
14. Mohammad Sultan Khuroo, SaleemKamii, ShahidJameel -Vertical transmission of hepatitis E virus
15. Patrick G. Halbur, ChaiyanKasorndorkbua, Xiang-JinMeng- hepatitis E virus
16. Scientific Committee on Enteric Infections and Foodborne Diseases Epidemiology and Prevention of Hepatitis E
17. Toshinori Tanaka, Masaharu Takahashi, EijiKusano- Development and evaluation of an efficientcell-culture system for Hepatitis E virus
18. SerkanOncu, SelcenOncu, Pinar Okyay- Prevalence and risk factors for HEV infection in pregnant women

19. Dr. Udayakumar Navaneethan, MD, Dr. Mayar Al Mohajer, MD, - Hepatitis E and Pregnancy- Understanding the pathogenesis
20. A. Kumara,*, M. Beniwala, P. Karb, J.B. Sharmaa, N.S. Murthyc- Hepatitis E in pregnancy22 July 2003.
21. Persistent Carriage of Hepatitis E Virus in Patientswith HIV Infection
22. The Two Faces of Hepatitis E Virus-22 July 2003
23. Andrew A Adjei, Yao Tettey1, John T Aviyase -Hepatitis E virus infection is highly prevalent among pregnantwomen in Accra, Ghana
24. Alain B. Labrique, Shegufta S. Sikder, Lisa J. Krain, Keith - Hepatitis E, a Vaccine-PreventableCause of Maternal Deaths
25. Silvia Sookoian-Symposium on liver & pregnancyLiver disease during pregnancy:acute viral hepatitis Annals of Hepatology 2006; 5(3): July-September: 231-236Annals of Hepatology
26. WHO Prevention &Control of Viral Hepatitis Infection : Framework for Global Action
27. Zakim and boyer's text book of liver diseases-Diagnosis and Detection of HEV
28. Padma billam et al- mechanism of pathogenesis and replication of an Avian strain of the hepatitis E virus in a chicken model.
29. HEV - background

30. Monica Mateus, ZekiaNeves, Ana Arauja-fulminant hepatitis E in a pregnant women-
31. Hepatitis E- august 2009 TRANSFUSION
32. Sandra Rodrigez, William D. Carey-Hepatitis E an evolving disease
33. Min Liu1, Yili Chen1, ZhengyuShenandHongxu Xu-Comparative clinical study on diagnostic detection of hepatitis E virus between nested polymerase chain reaction (PCR) and serological tests
34. M. Herremans,* E. Duizer, E. Jusic, and M. P. G. Koopmans-Detection of Hepatitis E Virus-Specific Immunoglobulin A in Patients Infected with Hepatitis E Virus Genotype 1 or 3
35. Hepatitis E Virus (HEV) Strains in Serum Samples Can Replicate Efficiently in Cultured Cells Despite the Coexistence of HEV Antibodies: Characterization of HEV Virionsin Blood Circulation
36. Hepatitis E Virus Antibodies in Patients with Chronic Liver Disease
37. Identification of Genotype 3 Hepatitis E Virus (HEV) in Serum and Fecal Samples from Pigs in Thailand and Mexico, Where Genotype 1 and 2 HEV Strains Are Prevalent in the Respective Human Populations

38. Prevalence of Antibodies to Hepatitis E Virus in Veterinarians Working with Swine and in Normal Blood Donors in the United States and Other Countries
39. Silent hepatitis E virus infection in Dutch blood donors, 2011 to 2012
40. Use of Serological Assays for Diagnosis of Hepatitis EVirus Genotype 1 and 3 Infections in a Setting of Low Endemicity
41. Jun Inoue- Analysis of human and swine hepatitis E virus(HEV) isolates of genotype 3 in Japan that are only81–83 % similar to reported HEV isolates of thesame genotype over the entire genome
42. Czech Republic Petra Vasickova, Michal Slany, Pavel Chalupa, Michal Holub, Radek Svoboda, and Ivo Pavlik -Detection and Phylogenetic Characterization of Human Hepatitis E Virus Strains.
43. Detection and characterization of infectiousHepatitis E virusfrom commercial pig livers sold inlocal grocery stores in the USA
44. Rakesh AggarwalÆShahidJameel- Hepatitis E vaccine
45. Pathogenesis and Treatment of Hepatitis E Virus Infection
46. Sporadic acute or fulminant hepatitis E inHokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food
47. Rabbit as a Novel Animal Model for Hepatitis E Virus Infection and Vaccine Evaluation

48. Swine Fact Sheet
49. Open reading frame structure analysis as a novel genotyping tool for hepatitis E virus and the subsequent discovery of an inter-genotype recombinant
50. Zoonotic hepatitis E: animal reservoirs and emerging risks
51. A serological immunoassay for Hepatitis E virus (HEV) diagnosis 1 based on genotype 3 Open Reading frame-2 (ORF-2) recombinant 2 proteins produced in Trichoplusianilarvae.
52. Challenges in diagnosis of Hepatitis E virus infections
53. Diagnosis of HEV infection by serological and real-time PCR assays: a study on acute non-A-C hepatitis collected from 2004 to 2010 in Italy
54. Serologic Assays Specific to Immunoglobulin M Antibodies against Hepatitis E Virus: Pangenotypic Evaluation of Performances
55. Cosme Alvarado-Esqivel, luis F - Hepatitis E virus exposure in pregnant women in rural Durango, Mexico.Annals of hepatology
56. Alain B. Labrique, * K. Zaman , Zahid Hossain , ParimalenduSaha, Mohammad Yunus-Population Seroprevalence of Hepatitis E Virus Antibodies in Rural Bangladesh
57. Christopher M .Howard , * Thomas Handzel , Vincent R . Hill , Scott - The American Society of Tropical Medicine and Hygien

Novel Risk Factors Associated with Hepatitis E Virus Infection in a Large Outbreak in Northern Uganda: Results from a Case-Control Study and Environmental Analysis

58. el-Zimaity DM[1], Hyams KC, Imam IZ, Watts DM, Bassily S, Naffea EK, Sultan Y, Emara K, Burans J, Purdy MA, et al-Acute sporadic hepatitis E in an Egyptian pediatric population.

59. Surajudeen A. Junaid1,2, Samuel E. Agina1 and Khadijah A. Abubakar- Epidemiology and Associated Risk Factors of Hepatitis E Virus Infection in Plateau State, Nigeria 2001 Jul-Sep;13(3):31-5.

60. Shams R[1], Khero RB, Ahmed T, Hafiz A-Prevalence of hepatitis E virus (HEV) antibody in pregnant women of Karachi.

ANNEXURES

PROFORMA

DATE:

NAME: AGE: OP No:

OBSTETRIC CODE:

LMP: EDD:

ADDRESS & CONTACT NO:

PRESENTING COMPLAINTS:

MENSTRUAL HISTORY :

Married since:

OBSTETRIC HISTORY:

PAST HISTORY:

PERSONAL HISTORY:

Vegetarian/ Non vegetarian:

Habit of hand washing:

Personal hygiene:

SOCIO-ECONOMIC HISTORY:

Place of residence:

Duration of settlement:

Source of water:

Sewage disposal:

Educational level:

Profession:

Per capita income:

Socio-Economic Status:

GENERAL EXAMINATION:

HT: WT: TEMP: PR: BP:

PALLOR: ICTERUS: PEDAL EDEMA:

CVS: RS:

P/A:

P/V:

INVESTIGATIONS:

Hb:

RBS:

Urine albumin:

BLOOD GROUP:

RFT:

Urea

Creatinine

Na

K

LFT:

Br/D

SGOT/PT

ALP

PT/INR

IgM Anti-HEV ELISA:

IgG anti-HEV ELISA:

Quantitaive Real time PCR of HEV RNA

96 well MICROTITRE PLATE

DATA SHEET

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC	S6	S14	S22	S30	S38	S46	S54	S62	S70	S78	S86
B	NC	S7	S15	S23	S31	S39	S47	S55	S63	S71	S79	S87
C	NC	S8	S16	S24	S32	S40	S48	S56	S64	S72	S80	S88
D	S1	S9	S17	S25	S33	S41	S49	S57	S65	S73	S81	S89
E	S2	S10	S18	S26	S34	S42	S50	S58	S66	S74	S82	S90
F	S3	S11	S19	S27	S35	S43	S51	S59	S67	S75	S83	S91
G	S4	S12	S20	S28	S36	S44	S52	S60	S68	S76	S84	S92
H	S5	S13	S21	S29	S37	S45	S53	S61	S69	S77	S85	S93

PC – Positive control

NC – negative control

IC – Internal control

NATURE ONATURE OF THE REAGENTS:

HEV Ag coated strips:

Polystyrene stripped 96 wells plate (breakable) coated with mix of recombinant antigens of HEV. Stored at 2-8° C until expiration date.

Sample diluent:

Transparent or slightly opalescent liquid, violet-blue coloured, sediment may form which completely dissolves at shaking. Preserving agent: 0.01% thimerosal. Store at 2-8° C until expiration date.

Conjugate concentrated 21-fold:

Monoclonal mouse antibodies against human IgM, labled horseradish peroxidase. Transparent or slightly opalescent liquid, light yellow colored. Preserving agent: 0.04% ProClin 300, 0.04% gentamycin sulfate. Store at 2-8° C until expiration date in a tightly sealed vial

Conjugate diluent:

Transparent, yellow liquid at temperature of 2-8° C, opalescent yellow color liquid at temperature of 18-24°C. preserving agent: Preserving agent: 0.01% thimerosal. Store at 2-8° C until expiration date in a tightly sealed vial.

Positive control:

Heat inactivated human serum positive for anti-HEV-IgM, negative for anti-HIV-1,2, HBsAg and anti-HCV. Transparent or slightly opalescent liquid, red colored. Preserving agent: 0.04% ProClin 300, 0.1% sodium azide. Store at 2-8°C until expiration date in a tightly sealed vial.

Negative control:

Heat inactivated human serum positive for anti-HEV-IgM, negative for anti-HIV-1,2, HBsAg and anti-HCV. Transparent or slightly opalescent liquid, green colored. Preserving agent: 0.01% thiomersal , 0.1% sodium azide. Store at 2-8°C until expiration date in a tightly sealed vial.

Washing solution (concentrated 25 fold)

Transparent or slightly opalescent liquid, colorless, or pale yellow, sediment may form that dissolves at 35-39°C and shaking. Store at 2-8°C until expiration date in a tightly sealed vial.

Substrate buffer:

Citric acid and sodium acetate solution, pH 4.1-4.3, containing H_2O_2 . Transparent colorless liquid. Preserving agent: 0.04% ProClin 300. Store at 2-8°C until expiration date in a tightly sealed vial.

TMB concentrated 21- folds:

Solution containing Tetramethylbenzidine (TMB). Transparent colorless liquid. Store at 2-8°C until expiration date in a tightly sealed vial.

Stopping reagent:

0.2M/L sulphuric acid solution. Transparent colorless liquid. Store at 2-8°C until expiration date in a tightly sealed vial.

DS-EIA-ANTI-G

NATURE OF THE REAGENTS:

HEV Ag coated strips:

Polystyrene stripped 96 wells plate (breakable) coated with mix of recombinant antigens of HEV. Stored at 2-8° C until expiration date.

Sample diluent:

Transparent or slightly opalescent liquid, violet-blue coloured, sediment may form which completely dissolves at shaking. Preserving agent: 0.01% thimerosal. Store at 2-8° C until expiration date.

Conjugate concentrated 21-fold:

Monoclonal mouse antibodies against human IgG, labled horseradish peroxidase. Transparent or slightly opalescent liquid, light

yellow colored. Preserving agent: 0.04% ProClin 300, 0.04% gentamycin sulfate. Store at 2-8° C until expiration date in a tightly sealed vial

Conjugate diluent:

Transparent, yellow liquid at temperature of 2-8° C, opalescent yellow color liquid at temperature of 18-24°C. Preserving agent: Preserving agent: 0.01% thimerosal. Store at 2-8° C until expiration date in a tightly sealed vial.

Positive control:

Heat inactivated human serum positive for anti-HEV-IgG, negative for anti-HIV-1,2, HBsAg and anti-HCV. Transparent or slightly opalescent liquid, red colored. Preserving agent: 0.04% ProClin 300, 0.1% sodium azide. Store at 2-8°C until expiration date in a tightly sealed vial.

Negative control:

Heat inactivated human serum positive for anti-HEV-IgG, negative for anti-HIV-1,2, HBsAg and anti-HCV. Transparent or slightly opalescent liquid, green colored. Preserving agent: 0.01% thiomersal, 0.1% sodium azide. Store at 2-8°C until expiration date in a tightly sealed vial.

Washing solution (concentrated 25 fold)

Transparent or slightly opalescent liquid, colorless, or pale yellow, sediment may form that dissolves at 35-39°C and shaking. Store at 2-8°C until expiration date in a tightly sealed vial.

Substrate buffer:

Citric acid and sodium acetate solution, pH 4.1-4.3, containing H_2O_2 . Transparent colorless liquid. Preserving agent: 0.05% ProClin 300. Store at 2-8°C until expiration date in a tightly sealed vial.

TMB concentrated 21- folds:

Solution containing Tetramethylbenzidine (TMB). Transparent colorless liquid. Store at 2-8°C until expiration date in a tightly sealed vial.

Stopping reagent:

0.75 M/L sulphuric acid solution. Transparent colorless liquid. Store at 2-8°C until expiration date in a tightly sealed vial.

Kit contents

Σ 96

1. HEV-Ag Coated Strips		1 plate
2. Conjugate (concentrated 21-fold)	0.75 ml	1 vial
3. Positive Control, Inactivated	1.2 ml	1 vial
4. Negative Control, Inactivated	2.5 ml	1 vial
5. Sample diluent	12.5 ml	1 vial
6. Conjugate diluent	13.5 ml	1 vial
7. Washing Solution (concentrated 25-fold)	50.0 ml	1 vial
8. Substrate Buffer	25.0 ml	1 vial
9. T M B	2.5 ml	1 vial
10. Stopping Reagent	25.0 ml	1 vial

"DS-EIA-ANTI-HEV-M"

EMZYME IMMUNOASSAY FOR THE DETECTION OF IgM ANTIBODIES TO HEPATITIS E VIRUS

LOT 040055

REF E-152

Code: 2.1.GB

2016-05-30

INSTITUTIONAL ETHICAL COMMITTEE, STANLEY MEDICAL COLLEGE, CHENNAI-1

Title of the Work : Seroprevalance of Subclinical Hepatitis E in Pregnancy.

Principal Investigator : Dr. Vinitra.D

Designation : PG MS (O & G)

Department : Department of O & G
Government Stanley Medical College,
Chennai-01

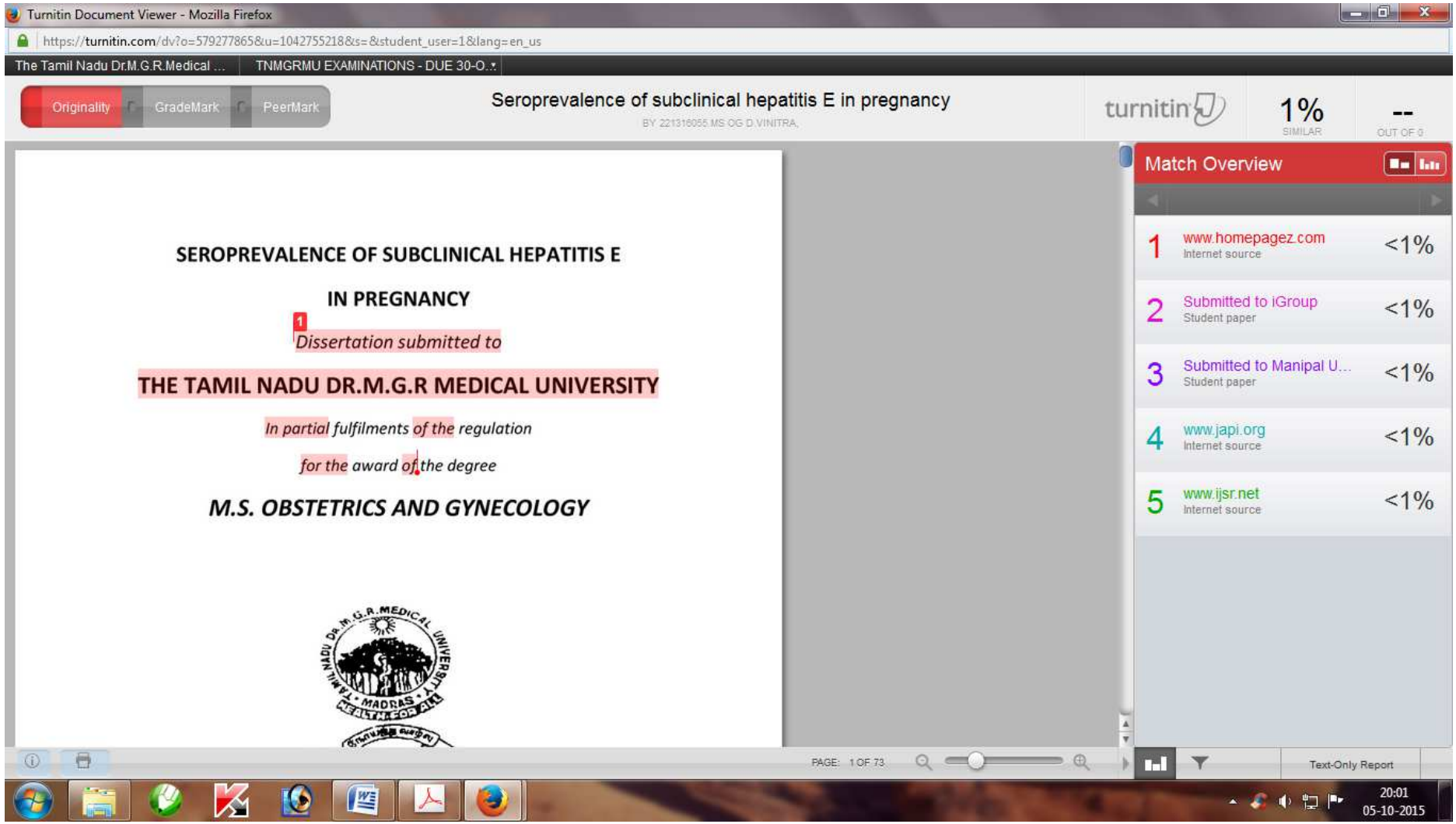
The request for an approval from the Institutional Ethical Committee (IEC) was considered on the IEC meeting held on 10.06.2015 at the Council Hall, Stanley Medical College, Chennai-1 at 2PM

The members of the Committee, the secretary and the Chairman are pleased to approve the proposed work mentioned above, submitted by the principal investigator.

The Principal investigator and their team are directed to adhere to the guidelines given below:

1. You should inform the IEC in case of changes in study procedure, site investigator investigation or guide or any other changes.
2. You should not deviate from the area of the work for which you applied for ethical clearance.
3. You should inform the IEC immediately, in case of any adverse events or serious adverse reaction.
4. You should abide to the rules and regulation of the institution(s).
5. You should complete the work within the specified period and if any extension of time is required, you should apply for permission again and do the work.
6. You should submit the summary of the work to the ethical committee on completion of the work.

MEMBER SECRETARY,
IEC, SMC, CHENNAI


Turnitin Document Viewer - Mozilla Firefox
https://turnitin.com/dv?o=579277865&u=1042755218&s=&student_user=1&lang=en_us
The Tamil Nadu Dr.M.G.R.Medical ...
TNMGRMU EXAMINATIONS - DUE 30-O..
Originality
GradeMark
PeerMark
Seroprevalence of subclinical hepatitis E in pregnancy
BY 221316055.MS OG D.VINITRA,
turnitin
1%
SIMILAR
--
OUT OF 0
SEROPREVALENCE OF SUBCLINICAL HEPATITIS E
IN PREGNANCY
Dissertation submitted to
THE TAMIL NADU DR.M.G.R MEDICAL UNIVERSITY
In partial fulfilments of the regulation
for the award of the degree
M.S. OBSTETRICS AND GYNECOLOGY
Match Overview
1 www.homepagez.com Internet source <1%
2 Submitted to iGroup Student paper <1%
3 Submitted to Manipal U... Student paper <1%
4 www.japi.org Internet source <1%
5 www.ijsr.net Internet source <1%
PAGE: 1 OF 73
Text-Only Report
20:01
05-10-2015

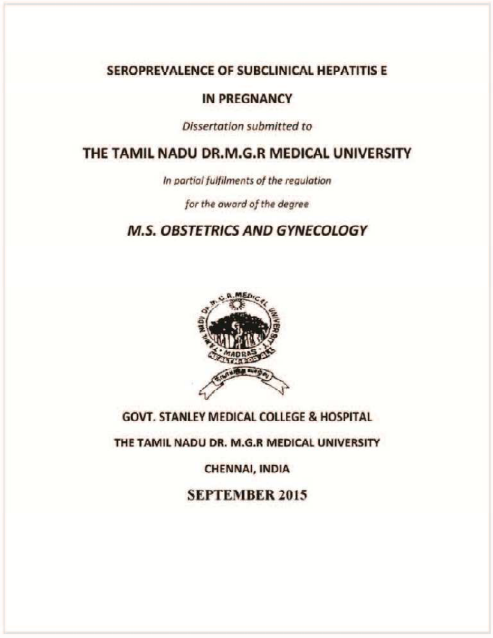
turnitin

Digital Receipt

This receipt acknowledges that Turnitin received your paper. Below you will find the receipt information regarding your submission.

The first page of your submissions is displayed below.

Submission author:	221316055.ms Og D.VINITRA,
Assignment title:	TNMGRMU EXAMINATIONS
Submission title:	Seroprevalence of subclinical hepa...
File name:	vinitrathesisfinal.docx
File size:	333.88K
Page count:	73
Word count:	10,669
Character count:	59,039
Submission date:	03-Oct-2015 07:52PM
Submission ID:	579277865



SEROPREVALENCE OF SUBCLINICAL HEPATITIS E

IN PREGNANCY

Dissertation submitted to

THE TAMIL NADU DR.M.G.R MEDICAL UNIVERSITY

In partial fulfilments of the regulation

for the award of the degree

M.S. OBSTETRICS AND GYNECOLOGY

GOVT. STANLEY MEDICAL COLLEGE & HOSPITAL

THE TAMIL NADU DR. M.G.R MEDICAL UNIVERSITY

CHENNAI, INDIA

SEPTEMBER 2015

CONSENT FORM

I agree to participate in the study entitled “A study on the assessment of prevalence and severity of Hepatitis E infection in pregnancy”

I confirm that I have been told about this study in my mother tongue and have had the opportunity to clarify my doubts.

I understand that my participation is voluntary and I may refuse to participate at any time without giving any reasons and without affecting my benefits.

I agree not to restrict the use of any data or results that arise from this study.

Name of the participant :

Sign / Thumb print:

Sign of Investigator :

MASTER CHART

sno	age	duration	p/m	graida	para	live	abortion	married since m	weeks	trimester	v/nv	handwash	metro/bw/pur	boiling	sewage	occupation	e/h/s/coll	sestatus	hb	rbs	ababo12345678	urea	cr	br	ot	pt	alp	pt/inr	igm	igg
1	23	1	1	1	0	0	0	12	10	1	1	1	1	1	1	1	1	3	9.9	100	1	12	0.6	0.4	13	17	199	10	0	0
2	24	0.33	1	1	0	0	0	4	11	1	2	1	1	1	1	1	1	4	9.5	145	3	34	0.8	0.7	33	23	340	16	0	0
3	20	0.66	1	1	0	0	0	8	8	1	2	1	1	1	1	1	3	4	9.4	120	3	11	0.9	0.9	34	12	360	15	0	0
4	25	10	2	2	1	1	0	60	4	1	2	1	1	0	1	1	4	3	14	100	1	23	0.8	0.5	23	56	340	14	0	0
5	27	27	1	1	0	0	0	3	5	1	2	0	1	0	0	1	1	4	8.8	120	1	33	0.9	0.8	56	23	452	12	1	1
6	19	0.41	1	1	0	0	0	9	14	2	2	1	1	0	1	1	3	4	11	94	3	44	0.8	0.6	22	24	233	18	0	0
7	21	1	2	3	0	0	2	12	12	1	2	1	1	1	1	1	3	3	12	99	7	44	0.8	0.9	44	54	340	16	0	0
8	23	1	1	1	0	0	0	12	15	2	2	1	2	0	1	1	5	3	9.9	90	1	22	0.9	0.8	22	33	440	14	0	0
9	22	0.66	1	1	0	0	0	8	26	3	1	1	3	1	1	2	4	2	10	86	1	22	0.4	0.8	43	46	390	12	0	0
10	19	1	1	1	0	0	0	12	22	2	1	1	1	1	1	1	1	3	9.4	92	1	21	0.6	0.9	35	23	400	14	0	0
11	29	29	2	3	1	1	1	120	28	3	1	0	1	1	1	1	1	3	12	108	3	20	0.5	0.4	28	62	340	11	0	0
12	22	0.83	1	1	0	0	0	10	21	2	1	1	3	1	1	1	4	3	11	86	7	14	0.5	0.3	22	47	280	12	0	0
13	31	5	2	2	1	1	0	108	18	2	1	1	3	1	1	1	3	3	9.6	88	3	16	0.5	0.8	22	34	380	10	0	0
14	22	2.5	2	2	1	1	0	36	26	3	1	1	3	1	1	1	4	3	9.8	89	8	26	0.3	0.4	46	22	330	12	0	0
15	29	1	1	1	0	0	0	12	26	3	1	0	3	1	1	1	3	3	9.3	85	7	16	0.4	0.8	33	24	400	10	0	0
16	23	3	2	3	2	2	0	84	28	3	2	1	3	1	1	1	2	3	10	80	3	22	0.8	0.3	34	58	342	12	0	0
17	24	5	2	2	1	1	0	60	30	3	2	1	1	0	1	1	2	3	9.7	78	7	23	0.7	0.5	23	21	289	11	0	0
18	21	3	2	2	1	1	0	36	6	1	2	1	1	0	1	1	2	3	9.8	76	7	24	0.6	0.9	22	34	390	10	0	0
19	25	3	1	1	0	0	0	3	8	1	1	0	1	1	1	1	4	2	10	90	3	40	0.3	0.4	37	29	420	10	0	1
20	21	10	1	1	0	0	0	12	22	2	2	1	2	1	1	1	1	3	10	75	3	18	0.4	0.3	72	38	310	11	0	0
21	26	4	2	2	1	1	0	48	26	3	1	1	2	1	1	1	4	3	10	85	3	24	0.5	0.9	37	24	390	11	0	0
22	20	1	1	1	0	0	0	9	30	3	1	1	1	1	1	1	1	4	11	86	7	14	0.5	0.8	28	30	400	10	0	0
23	23	4	2	2	1	1	0	48	22	2	1	1	1	1	1	1	2	4	11	92	7	20	0.9	0.6	22	46	450	10	0	0
24	21	22	1	1	0	0	0	12	23	2	1	0	1	0	0	1	2	4	10	91	3	15	0.4	1.1	23	45	330	13	0	0
25	22	22	2	3	2	0	0	36	23	2	1	1	1	0	0	1	1	4	9.8	96	7	20	0.7	0.8	39	35	390	10	0	0
26	22	3	2	3	2	1	0	36	24	2	2	1	1	0	1	1	2	3	9.8	80	3	16	0.5	0.5	24	28	400	10	0	0
27	22	1	1	1	0	0	0	12	26	3	1	0	2	0	1	1	2	3	10	100	7	12	0.8	0.7	23	12	290	12	0	1
28	24	3	1	1	0	0	0	24	36	3	2	1	1	0	1	1	2	3	11	69	3	25	0.6	0.5	65	45	420	12	0	0
29	22	2	1	1	0	0	0	24	38	3	1	1	2	0	1	3	3	4	9.8	90	7	20	0.9	0.7	25	10	480	11	0	0
30	27	10	2	2	1	1	0	10	37	3	2	0	1	0	1	1	2	4	10	62	7	14	0.2	0.8	22	45	450	10	0	0
31	20	1	1	1	0	0	0	6	24	2	1	1	1	1	1	1	2	4	9.2	88	7	22	0.8	0.4	32	46	280	10	0	0
32	28	6	2	3	1	1	1	60	38	3	1	1	2	0	1	2	1	4	10	90	1	30	0.7	0.6	33	43	320	11	0	0
33	27	1	1	1	0	0	0	12	32	3	2	1	1	1	1	1	1	2	8.9	62	1	12	0.6	0.8	24	30	208	12	0	0
34	27	1	2	2	1	1	0	72	24	2	2	1	3	0	1	1	0	4	8.6	58	1	16	0.5	0.6	24	26	301	10	0	0
35	28	1	1	1	0	0	0	18	38	3	1	1	2	1	1	1	3	4	10	98	7	28	0.7	0.9	28	36	256	11	0	0
36	25	7	2	2	1	1	0	84	30	3	2	1	1	1	1	1	2	4	9.2	78	1	24	0.8	0.6	20	16	268	10	0	0
37	33	11	2	3	2	2	0	120	28	3	2	1	1	0	1	1	2	4	12	79	3	14	0.5	0.9	28	36	362	12	0	0
38	27	10	1	1	0	0	0	24	24	2	1	0	1	1	1	1	0	4	9.6	80	2	14	0.5	0.4	32	40	262	10	0	0
39	36	2	2	2	1	1	0	36	38	3	1	1	1	1	1	1	3	3	9.8	89	7	27	0.5	0.5	34	52	293	11	0	0
40	22	2	1	1	0	0	0	6	14	2	2	1	1	0	1	1	2	3	11	99	3	23	0.4	0.3	21	22	190	10	0	0
41	23	3	1	1	1	0	0	2	38	3	1	1	2	0	1	2	4	3	9	89	3	18	0.4	0.2	19	18	200	11	0	0
42	29	3	1	1	1	0	0	12	38	3	1	1	1	1	1	1	1	3	9.8	100	1	19	0.8	0.3	11	17	190	11	0	0
43	23	3	2	2	1	1	0	60	38	3	1	1	2	1	1	1	3	4	9.4	67	1	29	0.3	0.7	26	36	256	10	0	0
44	27	10	2	4	2	1	1	96	30	3	2	1	1	0	0	1	1	4	9.4	97	1	19	0.8	0.6	43	53	264	13	1	1
45	28	2	1	1	1	0	0	12	36	3	1	1	1	1	1	1	2	4	9.7	99	1	22	0.7	0.8	12	11	180	9	0	0
46	30	7	2	3	2	2	0	84	36	3	1	1	1	0	1	1	1	4	8.7	67	1	19	0.5	0.7	23	24	190	11	0	0
47	24	10	1	1	1	0	0	12	32	3	1	1	1	1	1	1	2	4	9.7	102	3	20	0.8	0.5	12	17	200	10	0	0
48	18	3	1	1	0	0	0	12	14	2	1	0	2	1	1	1	0	4	9.6	100	3	10	0.5	0.1	45	29	400	11	0	0
49	34	4	2	2	1	1	0	48	36	3	1	1	3	0	1	1	1	4	9	89	1	20	0.9	0.7	23	20	380	10	0	0
50	18	2	1	1	0	0	0	24	10	1	1	1	2	1	1	2	5	3	9	88	3	20	0.8	0.3	28	22	190	10	0	0
51	28	2	1	1	0	0	0	24	26	2	2	0	3	1	0	1	2	5	9.4	145	3	12	0.7	0.6	23	16	293	10	1	0
52	24	20	1	1	0	0	0	12	28	2	2	1	3	1	1	1	3	5	14	120	3	34	0.9	0.9	12	14	190	11	0	0
53	27	2	1	1	0	0	0	12	12	1	1	1	3	1	1	1	3	5	8.8	100	7	11	0.5	0.8	56	23	200	11	0	0
54	24	2	1	1	0	0	0	18	10	1	2	1	3	1	1	1	3	5	11	120	7	23	0.8	0.8	23	24	190	13	0	0
55	26	5	1	1	0	0	0	8	12	1	2	0	3	0	0	1	0	5	12	94	7	33	0.6	0.9	24	25	256	12	1	0
56	24	2	1	1	0	0	0	36	36	3	2	0	1	1	1	1	3	3	9.9	99	7	44	0.9	0.4	54	21	264	11	0	0
57	25	6	2	2	0	1	0	84	24	2	1	0	1	1	1	1	3	3	10	90	7	44	0.8	0.3	33	24	180	10	0	0
58	21	3	1	1	1	0	0	12	36	3	1	1	1	1	1	1	3	3	9.4	86	7	22	0.8	0.8	46	35	190	9	0	0
59	25	10	2	2	1	1	0	96	28	2	1	1	1	1	1	1	3	3	12	92	7	22	0.9	0.4	23	26	200	11	0	0
60	28	3	1	1	0	0	0	12	36	3	1	1	1	1	1	1	3	3	11	108	7	21	0.4	0.8	62	45	400	12	0	0
61	22	5	1	1	0	0	0	12	36	3	2	0	2	0	1	1	3	5	9.6	86	4	20	0.3	0.3	47	34	380	12	0	1
62	23	6	2	2	1	1	0	60	36	3	1	1	2	1	0	1	3	3	9.8	88	3	14	0.8	0.5	34	22	190	12	0	0
63	30	12	2	2	1	0	0	108	12	1	2	0	2	0	1	1	3	5	9.3	89	7	16	0.4	0.9	22	12	340	15	1	1
64	30	10	2	2	0	1	0	96	28	2	1	1	3	1	1	1	3	3	10	85	7	26	0.8	0.4	24	13	280	12	0	0
65	25	8	2	2	1	1	0	72	32	3	1	1	3	1	1	1	3	3	9.7	80	7	16	0.3	0.3	58	15	380	11	0	0
66	22	6	2	2	1	1	0	60	34	3	1	1	3	1	1	1	2	3	9.8	78	7	22	0.5	0.9	21	23	330	11	0	0
67	21	1	1	1	0	0	0	12	27	2	1	1	3	1	0	1	2	3	10	76	2	23	0.9	0.8	34	21	400	13	0	0
68	23	15	2	3	1	1	1	120	39	3	1	1	3	1	0	1	2	3	10	90	2	24	0.4	0.6	29	54	342	13	0	0

69	27	1	1	1	0	0	0	12	38	3	1	1	3	1	0	1	4	3	10	75	1	40	0.3	1.1	38	34	289	12	0	0
70	22	3	1	1	0	0	0	24	40	3	1	1	3	1	0	1	4	3	11	85	1	18	0.9	0.8	24	24	390	10	0	0
71	21	3	1	1	0	0	0	24	28	2	1	1	3	1	1	1	4	4	11	86	3	24	0.8	0.5	30	27	420	11	0	0
72	26	5	1	1	0	0	0	12	38	3	2	0	1	0	0	2	3	4	10	92	7	14	0.6	0.7	46	23	310	11	1	0
73	23	14	2	2	1	1	0	60	38	3	2	1	3	1	1	1	4	2	9.8	91	7	20	1.1	0.5	45	12	390	13	0	0
74	28	4	1	1	0	0	0	18	32	3	2	1	3	1	1	1	4	2	9.8	96	7	15	0.8	0.7	35	34	400	12	0	0
75	28	9	2	2	1	1	0	96	28	2	2	1	3	1	1	1	4	2	10	80	7	20	0.5	0.8	28	35	450	11	0	0
76	26	2	1	1	0	0	0	18	39	3	1	1	3	1	1	1	4	2	11	100	7	16	0.7	0.4	12	33	330	13	0	0
77	22	3	2	2	1	1	0	60	38	3	1	1	3	1	1	1	4	2	9.8	69	3	12	0.5	0.6	45	33	390	12	0	0
78	18	2	1	1	0	0	0	12	38	3	1	1	3	1	1	1	3	3	10	90	3	25	0.7	0.8	10	35	400	11	0	0
79	23	5	1	1	0	0	0	30	39	3	1	1	3	1	1	1	3	3	9.2	62	3	20	0.8	0.6	45	37	290	10	0	0
80	22	2	1	1	0	0	0	8	28	2	2	1	3	1	1	1	3	3	10	88	3	14	0.4	0.9	46	12	420	14	0	0
81	22	5	1	1	0	0	0	36	39	3	2	1	1	1	1	1	3	3	8.9	90	3	22	0.6	0.6	43	15	480	12	0	0
82	25	15	2	3	2	2	0	120	28	2	2	0	1	0	0	2	3	4	8.6	62	3	30	0.8	0.9	30	19	450	11	0	1
83	30	9	2	2	1	1	0	84	20	2	2	0	1	1	1	1	2	3	10	58	1	12	0.4	0.4	26	34	280	10	0	0
84	28	3	2	2	0	0	1	24	24	2	2	0	1	1	0	1	2	3	9.2	98	3	16	0.4	0.5	36	56	320	11	0	0
85	21	4	1	1	0	0	0	36	26	2	2	1	1	1	0	1	2	3	12	78	2	28	0.8	0.3	16	34	208	12	0	0
86	24	6	2	3	1	1	1	60	20	2	1	1	2	1	1	1	2	3	9.6	79	4	24	0.3	0.2	36	35	301	13	0	0
87	19	1	1	1	0	0	0	12	39	3	1	0	2	1	1	1	2	3	9.8	80	3	14	0.8	0.6	40	45	256	14	0	0
88	35	20	2	3	2	2	0	120	36	3	1	0	2	1	0	1	2	3	11	89	3	14	0.7	0.9	52	46	268	11	0	0
89	25	2	1	1	0	0	0	18	39	3	1	1	1	1	1	1	2	3	9	99	2	27	0.5	0.8	22	19	362	11	0	0
90	32	7	2	2	1	1	0	72	37	3	1	0	1	1	1	1	2	3	9.8	89	1	23	0.8	0.8	18	20	262	12	0	0
91	23	3	1	1	0	0	0	30	36	3	1	0	1	1	0	1	2	3	9.4	100	1	18	0.5	0.9	17	34	254	12	0	0
92	30	1	1	1	0	0	0	12	34	3	2	1	1	0	1	3	2	5	9.4	67	1	19	0.9	0.4	36	12	342	11	0	1
93	21	4	1	1	0	0	0	24	26	2	2	1	1	1	0	1	3	3	9.7	97	1	29	0.8	0.3	53	90	134	10	0	0
94	27	8	1	1	0	0	0	24	36	3	2	1	1	1	1	1	3	3	8.7	99	1	19	0.7	0.8	11	12	34	10	0	0
95	25	3	1	1	0	0	0	12	16	2	1	0	1	1	1	1	3	3	9.7	67	1	22	0.9	0.4	24	12	235	11	0	0
96	23	3	1	1	0	0	0	12	38	3	1	0	1	1	1	1	3	3	9.6	102	1	19	0.5	0.8	17	12	342	12	0	0
97	28	3	2	2	1	1	0	6	26	2	2	0	1	1	1	1	0	2	9	100	1	20	0.8	0.3	29	12	124	11	1	0
98	20	10	2	2	1	1	0	96	34	3	2	0	2	1	0	1	3	3	9	89	7	10	0.6	0.5	20	11	345	11	0	1
99	23	3	1	1	0	0	0	39	30	3	1	1	2	0	1	1	3	3	9.4	88	3	20	0.9	0.9	22	14	234	11	0	0
100	19	6	2	3	1	1	0	84	27	2	1	1	1	0	1	1	3	3	14	145	1	20	0.8	0.4	25	23	433	11	0	0
101	21	8	2	2	1	1	0	84	39	3	1	1	1	0	1	1	3	3	8.8	120	7	12	0.8	0.3	22	12	342	11	0	0
102	23	1	1	1	0	0	0	6	18	2	1	1	1	0	1	1	4	3	11	100	8	34	0.9	0.9	23	13	123	13	0	0
103	22	4	2	2	1	1	0	72	39	3	1	1	1	1	1	1	3	3	12	120	3	11	0.4	0.8	24	24	222	13	0	0
104	27	2	1	1	0	0	0	18	30	3	1	1	1	1	1	1	3	3	9.9	94	5	23	0.3	0.6	23	12	345	12	0	0
105	26	2	1	1	0	0	0	24	34	3	1	1	1	1	1	1	3	3	10	99	7	33	0.8	1.1	22	13	345	13	0	0
106	21	3	1	1	0	0	0	24	39	3	2	1	1	0	0	1	3	3	9.4	90	5	44	0.4	0.8	23	14	344	12	0	1
107	30	5	2	2	1	1	0	48	40	3	1	1	1	0	1	2	3	4	12	86	2	44	0.8	0.5	13	13	666	12	0	0
108	19	10	1	1	0	0	0	12	36	3	1	1	1	0	1	1	4	4	11	92	2	22	0.3	0.7	16	23	233	12	0	0
109	27	8	2	2	1	1	0	72	37	3	1	1	1	1	1	3	3	3	9.6	108	1	22	0.5	0.5	15	25	455	12	0	0
110	27	7	2	4	2	2	1	72	38	3	1	1	1	0	1	3	3	3	9.8	86	1	21	0.9	0.7	23	22	333	12	0	0
111	22	9	2	2	1	1	0	84	32	3	1	1	1	1	1	1	3	3	9.3	88	1	20	0.4	0.8	27	23	453	12	0	0
112	22	8	2	3	1	1	1	84	38	3	2	1	1	0	1	1	3	3	10	89	1	14	0.3	0.4	22	24	233	12	0	0
113	28	10	2	2	1	1	0	96	39	3	1	1	1	0	1	1	3	3	9.7	85	7	16	0.9	0.6	23	23	345	12	0	0
114	26	5	1	1	0	0	0	12	37	3	2	1	1	1	1	1	3	2	9.8	80	7	26	0.8	0.8	23	22	343	11	0	0
115	23	2	1	1	0	0	0	24	30	3	1	1	1	0	1	1	2	3	10	78	7	16	0.6	0.6	25	23	333	11	0	0
116	32	10	1	1	0	0	0	120	35	3	2	1	1	0	1	1	2	3	10	76	3	22	1.1	0.9	32	13	345	11	0	0
117	19	4	1	1	0	0	0	24	36	3	1	1	1	1	1	1	2	3	10	90	3	23	0.8	0.6	16	16	345	12	0	0
118	24	4	1	1	0	0	0	24	26	2	1	1	1	1	1	1	2	2	11	75	3	24	0.5	0.9	45	15	123	11	0	0
119	23	12	2	2	1	1	0	72	20	2	2	1	1	1	1	1	2	2	11	85	3	40	0.7	0.4	43	23	231	10	0	0
120	20	3	1	1	0	0	0	18	37	3	2	1	1	1	1	1	2	2	10	86	6	18	0.5	0.5	47	27	231	13	0	0
121	22	8	2	2	1	1	0	84	36	3	2	1	1	1	0	1	2	2	9.8	92	6	24	0.7	0.3	44	22	222	12	0	0
122	29	9	2	2	1	1	0	84	32	3	2	1	1	1	0	1	2	2	9.8	91	3	14	0.8	0.2	45	23	123	13	0	0
123	20	3	1	1	0	0	0	12	25	2	2	1	1	1	0	3	2	2	10	96	3	20	0.4	0.4	46	23	234	12	0	0
124	21	2	1	1	0	0	0	18	39	3	2	1	1	0	1	1	2	2	11	80	3	15	0.6	0.6	47	12	252	11	0	0
125	22	2	1	1	0	0	0	24	35	3	2	1	1	0	0	1	2	3	9.8	100	3	20	0.8	0.8	44	56	245	11	0	0
126	19	5	1	1	0	0	0	24	38	3	2	1	1	0	0	1	2	2	10	69	3	16	0.4	0.6	41	23	213	10	0	0
127	19	2	1	1	0	0	0	12	10	1	2	1	1	1	0	1	2	4	9.2	90	3	12	0.4	0.9	24	24	218	8	0	1
128	21	4	2	2	1	1	0	60	20	2	1	1	1	0	1	1	2	4	10	62	3	25	0.8	0.6	34	54	229	10	0	0
129	22	8	2	2	1	1	0	72	16	2	1	1	2	0	1	1	1	4	8.9	88	4	20	0.3	0.9	56	33	256	11	0	0
130	19	6	1	1	0	0	0	12	24	2	2	1	1	0	1	1	1	4	8.6	90	7	14	0.8	0.4	34	46	289	12	0	0
131	23	4	2	3	1	1	1	48	12	1	2	1	3	1	1	1	1	4	10	62	7	22	0.7	0.5	38	23	267	13	0	0
132	32	6	2	2	1	1	0	60	24	2	1	1	1	1	1	1	1	4	9.2	58	7	30	0.5	0.3	39	62	280	14	0	0
133	22	5	2	2	1	1	0	48	8	1	1	1	1	1	1	3	2	4	12	98	1	12	0.8	0.2	23	47	230	12	0	0
134	23	3	1	1	0	0	0	24	26	2	1	1	1	1	0	1	1	4	9.6	78	1	16	0.5	0.6	39	34	134	11	0	0
135	26	9	2	3	1	1	1	84	30	3	1	1	1	1	1	1	2	4	9.8	79	1	28	0.9	0.9	22	22	234	10	0	0
136	25	15	2	3	2	2	0	120	28	2	1	1	1	1	1	1	2	4	11	80	7	24	0.8	0.8	33	24	160	12	0	0
137	29	5	2	2	1	1	0	42	14	2	1	0	1	0	1	1	2	4	9	89	7	14	0.7	0.8	44	58	180	12	0	1
138	26	2	1	1	0	0	0	12	16	2	2	1	2	1	0	1	2	4	9.8	99	7	14	0.9	0.9	34	21	149	11	0	0
139	25	3	1	1	0	0	0	24	19	2	2	1	1	1	0	1	3	3	9.4	89	7	27	0.5	0.4	23	34	230	12	0	0
140	21	8	2	2	1	1	0	72	20	2	2	1	1	1	0	1	3	3	9.4	100	7	23	0.8	0.3	33	29	222	13	0	0
141	24	9	2	3	1	1	1	96	12	1	2	1	1	1	0	1	3	3	9.7	67	4	18	0.6	0.8	56	38	234	12	0	0
142	21	10	2	2	1	1	0	84	18	2	2	1	1	1	0	1	3	3	8.7	97	3	19	0.9	0.4	45	24	234	11	0	0

143	21	4	1	1	0	0	0	24	22	2	2	1	1	1	0	1	2	3	9.7	99	7	29	0.8	0.8	61	52	213	10	0	0
144	21	14	1	1	0	0	0	12	24	2	2	0	1	1	0	1	2	3	9.6	67	7	19	0.8	0.9	12	22	234	11	0	0
145	20	4	1	1	0	0	0	42	36	3	2	0	1	1	0	1	3	3	9	102	7	22	0.9	0.6	22	18	234	12	0	0
146	22	4	1	1	0	0	0	36	26	2	1	0	1	1	0	3	3	4	9	100	7	19	0.4	0.9	15	17	213	11	0	0
147	25	10	2	2	1	1	0	120	28	2	1	0	1	1	0	1	3	4	9.4	89	2	20	0.3	0.4	33	36	209	13	0	0
148	30	9	2	3	2	2	0	120	26	2	1	1	1	1	1	1	2	3	14	88	2	10	0.8	0.5	33	53	218	13	0	0
149	25	4	1	1	0	0	0	36	32	3	1	1	1	1	1	1	2	3	8.8	145	1	20	0.4	0.3	33	11	134	13	0	0
150	22	4	1	1	0	0	0	12	34	3	1	1	1	1	1	1	2	3	11	120	1	20	0.8	0.2	45	24	289	14	0	0
151	23	1	1	1	0	0	0	12	20	2	2	1	1	1	1	1	2	2	12	100	3	12	0.3	0.6	62	17	290	14	0	0
152	25	5	2	2	0	0	1	48	14	2	2	1	1	1	1	1	2	4	9.9	120	7	34	0.5	0.9	34	29	245	13	0	0
153	24	4	2	3	0	0	2	36	32	3	1	0	2	0	1	1	2	4	10	94	7	11	0.9	0.8	22	20	221	12	0	1
154	33	13	2	3	2	1	0	150	24	2	1	0	1	1	0	1	3	5	9.4	99	7	23	0.4	0.8	22	22	231	12	0	0
155	21	1	1	1	0	0	0	12	16	2	1	0	1	1	0	1	4	3	12	90	7	33	0.3	0.9	22	16	211	12	0	0
156	24	1	1	1	0	0	0	12	16	2	1	0	1	1	0	1	3	4	11	86	7	44	0.9	0.4	12	14	209	12	0	0
157	21	1	1	1	0	0	0	6	20	2	1	0	1	1	0	1	3	4	9.6	92	3	44	0.8	0.3	12	23	234	12	0	0
158	22	4	2	1	0	0	1	24	18	2	1	0	1	1	0	1	3	3	9.8	108	3	22	0.6	0.8	11	24	221	14	0	0
159	30	1	2	3	2	2	0	48	22	2	1	1	1	1	0	1	3	3	9.3	86	3	22	1.1	0.4	12	25	212	12	0	0
160	21	2	1	1	0	0	0	24	24	2	1	1	1	1	0	1	3	3	10	88	3	21	0.8	0.8	66	21	211	12	0	0
161	21	1	1	1	0	0	0	6	12	1	1	1	1	1	0	3	3	3	9.7	89	3	20	0.5	0.4	16	24	209	12	0	0
162	19	5	1	1	0	0	0	60	14	2	2	1	1	1	0	1	3	3	9.8	85	3	14	0.7	0.4	36	35	208	12	0	0
163	23	4	2	2	1	0	0	48	32	3	2	1	1	1	0	1	3	3	10	80	1	16	0.5	0.8	40	11	211	12	0	0
164	22	6	2	2	1	1	0	72	26	2	2	1	1	1	1	1	3	3	10	78	3	26	0.7	0.3	52	24	231	12	0	0
165	32	12	2	3	1	1	1	120	12	1	1	1	1	1	1	1	3	3	10	76	1	16	0.8	0.8	22	17	220	12	0	0
166	22	1	1	1	0	0	0	8	28	2	1	1	1	1	1	1	3	3	11	90	3	22	0.4	0.7	18	29	231	12	0	0
167	19	5	2	2	0	0	1	60	30	3	1	1	1	1	1	1	3	3	11	75	3	23	0.6	0.5	17	20	213	12	0	0
168	27	7	2	2	0	0	1	84	37	3	1	1	2	1	1	1	3	3	10	85	3	24	0.8	0.8	36	22	245	11	0	0
169	23	9	2	3	2	2	0	120	22	2	2	1	2	1	1	1	3	3	9.8	86	7	40	0.5	0.5	53	22	222	11	0	0
170	23	8	2	2	1	1	0	60	36	3	2	1	1	1	1	1	3	3	9.8	92	7	18	0.7	0.9	11	22	233	12	0	0
171	23	3	1	1	0	0	0	36	28	2	2	1	1	1	1	3	3	3	10	91	7	24	0.8	0.8	24	65	231	11	0	0
172	21	1	1	1	0	0	0	12	36	3	2	1	2	1	1	1	3	3	11	96	7	14	0.4	0.7	17	35	221	12	0	0
173	22	3	1	1	0	0	0	36	13	2	2	1	2	1	1	1	3	3	9.8	80	7	20	0.6	0.9	29	22	222	12	0	0
174	18	1	1	1	0	0	0	12	20	2	2	1	2	1	1	1	3	3	10	100	4	15	0.8	0.5	20	23	213	12	0	0
175	30	10	2	3	2	2	0	120	24	2	2	1	3	1	1	1	3	3	9.2	69	3	20	0.5	0.8	22	22	234	12	0	0
176	22	2	1	1	0	0	0	18	39	3	2	1	3	1	1	1	3	3	10	90	7	16	0.8	0.6	22	25	222	11	0	0
177	21	1	1	1	0	0	0	12	38	3	2	1	3	1	1	1	3	3	8.9	62	7	12	0.7	0.9	22	27	212	11	0	0
178	27	7	2	2	1	1	0	7	28	2	2	1	3	1	1	1	3	4	8.6	88	7	25	0.9	0.8	65	12	211	11	0	0
179	25	10	2	2	1	1	0	96	34	3	1	0	1	0	0	1	0	4	10	90	7	20	0.5	0.8	35	15	234	11	0	0
180	24	2	1	1	0	0	0	24	8	1	2	1	3	1	1	1	2	3	9.2	62	2	14	0.8	0.9	22	17	221	11	0	0
181	25	5	1	1	0	0	0	60	16	2	1	1	3	1	1	1	0	3	12	58	2	22	0.6	0.4	23	33	256	12	0	0
182	23	5	2	3	1	1	1	60	38	3	2	1	3	1	1	1	0	3	9.6	98	1	30	0.9	0.3	22	53	217	11	0	0
183	19	1	1	1	0	0	0	12	38	3	1	1	3	1	1	1	0	3	9.8	78	1	12	0.8	0.8	25	24	433	10	0	0
184	30	12	2	3	0	0	2	120	32	3	2	1	3	1	1	1	0	4	11	79	3	16	0.8	0.4	27	44	256	11	0	0
185	26	12	1	1	0	0	0	8	8	1	2	1	3	1	1	3	0	4	9	80	7	28	0.9	0.8	12	23	289	12	0	0
186	30	5	2	2	1	1	0	60	4	1	2	1	3	1	1	1	0	3	9.8	89	7	24	0.4	0.3	15	22	290	13	0	0
187	22	2	1	1	0	0	0	3	5	1	2	1	3	1	1	1	2	4	9.4	99	7	14	0.3	0.5	17	23	267	11	0	0
188	23	2	1	1	0	0	0	9	14	2	1	1	3	1	1	1	2	3	9.4	89	7	14	0.7	0.9	17	43	290	12	0	0
189	22	5	2	3	0	0	2	12	12	1	1	1	3	1	1	1	2	3	9.7	100	7	27	0.9	0.4	16	23	209	13	0	0
190	23	1	1	1	0	0	0	12	15	2	2	0	3	1	1	3	2	4	8.7	67	3	23	0.5	0.3	18	22	200	14	0	0
191	23	1	1	1	0	0	0	8	26	3	1	1	3	1	1	1	2	4	9.7	97	3	18	0.8	0.9	36	23	276	12	0	0
192	22	1	1	1	0	0	0	12	22	2	2	1	3	1	1	1	2	4	9.6	99	3	19	0.6	0.8	33	34	267	11	0	0
193	23	1	2	3	1	1	1	120	28	3	1	1	3	1	1	1	2	4	9	67	3	29	0.9	0.6	34	54	219	10	0	0
194	24	3	1	1	0	0	0	10	21	2	1	1	3	1	1	1	2	4	9	102	3	19	0.8	1.1	23	34	222	11	0	0
195	25	3	2	2	1	1	0	108	18	2	1	0	1	0	0	1	0	4	8.9	100	3	22	0.8	0.8	23	12	290	10	0	0
196	27	3	2	2	1	1	0	36	26	3	2	1	2	1	1	1	1	3	9	89	1	19	0.9	0.5	34	22	222	10	0	0
197	19	1	1	1	0	0	0	12	26	3	2	1	1	1	1	1	2	3	9.7	88	3	20	0.4	0.7	34	22	212	13	0	0
198	25	4	2	3	2	2	0	84	28	3	1	1	1	1	1	1	3	3	10	89	2	10	0.3	0.5	15	23	211	10	0	0
199	24	3	2	2	1	1	0	60	30	3	2	1	1	1	1	1	4	3	9	99	1	20	0.8	0.7	23	23	234	11	0	0
200	22	1	1	1	0	0	0	12	20	2	1	1	1	1	1	1	4	3	9	90	7	20	0.4	0.8	12	24	221	10	0	0