Chapter 1

INTRODUCTION

Viral hepatitis is a major public health problem worldwide. Hepatitis is a medical condition defined by the inflammation of the liver due to viral attack and characterized by the presence of inflammatory cells in the tissue of the organ. The condition can be self-limiting (healing on its own) or can progress to fibrosis (scarring) and cirrhosis. The morbidity and mortality associated with viral hepatitis in the developing world is high as compared to developed countries. Different types of viruses cause viral hepatitis, which includes, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Hepatitis D virus and Hepatitis E virus. Among these viruses, hepatitis A virus (HAV) and hepatitis E virus (HEV) are identified as etiologic agents of enterically transmitted hepatitis. HEV is now recognized as the principal cause of enterically transmitted non-A, non-B (ET-NANB) acute viral hepatitis (Krawczynski, 1993).

The hepatitis E virus (HEV) is known for being the cause of major outbreaks of waterborne hepatitis in Asia and Africa (Khuroo *et al.,* 1995). In the Indian subcontinent, it accounts for 30–60% of sporadic hepatitis. It causes enterically transmitted acute sporadic viral hepatitis (Singh *et al.,* 2001) and fulminant hepatic failure in areas of poor sanitary conditions where the virus is endemic (Arankalle *et al.*, 1994). South Asia is endemic for HEV, and it accounts for over 50% of cases of acute viral hepatitis in endemic countries (Aggarwal & Naik, 1997; Yarbough, 1999).

In developed countries, hepatitis E occurs sporadically and infections are more often food-borne than water-borne (Teo, 2006). Anti-HEV antibody prevalence rates vary from 1% to above 20% in developed countries (Dalton *et al.,* 2008 & Dawson *et al*., 1992).

First Infection with HEV was documented in 1955 during an outbreak in New Delhi, India (Gupta, 1957). Several other outbreaks have been reported from countries in the Indian subcontinent, in southeast and central Asia, Africa and Mexico (Table 1.1) (Khuroo, 1980; Wong *et al.*, 1980; Velazquez *et al.*, 1990; Naik *et al.*, 1992). Outbreaks of HEV are primarily due to contamination of drinking water by sewage (Kane *et al*., 1984). These epidemics last for a few weeks to a year and affect thousands of people (Vishwanathan 1957; Bali *et al*., 2008).

Table 1.1 Chronological occurrence of major water borne HEV epidemics and the number of reported cases from the Indian subcontinent, Sovient Union and South East and Central Asia.

Serial no.	Epidemic region	Country	Year of occurence	No. of reported cases
1	Delhi	India	1955–1956	29 000
2	Kirghiz Republic	Former Soviet Union	1957	10 000
3	Ahmedabad	India	1975–1976	2500
4	Kashmir	India	1978–1982	52 000
5	Hyderabad	India	1989	2000
6	Kanpur	India	1991	79 000
7	Kathmandu	Nepal	1973	10 000
8	Mandalay	Burma	1976–1977	20 000
9	Xinjiang Uighur	Northwest China	1986–1988	119 280
10	Greater Darfur	Sudan	2004	4000

HEV is the sole member of the genus Hepevirus in the family of Hepeviridae (Emerson *et al*., 2004). It is a non-enveloped, positive-sense, single-stranded RNA virus (Fig.1) (Reyes *et al.*, 1990), and has a 7.2-kb-long genome, which is capped and polyadenylated, HEV has been isolated and fully sequenced (Tam *et al*., 1991). The genome contains three open reading frames (ORFs). The longest ORF1 codes for non- structural proteins, the next longest ORF2 encodes the viral capsid, and the remaining ORF3, which overlaps the capsid gene, codes for a small protein of unknown function, which may have regulatory properties (Chandra *et al.*, 2008).

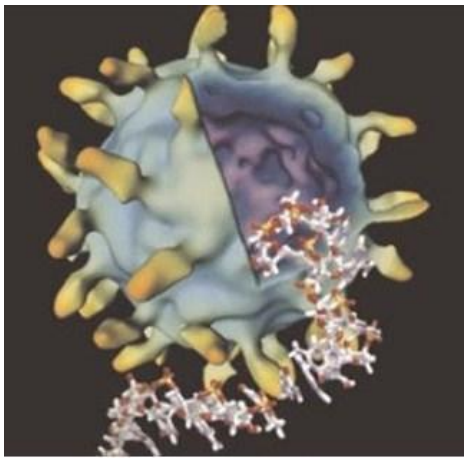


Fig.1.1: Hepatitis E Virus Particle. The three-dimensional structure of a self-assembled, recombinant HEV particle has been solved to 22A˚ resolution by cryo-electron microscopy and three-dimensional image reconstruction. (Cheng et al. http://www-ucdmag.ucdavis.edu/current/23:4;2006).

There are four genotypes according to HEV sequences, namely, genotypes 1, 2, 3, and 4 (Schlauder and Mushahwar, 2001) and only one serotype. Genotypes 1 and

2 are associated with large outbreaks and epidemics in developing countries with poor sanitation conditions and are restricted to humans only. Genotypes 3 and 4 mainly infects humans, pigs and other animal species and are responsible for sporadic cases of hepatitis E in both developing and industrialized countries . Genotype 1 particularly circulates among humans in tropical and subtropical countries in Asia and Africa, genotype 2 in Mexico and West and South Africa, and genotype 4 in East and Southeast Asia, whereas genotype 3 circulates worldwide (Fig.1.2) (Okamoto, 2007).

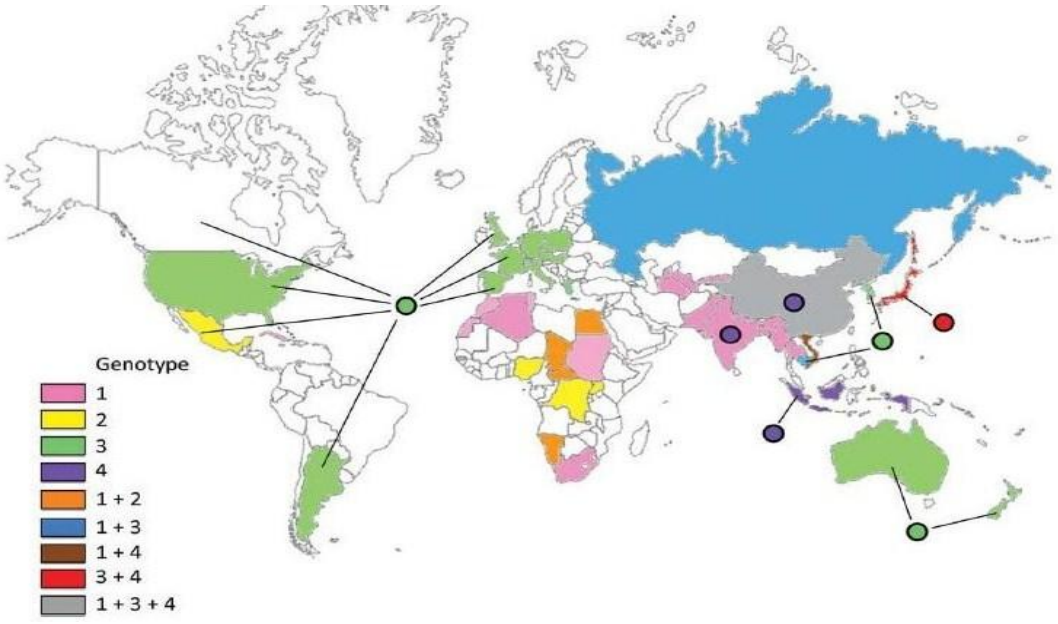


Fig.1.2: Distribution of HEV genotypes in viral isolates obtained from humans and animals (predominantly pigs). The colors used for a country and the circle associated with it represent the predominant HEV genotypes of human and animal

isolates, respectively, from that country. The figure is based on data from Okamoto, 2007.

Transmission of HEV is mainly waterborne due to contamination of drinking water. Other modes of transmission includes by consuming raw or undercooked meat of infected wild animals such as boars and deer and domestic animals such as pigs (zoonotic foodborne transmission), parenteral (bloodborne transmission), and vertical transmission from mother-to-child (perinatal transmission) (Fig.1.3). There is no evidence for sexual transmission of HEV, although in a report from Italy, 20% homosexual men had anti-HEV antibodies as compared to only 3% of intravenous drug users (Montella *et al.*, 1994).

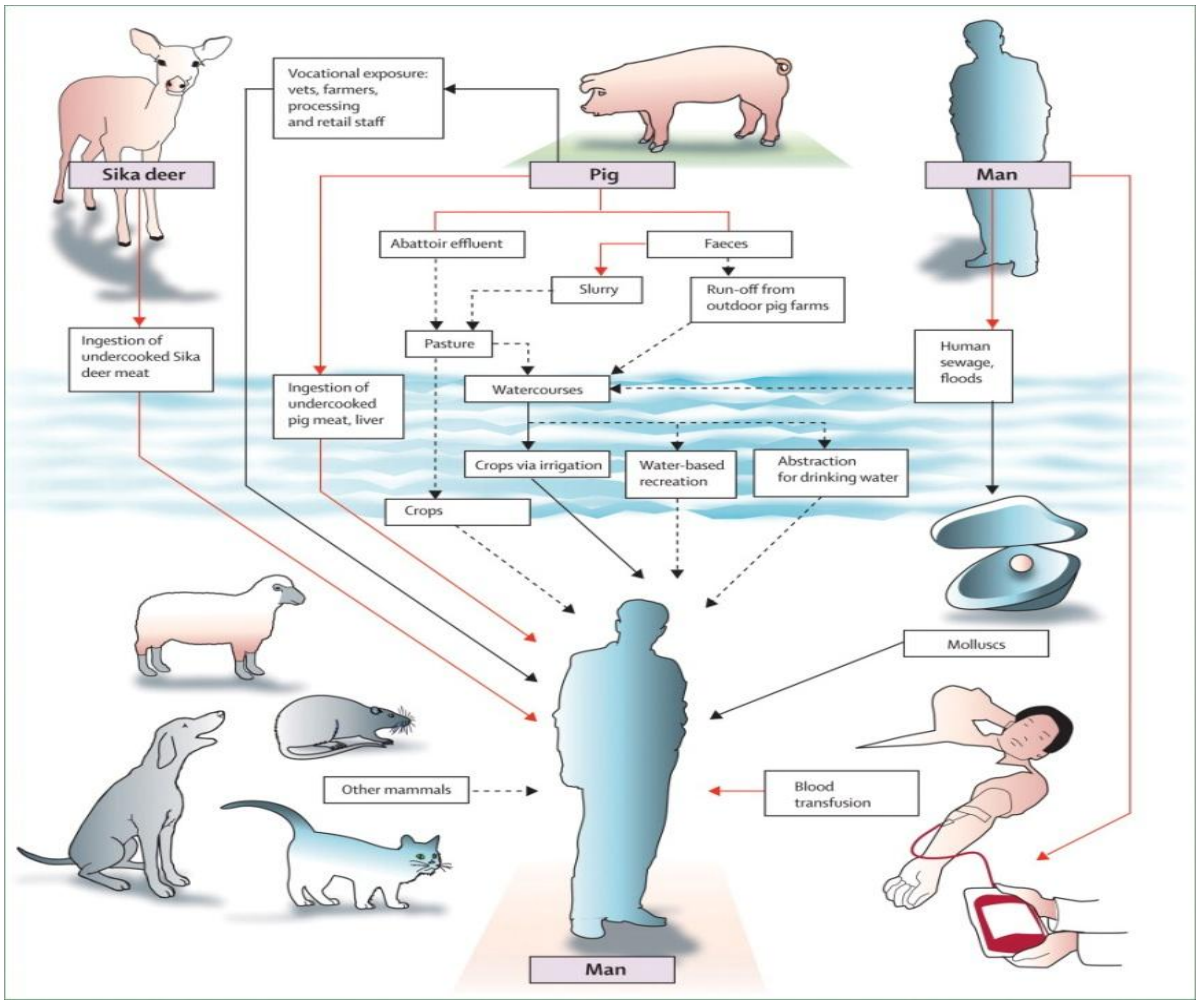


Fig.1.3: Confirmed, suspected, and potential transmission routes of HEV in developed regions.Red lines, confirmed route; continuous black lines, strong evidence for route; broken black lines, suspect or potential route (Harry *et al*, 2008).

Currently no specific vaccine and antiviral is available. The available HEV vaccine is made of a 56 kD pORF2 segment protein (genotype 1). It is a truncated protein produced in insect cells using a recombinant baculovirus efficiently self-assembles into virus-like particles (VLPs) that expose the dominant HEV

neutralization epitope (Robinsson *et al*., 1998) and elicit a protective antibody response in a monkey challenge model (Tsarev *et al*., 1994) .HEV causes a self-limiting illness with no chronic sequel. Improved sanitary conditions, proper disposal of human waste, higher standards for public water supplies and improved personal hygiene procedures can prevent spread of the disease.

IgM antibody to HEV is used as an acute phase marker of HEV infection and HEV IgG is used to study the exposure to HEV in a given population. Diagnosis is usually done in early acute-phase of the disease to avoid false negative results. A positive result for anti-HEV IgM indicates recent acute HEV infection. The presence of high or increasing titre of anti-HEV IgG support the diagnosis of acute HEV infection and in such cases acute hepatitis E can be presumed even in the absence of IgM anti- HEV. Several methods have been available for the diagnosis of hepatitis E. These include Immune electron microscopy (IEM), fluorescent antibody blocking assay, PCR, EIA and a recently developed immune-chromatographic assay.

Hepatitis E has clinical and morphological features of acute hepatitis which is similar to other forms of acute viral hepatitis. Hepatitis E causes a self-limiting viral infection usually followed by recovery. Prolonged viraemia, faecal shedding and chronic infection do not occur usually (WHO). Small occasional and food borne outbreaks have been reported. During outbreaks, 1%-15% of the population may be affected. Young adults are most often affected. Infection in children is more often

asymptomatic. Men usually outnumber women, possibly because of greater exposure to contaminated water. During the outbreaks, pregnant women have a higher disease attack rate and are more likely to develop fulminant hepatic failure (FHF) and die. Recently, a persistent HEV infection with chronic hepatitis and cirrhosis has been reported in patients with reduced immune surveillance as induced by chemotherapy or post-transplant immune suppression (Bihl & Negro, 2008).

Acute infection sometimes leads to more severe clinical fulminant hepatic failure in pregnant women and is associated with very high mortality particularly during the third trimester in endemic areas (Mushahwar, 2008).The incidence of HEV infection during the second and third trimesters of pregnancy (19.4% and 18.4%, respectively) is much higher than in the first trimester (8.8%) (Khuroo *et al*., 1981). A mortality rate of 80% caused by HEV-induced FHF during the third trimester of Pregnancy has been reported in men and non-pregnant women it has a low case-fatality rate (0.1%) (Krawczynski K, 2000). Hepatitis E in pregnancy is associated with high rates of spontaneous abortion, intrauterine death, and preterm labor (Dahiya *et al*., 2005).

In pregnancy normal levels of sex steroid hormones and immunity is altered. Abnormally high levels of pregnancy related hormones have been observed in case of FHF patients. Jilani *et al (2007)* found that HEV infected pregnant women with fulminant hepatic failure had lower CD4 count and higher CD8 counts. They also observed that the levels of estrogens, progesterone and beta-HCG were significantly higher in the HEV positive patients when compared to HEV negative patients or

control healthy pregnant females. Although the levels of hormones were physiologically high in the normal control population; patients with HEV infection seem to have significantly higher levels than controls, which probably explain the direct interaction of HEV with the immune system.

Progesterone is critical for the establishment and the maintenance of pregnancy, both for its endocrine and immunological effects. Progesterone receptors have been proposed to play a key role in human gestation, maintenance of human labor, and parturition (Alport *et al*., 2001 & Casey, 1997). Immunological recognition of pregnancy is categorized as an up regulation of progesterone receptors (PR) on natural killer (NK) cells in the decidual or on lymphocytes among placental cells. In the presence of progesterone, activated lymphocytes, and decidual CD56+ cells synthesize progesterone-induced blocking factor (PIBF), a 34-kDa protein, which exerts a substantial anti-abortive effect in vivo. Lower expression of PR and PIBF, as well as high viral load influences Hepatitis E disease severity and outcomes in pregnancy.

According to Khuroo (1981) these hormones are considered to play a pivotal role in the course of viral hepatitis. Hussaini *et al*., 1997 suggested that increased level of hormones might have a direct influence on viral replication through their effects on viral regulatory elements.

Although almost all studies to date demonstrate a high mortality rate associated with HEV infection during pregnancy, nothing is clear about the exact cause or mechanism(s) behind its occurrence. Keeping the above hypotheses in mind and knowing that hepatitis E is prevalent in our region and it causes high mortality in pregnant females, the present study is designed.

The aim of this study is

- To determine the levels of progesterone in the HEV sero-positive pregnant women population of low socioeconomic status,
- To compare progesterone levels in HEV positive and HEV negative population of low socioeconomic status.

Chapter 2

LITERATURE REVIEW

Viral hepatitis is a major public health concern worldwide. It was classified in the 20th century on the basis of various epidemiological studies, as either 'infectious' or 'serum' hepatitis (MacCallum, 1947). These two forms of hepatitis were distinguished by their mode of transmission as faeco-oral or parenteral, respectively.

It is caused by five distinct types of hepatotropic viruses A, B, C, D and E (Aggarwal, 2000). Hepatitis A and E spread through fecal (sewage) contamination of food or drinking water and was classified as infectious hepatitis (Feinstone *et al*, 1973). While B, C and D spread through transfusion of blood and body fluids, sexual Contact and by use of contaminated instruments and are termed as serum hepatitis (Blumberg *et al*, 1965). Except hepatitis A and B which has a very effective vaccine the rest of the types have no known vaccine for prevention.

Viral hepatitis can be of acute or chronic form:

1. Acute viral hepatitis (AVH)
2. Fulminant hepatitis/ Fulminant hepatic failure (FHF)

Acute viral hepatitis is defined as those cases which have acute self limited disease and a serum aspartate aminotransferase elevation of atleast five

fold or clinical jaundice or both (Smedile, 1982).Fulminant hepatic failure is considered when the patient after having a typical acute hepatitis, develops hepatic encephalopathy within four weeks without any history of pre-existing liver disease. It is characterized by mental changes progressing from confusion to stupor and coma as a result of severe impairment of hepatic function (Trey, 1970).

In Pakistan all five viruses are present in the community but in case of hepatitis A and E viral infections are endemic due to poor water and sewage systems. In India and Pakistan almost 100% of children under the age of 15 show evidence of exposure to HAV (Acharya, 2006). This early exposure to HAV is followed by long term, possibly lifelong, immunity to the virus (Wang, 1996). In Pakistan Hepatitis B Virus is prevalent in 4% of general population (Gideon, 2007) and India has a carrier frequency of 2%-4%. In India HBV genotypes A and D are prevalent which is similar to the HBV genotypes in the West. Hepatitis C Virus infection in India and Pakistan has a population prevalence of around 1% and 2.4% to 6.5% of general population (Gideon, 2008). It occurs predominantly through transfusion and the use of unsterile glass syringes. HCV genotypes 3 and 2 are prevalent in 60%-80% of the population in India (Acharya, 2006). HDV infection is infrequent in India and is present about 5%-10% of patients with HBV-related liver disease. In Pakistan it is prevalent in 36.8% of hepatitis B patients and 10% of healthy

carriers of hepatitis B in Karachi according to data published in 1995 (Gideon,1995).

Among all enterically transmitted viral hepatitis, Hepatitis E virus (HEV) is the most common cause of acute viral hepatitis. In developed countries, hepatitis E infections were traditionally thought to occur infrequently and only in individuals who had become infected while travelling in an area where the virus is endemic (Schwartz, 1999). South Asia is endemic for HEV, and it accounts for over 50% of cases of acute viral hepatitis in endemic countries (Aggarwal, 1997; Yarbough, 1999). Studies suggest that HEV is etiologically responsible for 10%–95% of admitted cases of hepatitis across South Asia (Khuroo *et al.,* 1985). In Pakistan due to poor sanitation system and excessive rainy and monsoon season seasonal epidemics of hepatitis E occurs frequently and sporadic cases occur continuously in areas with poor sanitation and weak public-health infrastructures. Among Children of age 14 to 15 HEV is 26% prevalent (Gideon, 2002-2004) and in adults prevalence is 62% (Gideon, 1995).

An enteric non-A, non-B agent was first suspected based on epidemiological investigations in an outbreak of viral hepatitis in 1978-1979 in Kashmir, India (khuroo, 1980) and retrospective analysis of a large waterborne outbreak in 1955-1956 in Delhi, India(Vishwanathan , 1957).This agent was initially known as the enterically transmitted non-A, non-B hepatitis virus and later on was renamed as hepatitis E virus (HEV) (Reyes *et al*., 1990) due to its

enteric transmission and association with hepatitis epidemics. HEV was molecularly characterized and cloning of its genome was done in 1990 (Reyes *et al*., 1990).

Initially, when It was first detected by Immune Electron Microscopy (Feinstone *et al.,* 1973) ET-NANBH was assumed to be an RNA virus and it was suggested to place this virus into the Picornaviridae family as hepatitis A type 2 virus (Balayan *et al.,* 1983;Miller, 1995).It was shown Later on that HEV was unrelated to the picornaviruses both antigenically and biophysically. On the basis of morphological similarities to Norwalk agent, HEV was classified into the family Caliciviridae, under a separate genus Hepevirus (kabrane-lazizi *et al,* 2001). There exists a resemblance between viruses belonging to the Caliciviridae family and that genome organization of HEV.

2.1 Epidemiology

HEV is endemic to tropical and subtropical countries in Asia, Africa, and Central America (Fig.2.1). Outbreaks of hepatitis E have been reported from the Indian subcontinent, China, Southeast and Central Asia, the Middle East, and northern and western Africa (Khuroo, 1980; Vishwanathan, 1955-56; Naik et al., 1992; Corwin et al., 1999) .Two small outbreaks were recorded in Mexico during 1986-1987, but none have been reported thereafter.

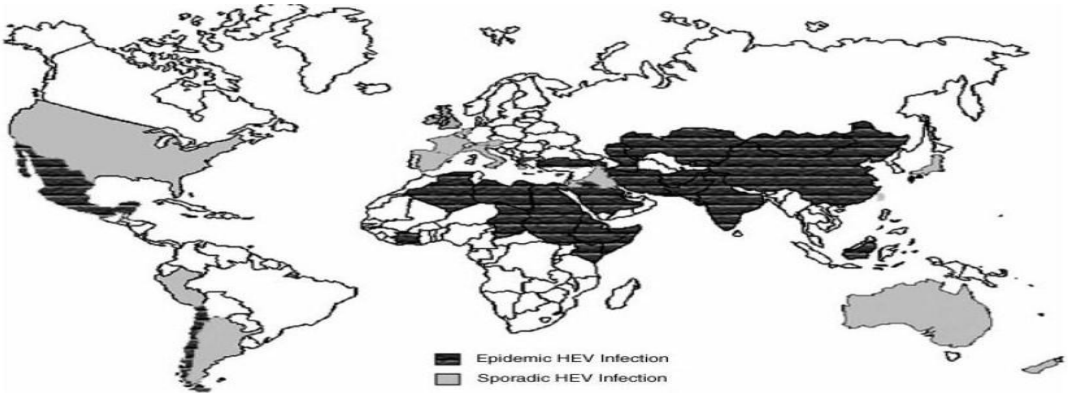


Fig.2.1: Geographical distribution of HEV. Countries of epidemic and sporadic HEV infections have been depicted**.**

The epidemics are usually related to contamination of drinking water with human excreta. These vary from small unimodal outbreaks lasting a few weeks to multipeaked epidemics lasting many months with several thousand cases (Vishwanathan, 1955-56; Naik *et al.,* 1992).Water contamination is often related to heavy rainfall and floods (Khuroo, 1980; Vishwanathan, 1955-56) diminution of water flow in rivers increasing the concentration of contaminants (Naik *et al.,* 1992; Corwin *et al.,* 1999)or leaky water pipes passing through sewage-contaminated soil.

In the United States, Western Europe, and developed countries of the Asia-Pacific, hepatitis E is quite infrequent . Initially, most such cases were considered as related to travel to high-endemic areas. However, in recent years, an increasing number of sporadic cases, and occasional small food borne outbreaks related to autochthonous (i.e., locally acquired) hepatitis E, has been reported from these regions (Kwo *et al*., 1997).

The reservoirs of HEV responsible for maintaining the disease in hyper endemic populations remain unclear. Protracted viremia and prolonged fecal shedding of HEV have been suggested; however, viral shedding in feces appears short-lasted (Aggarwal *et al.*, 2000).Frequent detection of HEV genomic sequences in sewage suggests a role for an environmental reservoir (Ippagunta *et al.,* 2007).

2.2 The disease

Clinical manifestations of HEV infection are similar to those of infection with other hepatitis viruses and encompass a wide spectrum of symptoms. The disease cause by HEV infection manifests as subclinical and sometimes it might leads to fulminant disease in humans. The infection may be entirely asymptomatic, or may resemble an acute viral febrile illness without any characteristic features. When symptomatic, it typically causes acute self-limiting viral hepatitis. This may run a prolonged cholestatic course. The incubation period ranges from 15 to 60 days with a mean of 40 days (Vishwanathan, 1957;Chauhan *et al*., 1993;Khuroo *et al*, 1980).

There are two main phases of the disease

1. The pre-icteric phase lasts for 1–10 days, (average 3–4 days) and gastrointestinal symptoms such as epigastric pain, nausea, and vomiting are frequently reported.
2. The icteric phase begins abruptly, with the appearance of jaundice, dark urine and clay coloured stools. In uncomplicated cases, this lasts 12–15 days, and complete recovery usually takes place within 1 month (Fig.2.2).

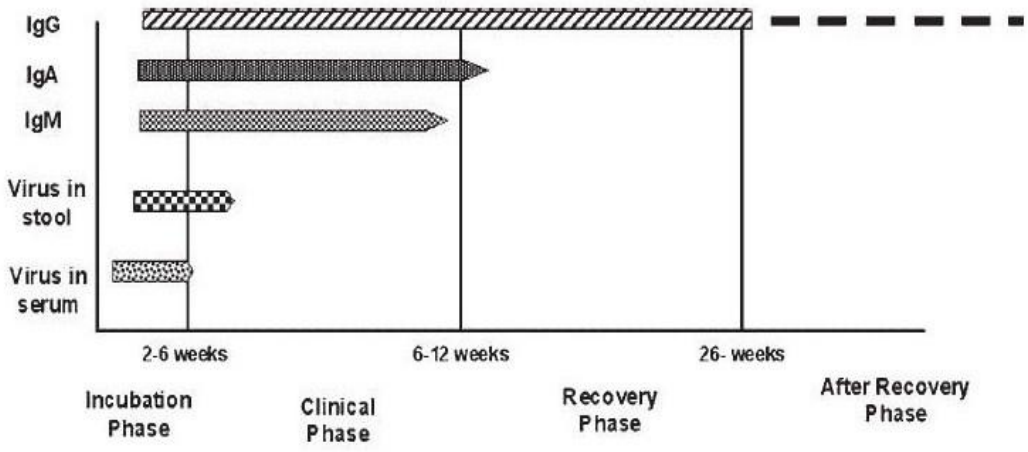


Fig.2.2: Hepatitis E virus infection, viremia and immune response.

Clinical studies have shown that the elevation of serum ALT levels occurs as a single peak preceding or coinciding with the onset of jaundice (khuroo *et al*., 1980; Balayan *et al*., 1983; Deinstag 1983) which is similar to most of the other forms of viral hepatitis.

Several methods have been available for the diagnosis of hepatitis E. These include Immune electron microscopy (IEM), fluorescent antibody blocking assay, PCR, EIA and a recently developed immunochromatographic assay. Amongst these methods, EIA and immunochromatography are most convenient for the detection of IgM and/or IgG anti-HEV. These are also inexpensive and suitable assays for routine diagnosis and seroepidemiological surveys. Anti-HEV IgM appears in the serum of infected patients with the onset of symptoms and remains detectable for variable period of 2 weeks to 3 months(Chauhan *et al*., 1993).Testing is recommended in early acute-phase of the disease to avoid false negative results. A positive result for anti-HEV IgM indicates acute HEV infection (Fig.2.2).

2.3 Viral Hepatitis and pregnancy

Viral hepatitis is the most common cause of jaundice in pregnancy (Smolenic & James, 1993). The course of most viral hepatitis infections (e.g., hepatitis A, B, C and D) is unaltered by pregnancy (Mishra & Seeff 1992; Snydman, 1985) However, a more severe course of viral hepatitis in pregnancy has been noted in patients with hepatitis E.

In most cases the pregnancy itself will not affect the severity of the hepatitis infection for the woman .HAV runs a course in the pregnant women without any severe and fulminant forms. While in case of virus B hepatitis, the pregnant women are threatened with the development of hepatic coma associated with a high maternal lethality but the good thing is that its been decreasing over the recent years (from

1.79% in 1956-1965 to 0.29% in 1966-1980 and to 0.21% in 1981-1989) (Beniwal *et al*., 2003).

A study of prevalence of viral hepatitis during pregnancy in Nepal Medical College Teaching Hospital was conducted and sample of 5602 pregnant women were taken and viral hepatitis was seen in 29 cases. HBV was 62.0%, HEV 20.6% and viral hepatitis of undetected serology was seen in 17.2% cases. Fulminant hepatitis with hepatic encephalopathy was the commonest cause of death which was due to hepatitis E infection during pregnancy (Shresta*et al*, 2009).

Another study in Nepal showed increased maternal and perinatal complications of hepatitis E infection like Preterm delivery, post partum haemorrhage , low birth weight contracted during pregnancy, especially so in those with fulminant hepatitis (Bista & Rana, 2006).In North India a study was conducted by Beniwal *et al* in 2003 on 97 pregnant patients in third trimester with acute viral hepatitis (AVH) or fulminant hepatic failure (FHF). Hepatitis E virus (HEV) was the causative agent in 47.4% of the cases of viral hepatitis and 52.6% were caused by non-E viruses (HAV-5.2%, HBV-7.2%, HCV-0%, non A-E 47.4%). HEV was responsible for 36.2% of the cases of AVH and 75% of the cases of FHF. The mortality rate was 39.1% in HEV group and 11.7% in non HEV group. It shows that HEV is involved in FHF related pregnancy which ultimately causes death of mother or fetus.

Chances of getting HEV infection in third trimester of pregnant females are high and are associated with FHF in pregnant women (Jaiswal, 2001 and Singh, 2001).

Prevalence of HEV infection is between 40-57% (Jaiswal, 2001; Singh, 2001).The mortality rate associated with FHF is in the range of 30-45% (Fig.10) (Jaiswal, 2001; Khuroo, 1995) and may be as high as 70%, woman mostly die undelivered (Singh *et al.*, 2001).

Multiple hepatitis virus infection during pregnancy has no more serious effect on the pregnant woman, but has worse effect on fetus than single hepatitis virus infection (liu et al., 2001). Ter (1990) studied viral hepatitides in the pregnancy and etiology of disease.In results of study of Ter, (1990) he concluded that Virus A hepatitis runs a favourable course in the pregnant, i.e. without severe and fulminant forms. In virus B hepatitis, the pregnant women are threatened with the development of hepatic coma associated with a high maternal lethality. however its noticeably been decreasing over the recent years (from 1.79% in 1956-1965 to 0.29% in 1966-1980 and to 0.21% in 1981-1989. Virus E hepatitis may be of the greatest mortal danger for mothers in conditions of water epidemic. In that case the lethality among pregnant women may reach 12.1% (in the Turkmenian SSR) and 15.6% (in the Kirghiz SSR). Super infection with HEV in patients of Chronic Liver Disease can cause severe hepatic decompensation leading to increased morbidity and mortality. Recent studies have shown that number of infectious agents that generally cause a mild, self-limiting illness can result in severe disease and a poor outcome in patients with underlying CLD (Vento, 1998 & Ducchini, 2000).

2.4 Hepatitis E and pregnancy

Hepatitis E has clinical and morphological features of acute hepatitis which is similar to other forms of acute viral hepatitis except in pregnant women in which course of its infection are more severe. complications which occurs normally during pregnancy includes death of the mother and fetus, abortion, premature delivery, or death of a live-born baby soon after birth (Hamid *et al.*, 1996).but in case of HEV infection during pregnancy FHF results which is associated with higher viral load of HEV as compared to AVH (Arankelle *et al*., 1998). Hepatitis E in pregnancy is also associated with high rates of spontaneous abortion, intrauterine death, and preterm labor (Dahiya *et al*., 2005). Intensive treatment of acute liver failure caused by HEV may reduce the high mortality reported in Asia (Hussaini *et al*., 1997).

Hepatitis E virus is the leading cause of acute sporadic and fulminant hepatitis in endemic areas. In certain geographic areas like subcontinent hepatitis E virus (HEV) is endemic and its outbreaks occur frequently during monsoon and rainy seasons. Although these outbreaks effect the general population including the children and adults but they recover as the disease is self limiting. However in pregnancy FHF results which is associated with a mortality rate of up to 20%.

Studies carried out in Iran, Africa and the Middle East have also found the incidence of fulminant hepatitis to be significantly higher during pregnancy (Table 2.1) (Balayan, 1997; Dilawari *et al.*, 1994; Arankelle *et al*., 1993; Tsega *et al*., 1993; Delons *et al*., 1962).In contrast, reports from Europe and the USA have shown that the course and severity of viral hepatitis during pregnancy is in no way different from that

in non-pregnant women(Cahill, 1962;Hsia *et al*., 1952; Adams &Combes, 1965;Mishra & Sheef, 1992).

Table 2.1: Studies on hepatitis E infection and pregnancy.

Studies on Hepatitis E infection and Pregnancy

Study	Patients (n)	Prevalence of HEV infection (%)	Prevalence of fulminant liver failure (%)	Mortality rate (%)
Jaiswal et al, 2001 (North India) 15	127	58	58	45
Singh et al, 2003 (North India) 14	60	37	64	64
Khuroo et al, 2003 (North India) 16	76	86	69	55
Beniwal et al, 2003 (North India) 8	97	47.4	75	39.1
Tsega et al, 1993 (Ethiopia) 10	32	59	-	42
Kumar et al, 2004 (North India) 12	65	45	32	73
Patra et al, 2007 (North India) 13	220	60	55	41
*Stoszek SK et al, 2006 (Egypt) 31	2428	84.3	0	0
*Rasheeda et al, 2008 (South India) 77	115	75	3.4	3.4

*Studies with low morbidity and mortality in pregnancy

The rate of FHF in sporadic AVH was 19.6% which is higher (61.8%) in pregnant women than in non pregnant women (10.1%) (Khuroo, 2003).Data showed that the higher rate of FHF in pregnant women was limited to those caused by HEV. Forty-five (69.2%) of the 65 pregnant women with sporadic AVH caused by HEV had developed FHF, whereas only two (18.2%) of the 11 pregnant women with sporadic AVH caused by viruses other than HEV (HBV ¼ 2 and HNAE ¼ 9) had developed FHF (Khuroo, 2003).

Jaiswal and others (2001) reported a high rate of infection with HEV (57.5%) causing acute hepatitis in pregnant women in India. Patra and kumar *et al* (2007) also reported a great difference in mortality rate between pregnant and non-pregnant women suffering acute hepatitis E.A recent large prospective study from Northern

India on the maternal and fetal outcomes of Hepatitis E infection, close to 60% of viral hepatitis in pregnant women was attributed to

hepatitis E infection (Patra *et al.*, 2007).

During an epidemic of non-A non-B hepatitis a prospective study was carried by Khuroo *et al*. (1981) for determining the incidence and severity of hepatitis in pregnant women, non pregnant women and men. In 17.3 percent of 208 pregnant women viral hepatitis developed, as compared to 2.1 percent of 3,350 non pregnant women and 2.8 percent of 3,822 men. The incidence of disease in pregnant women was higher than in the two control groups (Khuroo *et al*., 1981). In eight pregnant women (22.2 percent) with viral hepatitis, fulminant hepatic failure developed, as compared to its occurrence in three men (2.8 percent) and in no non pregnant women (Khuroo *et al*., 1981).This significantly increased incidence of fulminant hepatitis in pregnancy was indicative of a greater severity of hepatitis during pregnancy and was higher in last trimester (Khuroo *et al*., 1981).

According to study on the Outcome of hepatitis E virus infection in Indian pregnant women, they found out that those who were in the first trimester having acute viral hepatitis (AVH) they recovered completely. But those who were in 2^{nd} and 3^{rd} trimesters they developed FHF. The fatality rate in HEV infected patients was not different between the second and third trimesters (66.6% vs. 71.43%), respectively (Singh *et al*., 2001).

In Ghana, the prevalence of HEV was found to be higher in women in the third trimester of pregnancy (30.3%) compared with women in the second trimester of

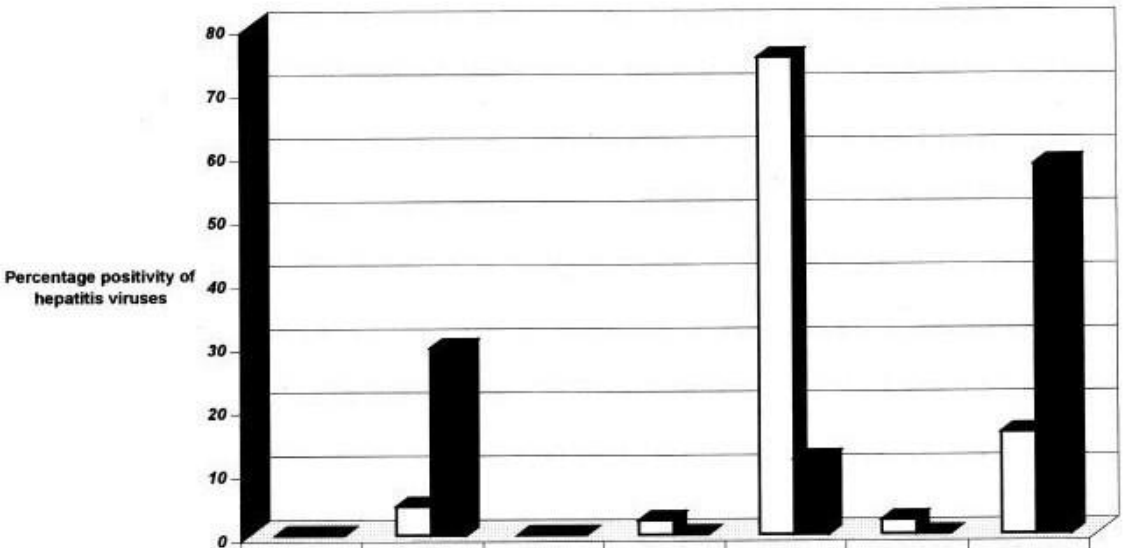
pregnancy (25.0%) (Adjei *et al*., 2009). Mushahwer (2008) also confirmed this that HEV is associated with high mortality particularly in the third trimester. Death usually occurs due to encephalopathy, haemorrhagic diathesis or renal failure. It has been shown that HEV causes intrauterine infection as well as substantial prenatal morbidity and mortality (Khuroo, 1995). A high degree of intravascular coagulation associated with hepatitis E in pregnant women is also well recognised (Hussaini *et al*, 1997).

The high risk of vertical transmission of HEV infection from mother to infant was investigated in a study of 469 pregnant women and reported a mother-to-infant transmission of 100%. In another study by Khuroo *et al*. (1995), vertical transmission was observed in five new borns whose mothers had developed hepatitis during an epidemic of water-borne HEV infection.

A study from Bangladesh reported an HEV prevalence of 58.3% among 144 patients and mortality rate of 80% caused by HEV-induced FHF during the third trimester of pregnancy (Mamun *et al*., 2009).Study on viral hepatitis in pregnant Asian women suggest that hepatitis E causes serious liver disease during late pregnancy in women (Khuroo *et al.*, 1981; Purdy, 1994; Balayan, 1997; Madan *et al*., 1998; Hamid *et al.*, 1996; Arankelle *et al*., 1998; Dilawari *et al* 1994; Cahill, 1962; Hussaini *et al*, 1997; Jameel *et al*., 1992; Reyes *et al*., 1990).However, the exact mechanism(s) are not clearly understood. Possible reasons behind this severity of disease in pregnancy have been studied well. Some of the reasons and causes of severity are discussed below.

2.5 Causes of severity of infection in pregnancy

From the above findings we can conclude that worse maternal and fetal outcomes of Hepatitis E have been observed in pregnant women with HEV infection by Khuroo *et al* (1983) as compared to the other types of viral hepatitis such as in case of HAV, HCV, HDV and HBV. However severity of disease was observed in Co-infection of HAV and HEV in both pregnant and non pregnant women (Fig.2.3) (Jaiswal *et al*., 2001).



	HAV	HBV	HCV	HDV	HEV	HAV+HEV	nA–nE
Series1	0	4,5	0	2,27	75	2,27	15,9
Series2	0	29,41	0	0	11,76	0	58,12

S1= Pregnant females S2= non-pregnant females

Fig.2.3: Incidence of viral hepatitis in pregnant and non pregnant fulminant females.

Pregnancy has no adverse effect on the course of hepatitis if proper nutrition and treatment is provided (Medhat *et al*., 1993; Dinsmoor, 1997). That is why fatality rate for pregnant and non-pregnant women of the developed world remains the same in case of any viral hepatitis. However in case of developing areas, due to poor prenatal care and maternal nutrition, during pregnancy the severity of HEV infection increases which means pregnancy is a key factor in establishment of HEV infection and worse disease outcomes (Krawczyuski, 1993; Khuroo *et al*., 1983).

This means that genetic and environmental factors that are found in certain low-resource countries contribute to development of HEV related infection (Navaneethan *et al*., 2008).It is also possible that a local and regional variation of host immune response to HEV is a contributing factor. Uptill now it remains unknown whether the hepatocyte damage in this disease is mediated primarily by the virus or by the host immune response to it. Fatal outcomes of the disease were evaluated by Purabi *et al* (2011). He studied the possible viral and host factors involved in this HEV related pregnancy outcome. Some of factors related to pregnancy are discussed below.

2.5.1 Steroid Hormones

According to studies hormones play an important role in causing high mortality rate during pregnancy. During pregnancy the normal hormone balance is disturbed, alteration in steroid hormone levels, in HEV infection provides incidence to

development of FHF, which ultimately leads to high mortality rate (Dilawari *et al*., 1994)

Reason behind this increased viral infection, replication and viral load is increase in steroid hormones. This increase in hormones plays a Pivotal role in the course of viral hepatitis (khuroo *et al*., 1981; Madan *et al*., 1998; Adams & Combes, 1965) by acting on viral regulatory regions. It is also possible that viral infection itself may induce steroid hormone secretion and these hormones may in turn enhance viral replication/expression leading to severity of disease in pregnancy (Nishat *et al*., 2007).

In a study an increase in the levels of hormones was seen in pregnant FHF patients when compared to the pregnant AVH patients or Controls. Similarly in comparison of HEV positive pregnant FHF patients to the HEV negative pregnant FHF patients increase in levels of estrogen, progesterone and B HCG was observed (Nishat *et al.,* 2007).

HCG which is a steroid hormone has an immuno regulatory role during pregnancy as its effect on cell mediated immunity has been observed by Lewis *et al.* (1966). The increase of Estrogens results in a decrease in bone-marrow B-cell production, mainly pre-B and immature Bone-marrow B-cells (Navaneethan *et al*., 2008). It also has a role in reduction of thymus weight, CD4 and CD8 populations (Boll & Reimann, 1996; Rijhisinghami *et al.,* 1996) while progesterone is associated in blocking the process of development of T cells (Tibbets *et al*., 1996).

2.5.2 Immunological changes

Immunological changes occur for the maintenance of antigenic fetus in maternal environment during early stages of pregnancy by suppression of T-cell-mediated Immunity. This reduction in cell mediated immunity increases the susceptibility of the mother to various infectious diseases (Balayan, 1997). Higher IL-10/IL-12 ratio (Th2-type immunity) is a pre requisite for successful pregnancy maintenance, which is altered maximally toward the Th1 biased state in HEV related pregnancy cases .This shift plays a role in the severe clinical course and mortality of HEV infection during late pregnancy (Rekha *et al*., 2005) as compared to healthy or HEV related AVH pregnancy cases.

2.5.3 Folate Deficiency

Folate deficiency occurs during pregnancy when proper nutritional intake is not followed. That's is why pregnant women in Asia generally suffer from folate deficiency (Jukes, 1983) which increases the risk of multiple viral infection and higher viral load due to reduced immune competence (Fig.2.4) (Chandra & Chandra, 1986; Dhar *et al.,* 1991). Possible reason may be due to effect of high hormone levels directly on hepatic cells which may predispose to hepatic dysfunction with toxin or pathogen exposure (Barbara *et al*., 2006).

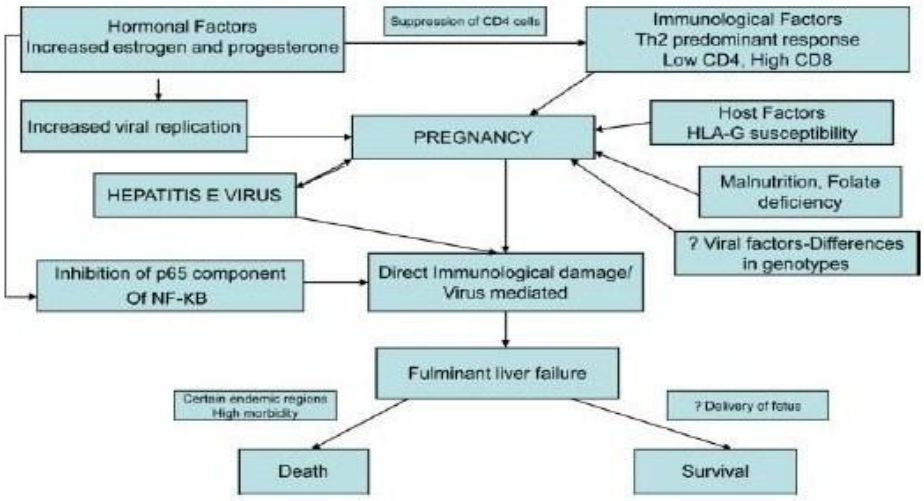


Fig.2.4 Pathogenisis of HEV during pregnancy.

2.5.4 Genotype

Kar *et al.* (2008) reported that apart from the host factors, viral factors also play a major role in HEV disease severity. However in comparison to HEV genotype with outcome of disease, these can't be co related as in a study only a single genotype ,Genotype 1, was detected in both the disease groups; One with FHF patients showing higher HEV viral load and other with AVH showing lesser viral load (139,994.0 ± 103,104.17 copies/ml) than (768.92 ± 1105.40 copies/ml).

2.5.5 Progesterone and its Receptor

Progesterone is critical for the establishment and the maintenance of pregnancy its levels increase during the latter half of pregnancy because of its endocrine and immunological effects. It was seen in case of HEV infection in FHF patients the levels of progesterone and other hormones increases dramatically as compared to normal pregnancy. This can be illustrated in the following graph (Fig.2.5).

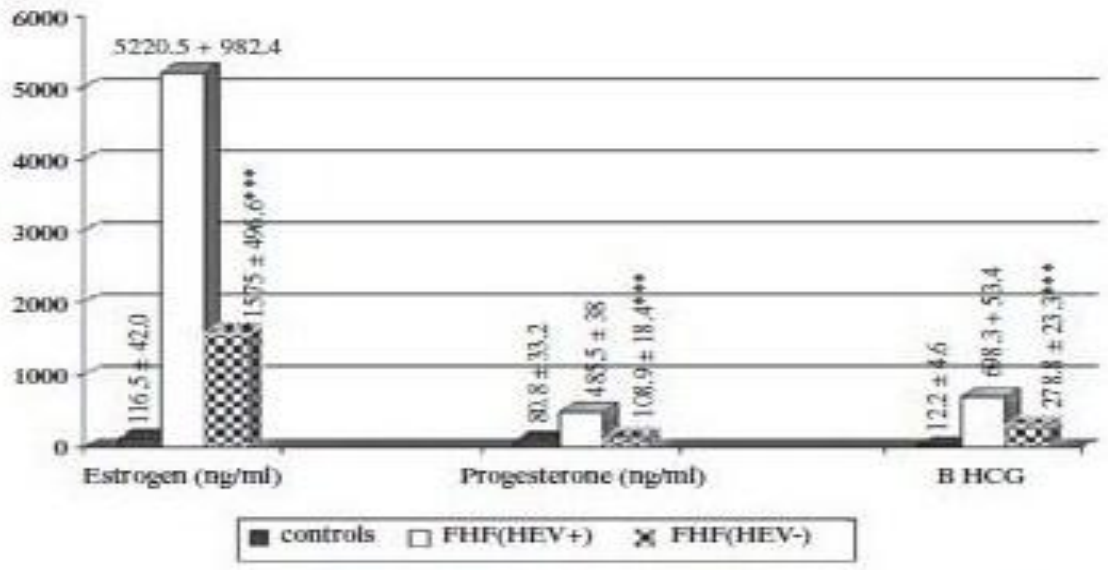


Fig.2.5: Bar diagram showing hormonal levels in controls, hepatitis E virus (HEV) positive and HEV negative pregnant fulminant hepatic failure (FHF) patients. *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant (P < 0.05) for comparison

between HEV positive and HEV negative pregnant FHF patients. Statistical analysis by Student's *t*-test (Nishat *et al.*, 2007).

The actions of progesterone are mediated by the intracellular progesterone receptors (Connely, 2000). Progesterone receptors and progesterone induced blocking factors (PIBF) have been proposed to play a key role in human gestation, maintenance of human labor, and parturition ((Druckmann & drukmann, 2005; Szekeres-Bartho *et al*., 2001). Immunological recognition of pregnancy takes place by an up regulation of progesterone receptors on natural killer (NK) cells in the decidua or on lymphocytes among placental cells (Roussev *et al.*, 1993).

When progesterone is present, activated lymphocytes, and decidual CD56+ cells synthesize progesterone-induced blocking factor (PIBF), a 34-kDa protein, which exerts a substantial anti-abortive effect in vivo (Roussev *et al.*, 1993). Women with threatened abortion, their lymphocytes fail to produce this factor. PIBF actually stabilizes the mRNA of interleukin-10 (IL-10) (Polgar *et al*., 2003). PIBF is anti-abortive, which is due to induction of a Th2 biased cytokine production and blocking of natural killer cell (NK) activity (Polgar *et al*., 2003; Szekeres *et al*., 1997). Neutralization of this blocking factor results in a Th1 shift (Szekeres *et al*., 1997).

Progesterone receptor (PR) signaling pathway plays an important role in the severity of hepatitis E during pregnancy. Expression analysis for the progesterone receptor showed gradient down regulated expression in HEV related pregnancy cases compared to healthy pregnant cases in a manner: Healthy pregnancy> AVH-E related pregnancy > FHF-E related pregnancy (Purabi *et al.*, 2011). This was further

confirmed by Purabi *et al*., (2011) by showing that high viral load, lower expression of PR and PIBF and higher NK cell activity results in reduced fetal protection and eventually fetal death occurs because of immunological injury. The HEV related pregnancy outcomes is illustrated in the Fig.2.6 (Purabi *et al.,* 2011).

A haplotype of progesterone receptor, PROGINS, is associated with reduced amounts of gene transcript and a lesser response to progesterone in case of HEV related pregnancy (Romano *et al.,* 2007). PROGINS may be considered as a candidate prognostic marker for HEV related pregnancy complications and outcomes); as it was seen that in FHF patient it was more prevalent (10/43, 23.26%) as compared to AVH (14/100, 14%) and the healthy pregnant (2/50, 4%) group.

Lower expression of PR and PIBF, PROGINS carriers and high viral load influences Hepatitis E disease severity and outcomes in pregnancy (Purabi *et al*., 2011).

During late pregnancy it is possible that viruses can spread more rapidly, attaining high titers in tissues and producing more severe disease. A study of Lassa fever in pregnant women showed an extremely high titer of the virus in placental tissues, suggesting that the placenta may be the preferred site of this viral replication (McCormick *et al.,* 1986). Some other infectious diseases such as malaria during pregnancy are also known to cause severe disease manifestation and higher mortality (Roberts *et al*., 2001).

Therefore, we can say that pregnancy appears to be a potential risk factor for viral replication and low immune status in Indian/Asian pregnant women (Nishat *et al*., 2007).

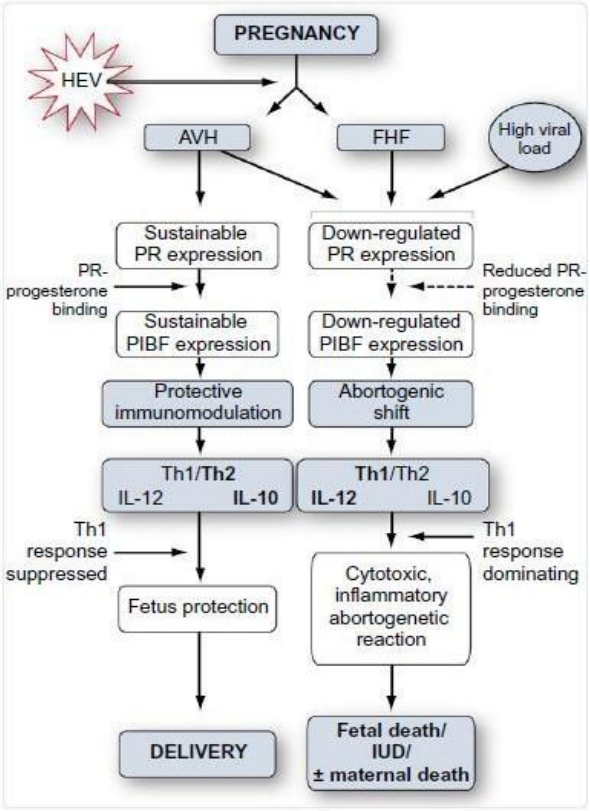


Fig.2.6: Model depicting the pathways of HEV modulated pregnancy outcome (Purabi *et al.*, 2011).

Chapter 3

MATERIALS AND METHODS

3.1 Sample collection

Sample Number: The research was conducted on 91 blood samples of pregnant women of low socioeconomic status.

Setting: The samples were taken from different clinics and hospitals of Rawalpindi and Islamabad.

Collection: BD syringes were used to collect 5 ml of blood samples in EDTA tubes till serum extraction.

Inclusion Criteria:

- Pregnant women of Low socioeconomic status

Exclusion Criteria: Women who had gone through:

- Hormonal Ova Induction
- In vitro fertilization

Consent: Informed consent was obtained at the time of blood sampling from every patient included in the study. The ethical committee of the institution approved the study protocol.

Questionnaire: The participants were asked to fill out a questionnaire on socio-demographic characteristics (age, trimester, gravida, family income, disease, place of previous delivery, contact number).

Sample Consent Form

Serial number:

Code:

"Prevalence of anti-HEV antibodies in pregnant women.

Informed Consent Form for Rabia Anwar

Degree: BS Applied Biosciences.

Supervisor: Dr. Saadia Andleeb

Name of Organization: Atta ur Rehman School of Applied Biosciences

Name of Project:

I have been asked to give consent to participate in this research study which will involve completing one questionnaire and blood sample collection. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate as a participant in this study.

Name of Participant _____

Signature of Participant _____

Date _____

Sample Questionnaire

Serial number:

Code:

"Prevalence of anti-HEV antibodies in pregnant women.

Personal Details:

Name of Participant:

Date of birth: ____/_____/_____dd/mm/yy

Income:

Contact number:

Medical History:

History of Jaundice:

Any other illness:

History of Blood transfusion:

Gravida:

Gestation period:

Mode of previous delivery: 1. Home 2. Hospital (Private Government)

3.2 Sample processing

Serum Extraction: Serum was extracted from the blood samples by centrifugation at 10,000rpm for 5 minutes in eppendorf 5424.

Storage: The extracted serum was stored at -80 degrees.

Equipment:

Eppendorf pipettes were employed during the procedures.

The ELISA readings were read on Biotek Elx800 and printed by Epsonlx-300+2.

3.3 Antibody detection by ELISA

HEV Anti -IgG ELISA

Chemicals and Materials provided in the kit:

- 96 Well microwell plate
- Negative Control

- Positive Control
- Specimen diluent
- HRP-Conjugate
- Wash Buffer
- Chromogen Solution A
- Chromogen Solution B
- Stop Solution
- Plate Cover

Assay Procedure

Preparation: The reagents and samples were allowed to reach room temperature for at least 15-30 minutes. The Wash buffer concentrate was checked for the presence of salt crystals and diluted 1:20 with distilled water. Three wells were marked as Negative control (A1, B1 and C1) and two wells as Positive control (D1, E1).

Addition of Diluent:100µl of Specimen Diluent were added into each well except including the controls.

Addition of Sample: 10µl of Positive control, Negative control, and Specimen were added into their respective wells and were mixed by tapping the plate gently.

Incubation (1): The plate was covered with the plate cover and incubated for 30 minutes at 37°C.

Washing (1): At the end of the incubation, the plate cover was removed and discarded. Each well was washed 5times with diluted Wash Buffer. Each time, the microwells

were allowed to soak for 30-60 seconds. After the final washing cycle, the plate was turned onto clean tissue papers, and tapped to remove any remainders.

Adding HRP-Conjugate:100µl of HRP-Conjugate were then added to each well.

Incubation (2): the plate was covered and incubated at 37 degree for 30 minutes.

Washing (2): After the end of the incubation, the plate cover was removed and discarded. And the wells were washed 5 times each with washing buffer. Each time, the microwells were allowed to soak for 30-60 seconds. After the final washing cycle, the plate was turned down onto clean tissue papers and tapped to remove any remainders.

Coloring: 50µl of Chromogen A and 50µl Chromogen B solutions were added into each well. The plate was incubated at 37°C for 15 minutes avoiding light. The enzymatic reaction between the Chromogen solutions and the HRP-Conjugate produced blue color in Positive control and HEV IgG positive sample wells.

Stopping Reaction: Using a multichannel pipette, 50µl of stop solution was added into each well and mix gently. Intensive yellow color developed in Positive control and IgG-HEV positive sample wells.

Measuring the Absorbance: The plate reader was calibrated and the absorbance was read at 450nm, the reference wavelength was set at 630nm as a dual filter instrument was used. The Cut-off value was calculated and the results were evaluated.

Calculation of the Cut-off value (C.O.) = Nc + 0.16

(Nc = the mean absorbance value for three negative controls).

Interpretations of the results: (A = the individual absorbance (OD) of each specimen).

Negative Results (A <1): Samples giving A value less than the Cut-off value were considered negative for this assay, indicating that no IgG to HEV have been detected with the HEV-IgG ELISA kit, therefore there are no serological indications for infection with HEV.

Positive Results (A ≥ 1): Samples giving A value which were equal to, or greater than the Cut-off value were considered reactive, which indicated that IgG to HEV were detected using the a HEV-IgG ELISA kit, and therefore the patient is infected with HEV.

HEV Anti- IgM ELISA

Chemicals and Materials provided in the kit:

- 96 Well microwell plate
- Negative Control
- Positive Control
- Specimen diluent
- HRP-Conjugate
- Wash Buffer
- Chromogen Solution A
- Chromogen Solution B
- Stop Solution
- Plate Cover

Assay Procedure

Reagents Preparation: The reagents and samples were allowed to reach room temperature for at least 15-30minutes.The Wash buffer concentrate was checked for the presence of salt crystals. The Wash buffer was diluted 1:19 with distilled water. Three wells were marked as Negative control (e.g. B1, C1, D1), two wells as Positive control (e.g. E1, F1).

Addition of Diluent: 100µl of Specimen Diluent was added into each well.

Addition of Sample: 10µl of samples and 10µl ofPositive and Negative controls were added into their respective wells.

Incubation (1): The plate was covered with the plate cover and incubated for 30minutes at 37°C.

Washing (1): After the end of the incubation, the plate cover was removed and discarded. Each well was washed 5 times with diluted Washing buffer and each time, the microwells were allowed to soak for 30-60 seconds. After the final washing cycle, the plate was turned down onto clean tissues and tapped to remove any remainders.

Addition of HRP-Conjugate: 100µl of HRP-Conjugate Reagent was added into each well.

Incubation (2): The plate was covered with the plate cover and incubates for 30minutes at 37°C.

Washing (2): The plate sealer was removed and discarded, the liquid was aspirated and each well was rinsed 5 times with Wash buffer. After the final washing cycle, the plate was turned and tapped out of any remainders.

Coloring: 50µl of Chromogen A and 50µl Chromogen B solution was added into each well. The plate was incubated at 37°C for 15minutes avoiding light. The enzymatic reaction between the Chromogen solutions and the HRP-Conjugate produced blue color in Positive control and HEV IgM positive sample wells.

Stopping Reaction: Using a multichannel pipette, 50µl of Stop solution was added into each well and mixed gently. Intensive yellow color developed in Positive control and HEV IgM positive sample wells.

Measuring the Absorbance: The plate reader was calibrated the absorbance was read at 450nm with the reference wavelength at 630nm. The Cut-off value was calculated and the results were evaluated.

Calculation of Cut-off value (C.O.) = Nc + 0.26

(Nc = the mean absorbance value for three negative controls.)

Interpretations of the results: (S = the individual absorbance (OD) of each specimen).

Negative Results (S<1): Samples giving absorbance less than the Cut-off value were negative for this assay, indicating that no IgM-class antibodies to hepatitis E virus were detected with the kit therefore there are no serological indications for current infection with HEV.

Positive Results (S ≥1): Samples giving an absorbance equal to, or greater than the Cut-off value were considered reactive, which indicated that IgM-class antibodies to hepatitis E virus have been detected using the HEV IgM ELISA kit, and the patient is recently infected with HEV.

3.4 ELISA for measurement of progesterone levels

Reagents and equipments needed:

- Precision pipettes to dispense: 5ul-40ul, 50ul-200ul and 1.0ml.
- Disposable pipette tips
- Distilled or deionized water
- Vortex mixer
- Absorbent paper or paper towel
- Microwell plate reader
- Linear –linear graph paper

Reagents and materials provided:

- Goat Anti-Rabbit IgG- Coated Microwell Plate-Break Apart Wells
- Progesterone -Horseradish Peroxidase (HRP) Conjugate Concentrate – (11x), 1.3 ml
- Progesterone -Horseradish Peroxidase (HRP) Conjugate Diluent, 13ml.
- Progesterone Reference Standards: 0, 0.5, 3.0, 10, 25 and 50 ng/ml. liquids, 0.5 ml each, ready to use.
- Wash Buffer Concentrate (50X), 15ml.
- Stop Solution, 12ml.
- TMB Substrate, 12ml.

Reagent preparation:

- All reagents were brought to room temperatures (18-22C) before use.

- Working Progesterone-HRP Conjugate Reagent was prepared by adding 0.1ml of Progesterone-HRP Conjugate Concentrate (11x) to 1.0ml of Progesterone-HRP Conjugate Diluent (1: 10 dilution) and then it was mixed well.
- Preparation of wash buffer: 1 volume of Wash Buffer (50x) was diluted with 49 volumes of distilled water and was mixed well before use.

Procedure:

All reagents were allowed to reach room temperature before the process. Calibrators, controls and specimen samples were assayed in duplicate. All the steps were completed without interruption once it was started.

Microtiter plate with coated wells was placed in holder to secure the plate.

Addition of calibrator, control and sample: 25 µl of each calibrator, control and specimen sample were dispensed into appropriate wells.

Addition of rabbit anti-progesterone reagent: 50ul of rabbit anti-progesterone reagent was dispensed to each well.

Addition of progesterone-HRP Conjugate Reagent: 100 µl of working progesterone-HRP Conjugate Reagent was added into each well using a multichannel pipette and then it was thoroughly mixed for 30 seconds.

Incubation 1: It was incubated for 90 min at room temperature (18-22C).

Washing: after Rinsing and flicking the microwells were washed for 5 times with washing buffer (1X).

Addition of substrate: 100 µl of TMB substrate was added into each well at time intervals and was mixed for 10 seconds.

Incubation 2: Incubation was done on a plate shaker for 10-20 minutes at room temperature.

Addition of stopping solution: 100 µl of stopping solution was added into each well at the same time intervals as in step 7.

Mixing: It was mixed gently for 30 seconds and all the blue colored wells which changed to yellow color completely were noted.

Reading: The plate was read on a microwell plate reader at 450nm within 20 minutes after addition of the stopping solution.

Calculations:

- The mean optical density (A450) for each set of reference standards, controls and samples was calculated.
- A standard curve on semi-log paper was Drawed with the mean optical densities obtained for each reference standard against its concentration in ng/ml on a linear-linear graph paper on the Y-axis and the calibrator concentrations on the X-axis.
- The mean optical density of each unknown duplicate was calculated.
- Reading of the values of the unknowns was taken directly off the calibrator curve.

3.5 Detection by chromatography assay

Detection of HCV

Materials supplied:

- Test strip
- Desiccant

Procedure:

- Serum specimens were allowed to equilibrate to the room temperature.
- The test device was removed from pouch and the test strip was labeled with patient identification
- The test device was placed in a smooth and clean surface.
- 5 µl of serum was transferred on the test device using a pipette.
- 2 drops of buffer were added and the timer was started.
- The result was read after 10 minutes, by the appearance of colored lines.

Interpretation of Results

Positive: The appearance of two colored lines, one in the control region and other in the test region, was considered as positive.

Negative: Appearance of a single colored line in the control region was considered a negative result.

Detection of HBV

Materials supplied:

- Test strip
- Disposible specimen droppers

Procedure

- Serum specimens were allowed to equilibrate to the room temperature.
- The test device was removed from pouch and the test strip was labeled with patient identification
- The test device was placed in a smooth and clean surface.
- 100µl of serum (3 drops) was added to the specimen well (S) of the test device using the given dropper and the timer was started.
- The result was read after 15 minutes, by the appearance of colored lines.

Interpretation of Results

Positive: The appearance of two colored lines, one in the control region and other in the test region, was considered as positive.

Negative: Appearance of a single colored line in the control region was considered a negative result.

3.6 Statistical Analysis

Statistical tests were applied using Pearson's correlation test, one way ANOVA and T –test. Graphs were drawn in Graph Pad Prism 5. P value less than 0.0005% were considered as statistically significant.

Chapter 4

RESULTS

4.1 Results of IgG and IgM ELISA

The total numbers of samples collected for the current seroprevalence study were 90. ELISA was performed on all the samples and of which 54 were found positive for IgG (had value greater than the cut off value of 0.196) thus giving a seroprevelance of 60% (54/90) (Fig.4.1). The IgM ELISA gave the results of 13.3% (12/90) seroprevelence in the sample population (considered positive in case of having value greater than 0.267) (Fig.4.2).

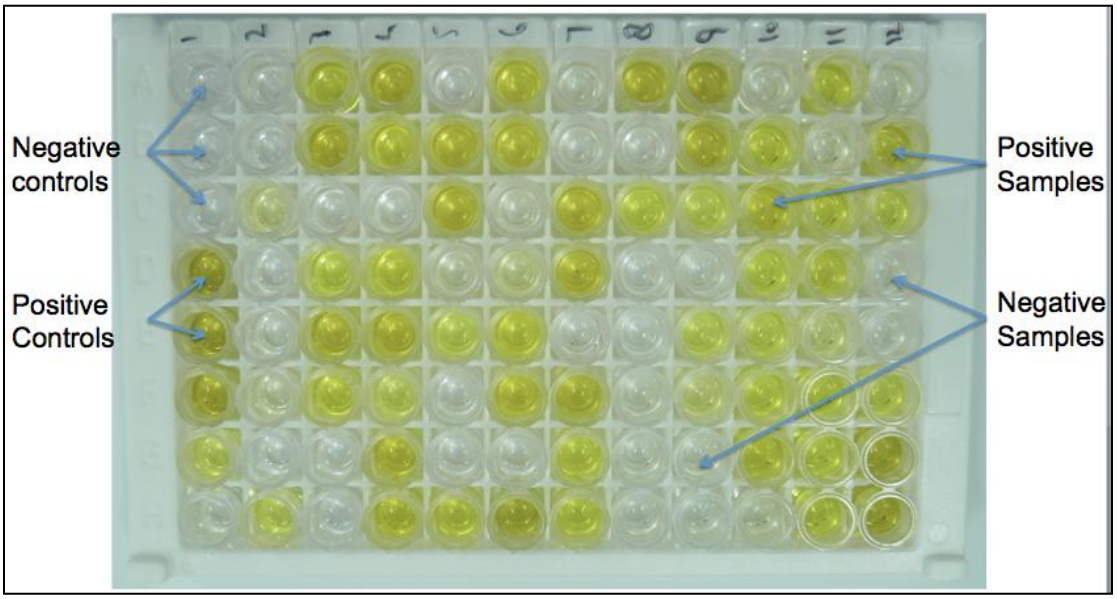


Fig. 4.1: ELISA micro well plate for detection of anti-HEV IgG.

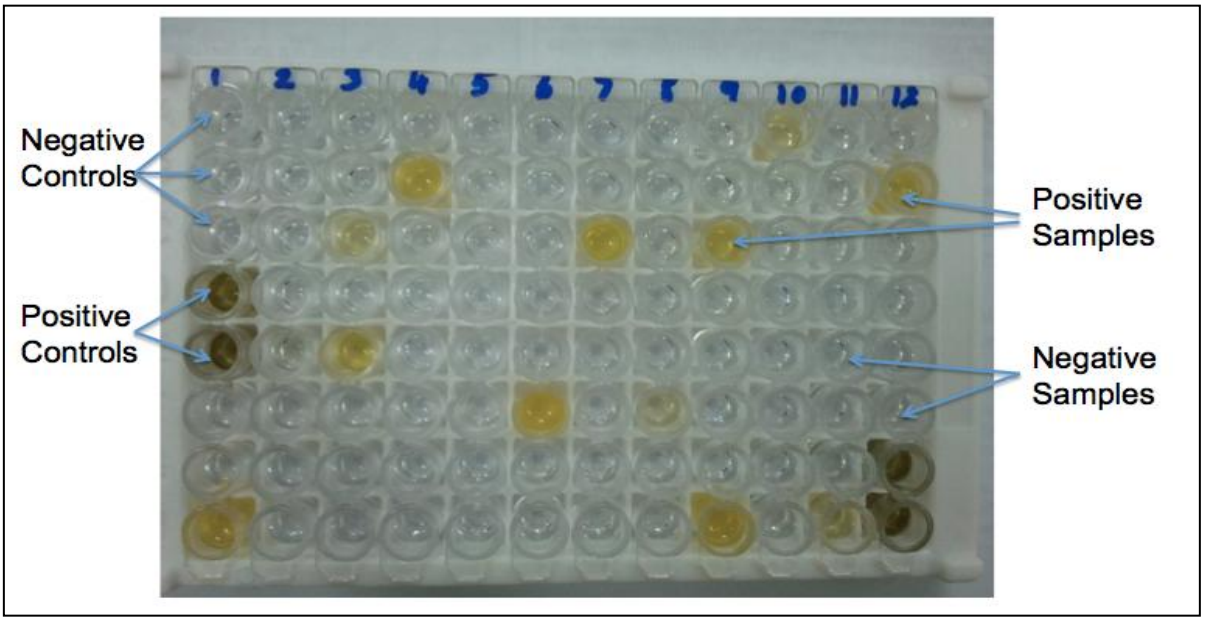


Fig.4.2: ELISA micro well plate for detection of anti-HEV IgM.

4.1.1 Trimester wise IgG and IgM prevalence.

The prevalence of IgG and IgM were determined in the three trimesters of pregnancy and varied between 58.3% in case of 1st trimester and 63.1% in case of 3rd trimester for IgG. Similarly, the results showed an increasing trend for seroprevelence of IgM from 1st to 3rd trimesters ranging between 5.55 to 21.05% respectively (Fig.4.3).

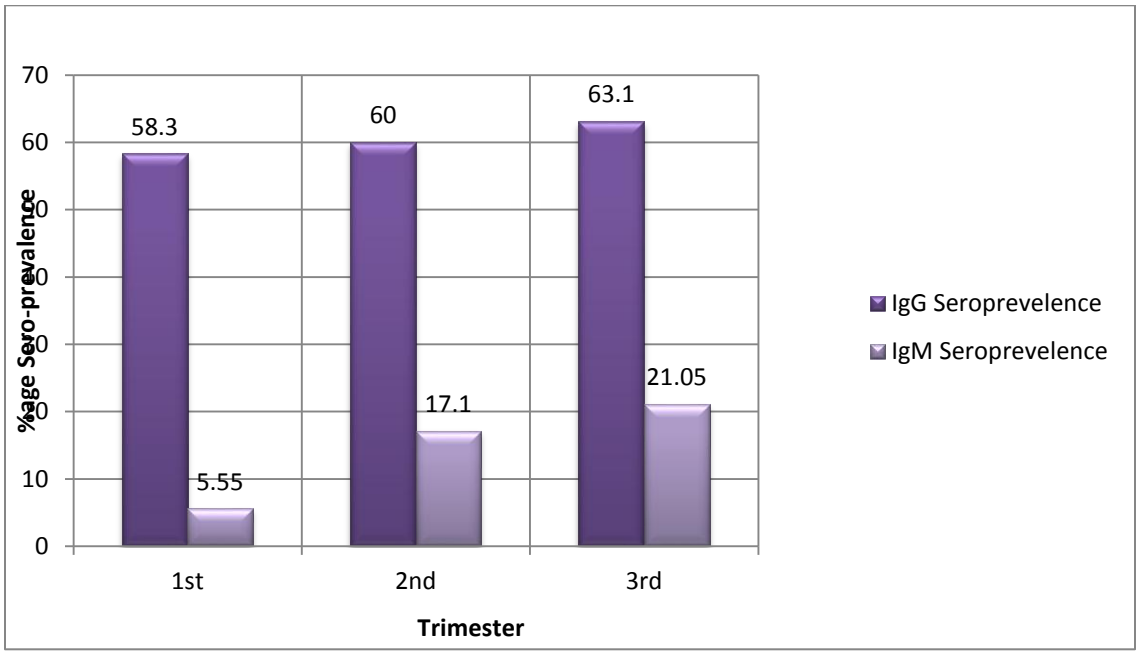


Fig.4.3: On X-axis trimesters of pregnancy and Y-axis shows prevalence of HEV IgG and IgM.

4.2 Results of Immunochromatography

- 4 (7%) out of 54 positive samples for anti-HEV showed positive results for HCV.
- None of the samples were found positive for HBV.

Fig.4.4: Strip test for detection of HCV and HBV.

4.3 Progesterone level measurement

Progesterone ELISA was done on all samples which were IgM and IgG positive and as well as on those which were negative for both (Fig.4.5). Different comparisons were made using statistical tests and the significance of the correlations was also made.

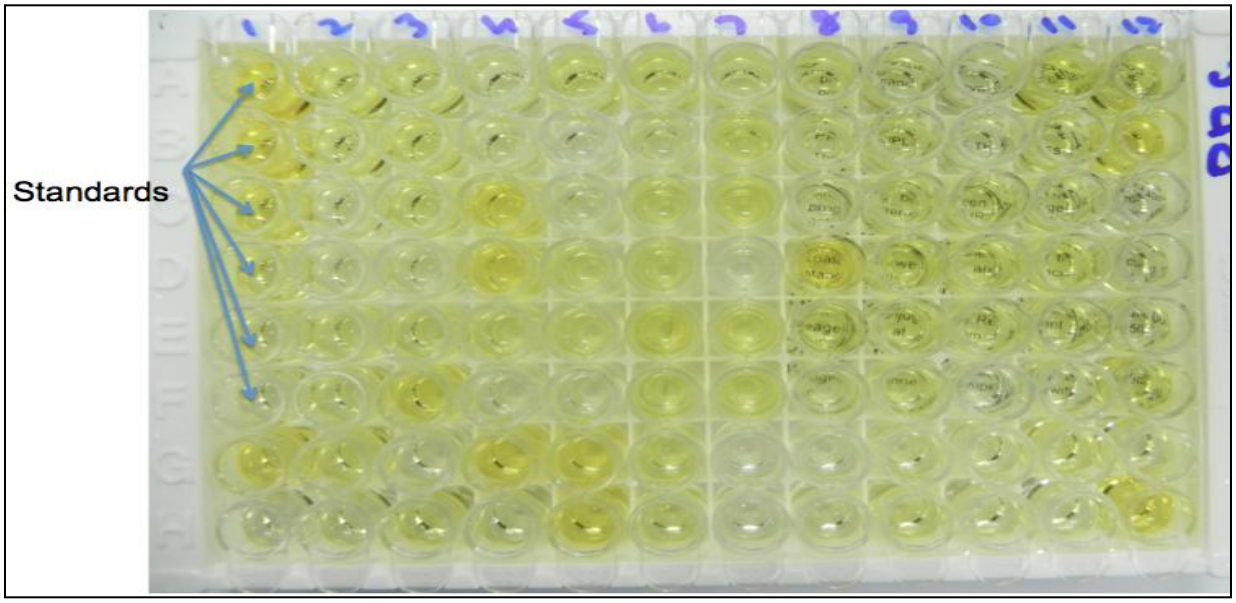


Fig.4.5: ELISA micro well plate for detection of Progesterone level.

4.3.1 Correlation between 1st trimester HEV positive and HEV negative patients

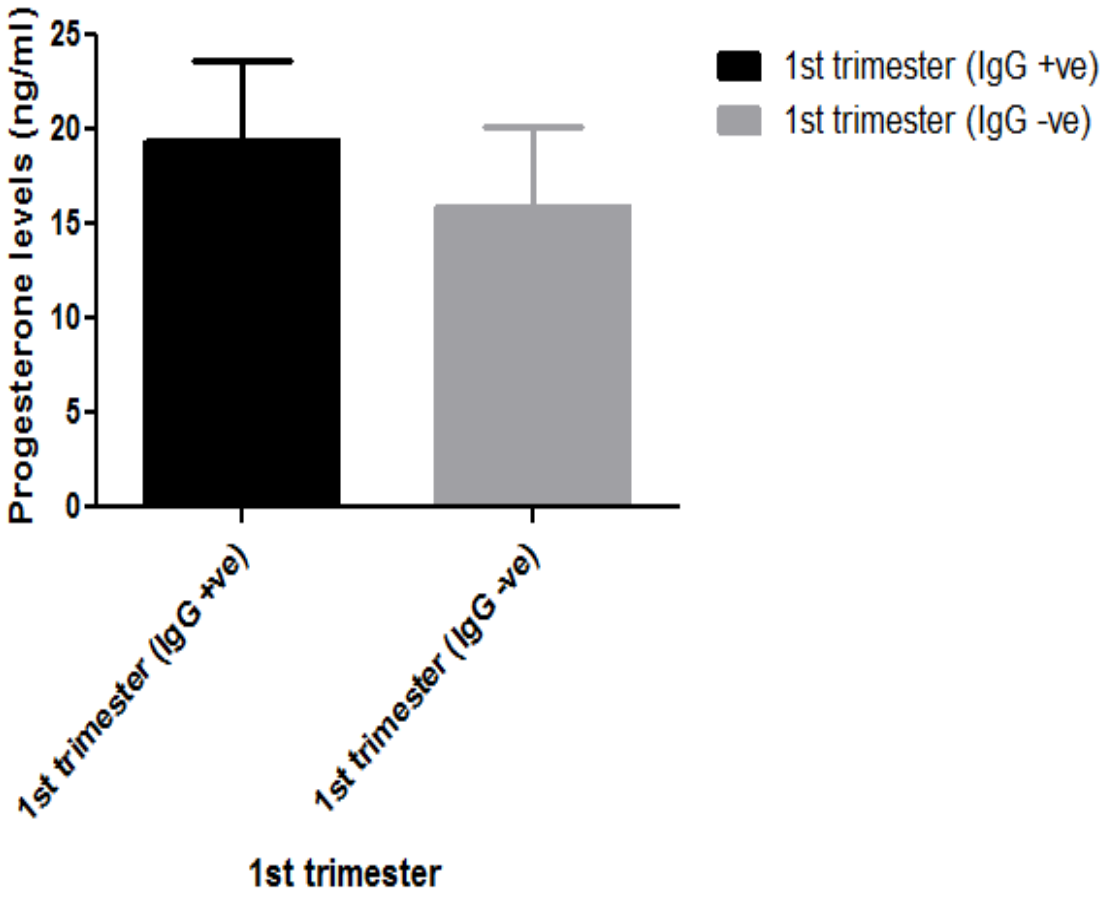


Fig. 4.6: Figure showing the levels of progesterone (ng/ml) in HEV-IgG +ve and IgG –ve patient population. Pearson correlation (one-tailed) test is applied and P value is <0.0001.

The trend observed in all the patients of 1st trimester shows higher progesterone levels in IgG +ve patients as compared to IgG –ve population (Fig.4.6). Total number of patients in each group is 15. Statistically significant results (P<0.0005%) were observed in this case.

4.3.2 Correlation between 2nd trimester HEV positive and HEV negative patients

In 2nd trimester IgG +ve patients are showing lower progesterone levels compared to HEV negative which shows progesterone levels in normal range (Fig.4.7). Total number of patients in each group is 13. The results are statistically significant (P<0.0005%).

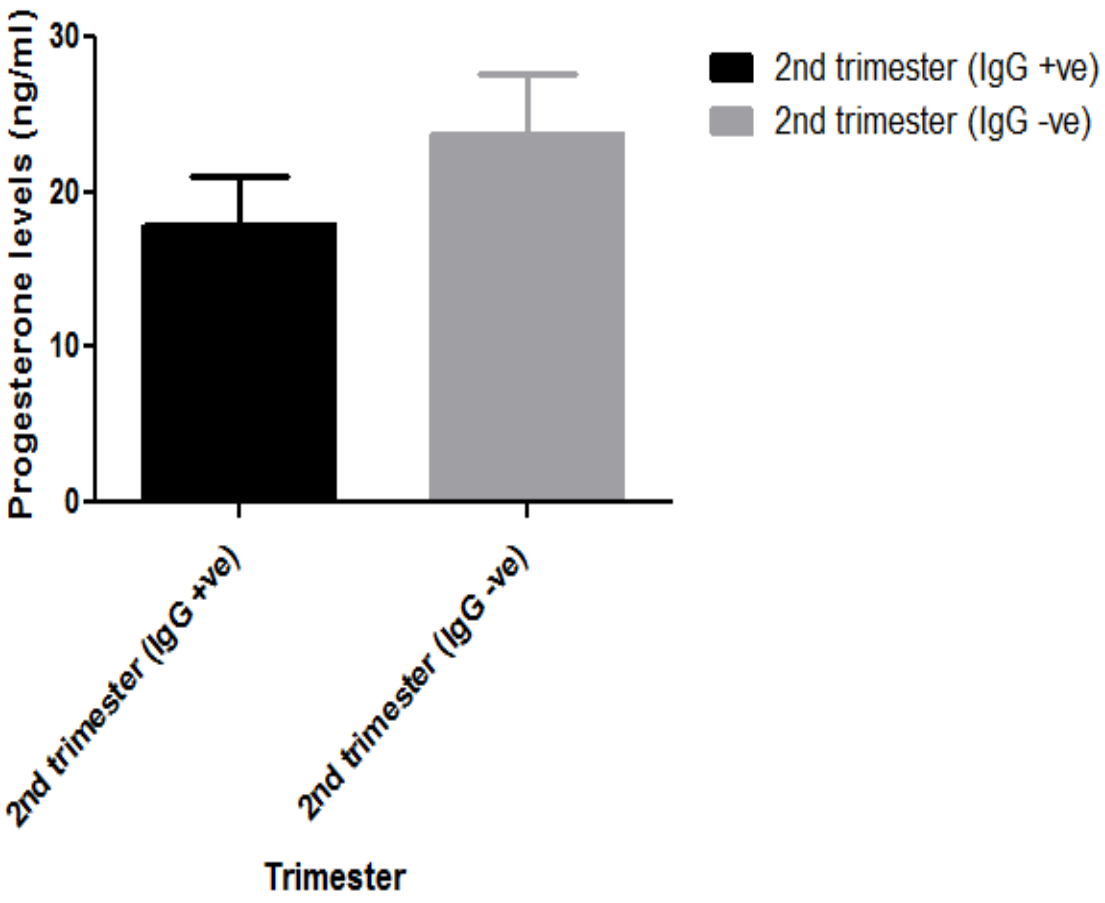


Fig.4.7: Levels of progesterone (ng/ml) in HEV IgG +ve and IgG –ve patient population. Pearson correlation (one-tailed) test is applied and P value is <0.0005.

4.3.3 Correlation between 3rd trimester HEV positive and HEV negative patients

In HEV positive pregnant women of third trimester the levels are too low as compared to the healthy population. Total number of patients in each group was 7. The results are statistically significant showing P<0.0001% (Fig.4.8).

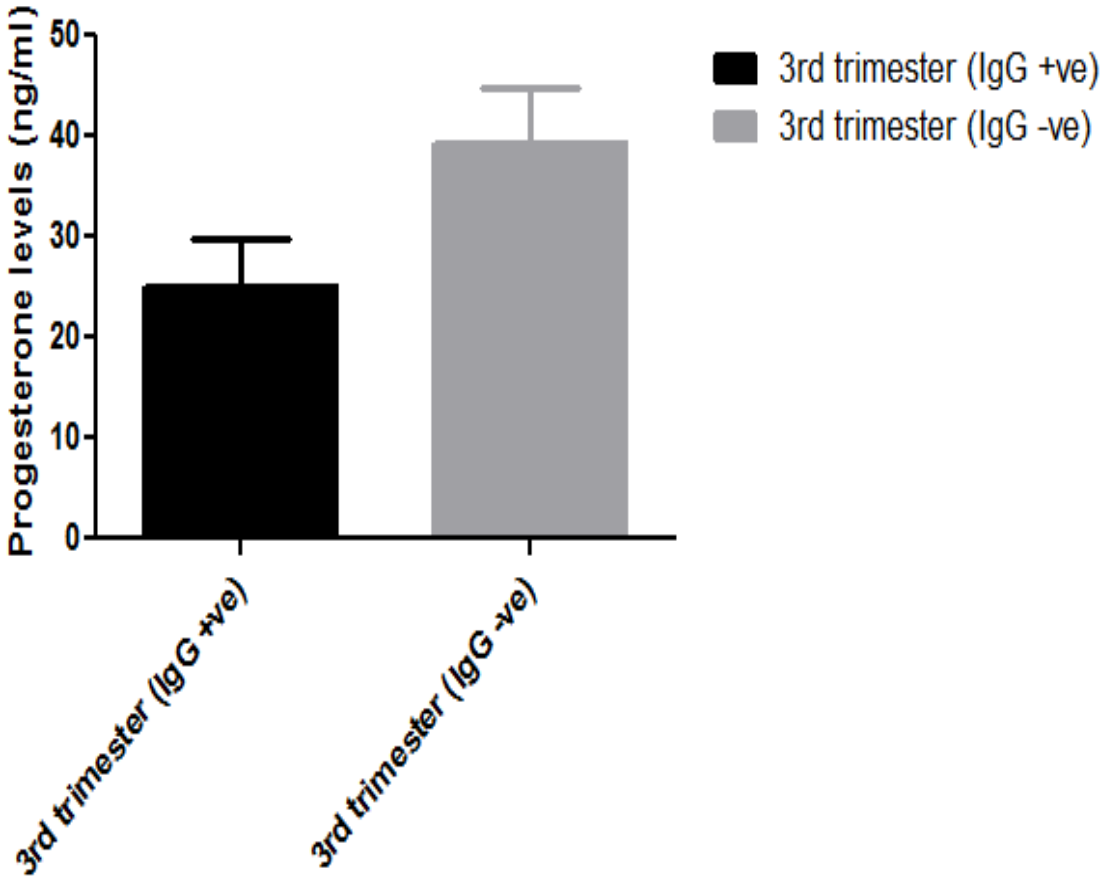


Fig.4.8: This graph shows the levels of progesterone on Y-axis in ng/ml and IgG +ve and IgG –ve patient population. Unpaired T-test (one-tailed) is applied and P value is <0.0001.

4.3.4 Progesterone levels in IgM and IgG positive patients

Comparison of progesterone levels of HEV IgM and HEV IgG indicated that in case of recent infection the levels are high as compared to past infection (IgG-+ve). Total number of patients in each group is 12. The results are statistically significant showing P<0.0002% value (Fig.4.9).

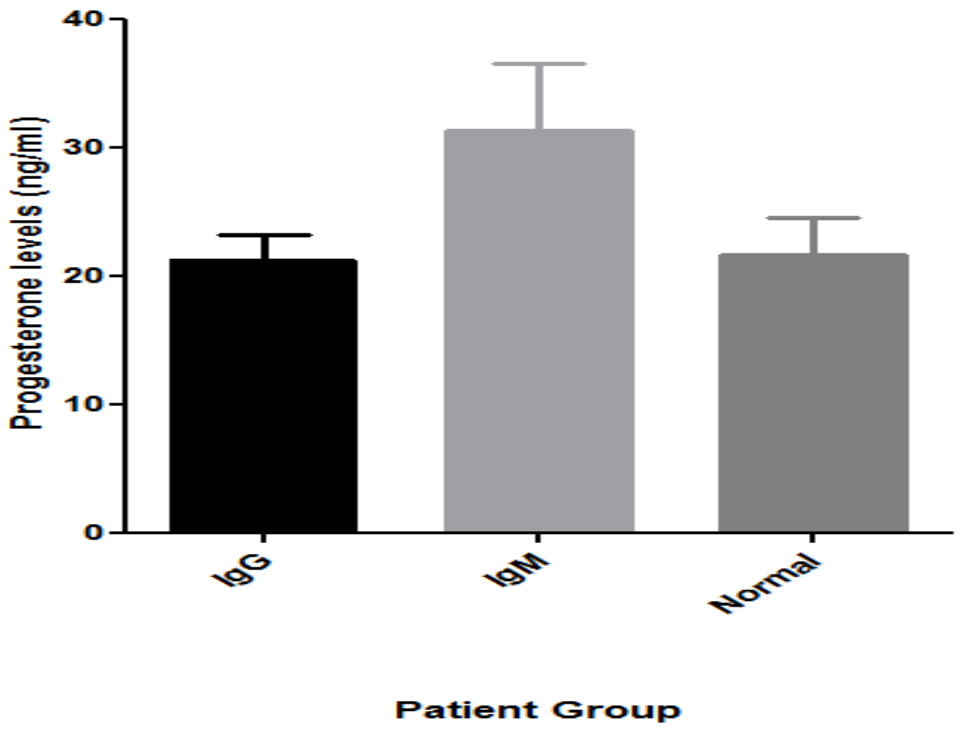


Fig.4.9: Figure showing levels of progesterone (ng/ml) in IgG +ve, IgM +ve patients and healthy population. One way ANOVA was applied and results are statistically significant P<0.0005%.

4.3.5 Progesterone levels in IgG positive patients and IgG negative patients in all trimesters

In HEV-IgG negative population the increase in hormonal levels are according to the normal increase during all three trimesters (Fig.4.11) as compared to IgG positive patients in which increased levels are detected in 1st trimester and lower levels in 2nd and 3rd trimester (Fig.4.10). Total number of patients in each group is 7. The results are statistically significant showing P<0.0005% in HEV-IgG positive population and P<0.0001% in normal population.

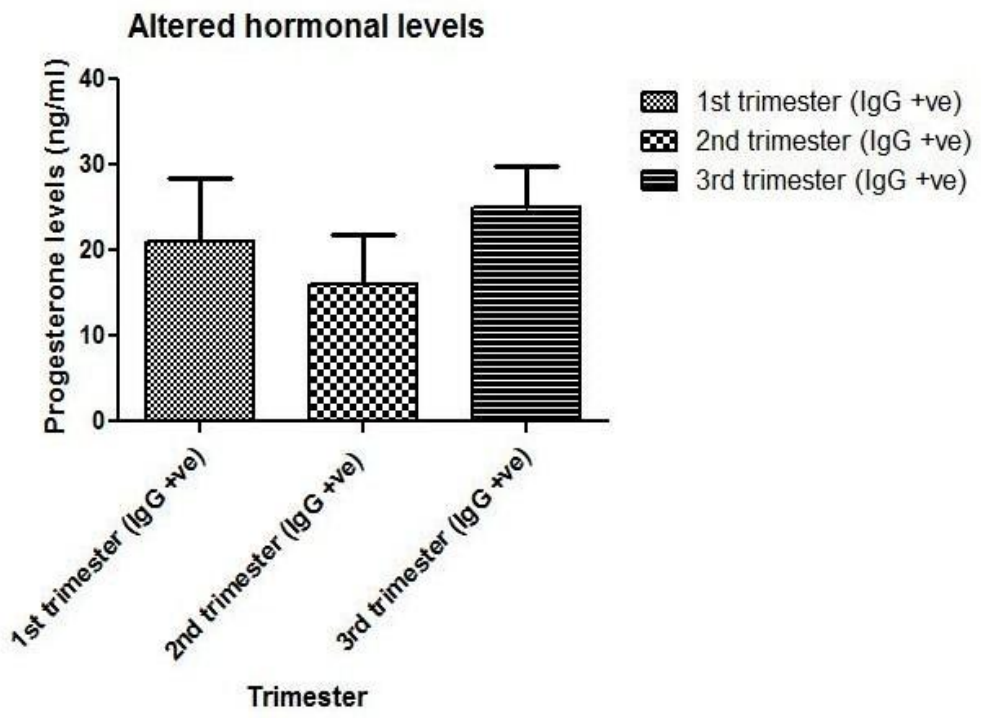


Fig.4.10: Figure showing altered levels of progesterone (ng/ml) in1st, 2nd and 3rd trimester in HEV-IgG patient population.

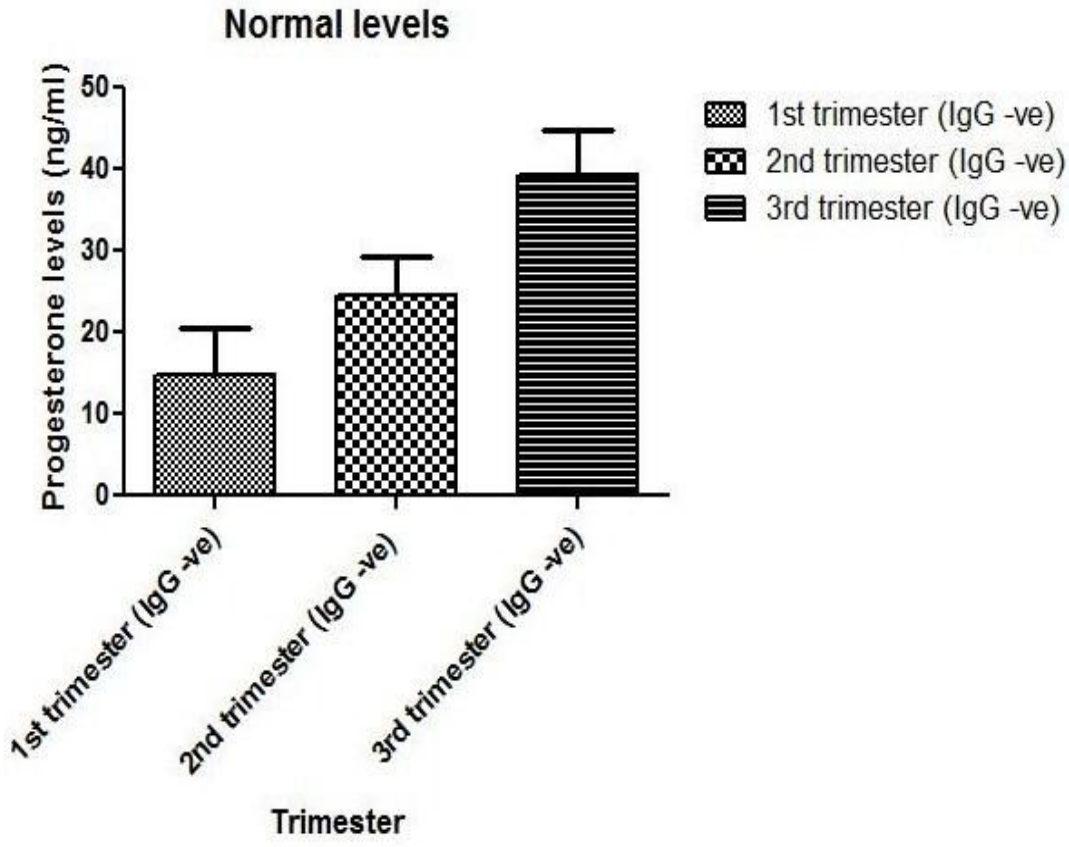


Fig.4.11: Figure showing normal levels of progesterone (ng/ml) in 1st, 2nd and 3rd trimester in HEV-IgG patient population.

Chapter 5

DISCUSSION

The present study shows the overall 60% prevalence of anti-HEV IgG in randomly collected pregnant women of Rawalpindi region as compared to 1.4% per cent in the general population of whole Pakistan (Fig.4.1). In 1994 in Karachi almost same prevalence i.e 61% was found in pregnant women who had acute viral hepatitis. The high prevalence rate indicates that HEV virus is endemic in Pakistan. According to a study, the presence of HEV in sewerage water of Rawalpindi region is very high i.e. 40% (Tahir *et al.,* 2010). HEV is enterically transmitted non-A, non-B hepatitis so the major waterborne outbreaks of acute hepatitis in developing countries, especially in the tropical and subtropical regions of the world are associated with fecal contamination (Tahir *et al.,* 2010). In Pakistan due to excessive rainy and monsoon seasons, poor sanitation system and close proximity of drinking water supply lines adjacent to or through the sewerage disposal system results in the high risk of the contamination of drinking water supplies (Tahir *et al.,* 2010).

South Asia is endemic for HEV and if we compare our results (Fig.4.1) to other countries like India, prevalence rate of HEV in pregnant women is 33.67% (Aggarwal, 1997; Yarbough, 1999). Although it is been reported from India that in sewerage water HEV prevalence is very high (41%) (Ippangunta *et al.,* 2007).We can conclude that their sanitary conditions in that particular region might be better than the

conditions of Rawalpindi region. Jaiswal and others (2001) reported a high rate of infection with HEV (57.5%) causing acute hepatitis in pregnant women in India.In Nepal prevalence of viral hepatitis during pregnancy is only 20.6 % (Shresta*et al*, 2009). A report from Bangladesh shows an HEV prevalence of 58.3% among 144 patients (Mamun *et al*., 2009).

In developed countries like spain very low prevelance of 3.6% was observed. The HEV genotypes and subtypes circulating in different countries can be responsible for the different geographical morbidity in pregnant women (Navaneethan et al., 2008). Also good hygienic measures and public health conditions in Spain may be the most important factors in maintaining the low prevalence rate of hepatitis E (Maria *et al.,* 2010).

Studies carried out in Iran, Africa and the Middle East have also found the incidence of hepatitis E significantly higher during pregnancy specially in third trimester (Balayan, 1997; Dilawari *et al.,* 1994; Arankelle *et al*., 1993; Tsega *et al*., 1993; Delons *et al*., 1962) as compared to Europe and the USA which are less endemic regions (Cahill, 1962; Hsia *et al*., 1952; Adams &Combes, 1965; Mishra & Sheef, 1992).

The number of HEV positive patients both with recent and past infection is found high in third trimester .It can be attributed that during third trimester the chances of getting HEV increases as studies to date on viral hepatitis in pregnant Asian women suggest that hepatitis E causes serious liver disease during late pregnancy in women (Krawczyuski, 1993; Khuroo *et al*., 1983).

Our results of the first trimester show that in case of IgG positive patients, lower levels were seen as compared to the normal range of progesterone in 1st trimester. In IgG negative patients progesterone level were mostly in the normal range, indicating that HEV might has a role in regulation of progesterone levels this could be due to viral factors involved in the down regulation of progesterone which ultimately leads to unsuccessful pregnancies and pre terms and abortions. The normal range of progesterone is critical for the establishment and the maintenance of safe pregnancy, both because of its endocrine and immunological effects (http://www.crinoneusa.com/patients/progesterone_role.html).

In some cases the levels were abnormally high which may be due to viral effect on PR receptors as it has been seen that PROGINS, a haplotype of progesterone receptor is associated with reduced amounts of gene transcript and a lesser response to progesterone (Lorenz *et al.*, 2001) and in case of HEV it was more prevalent in FHF (10/43, 23.26%) compared to AVH (14/100, 14%) and the healthy pregnant (2/50, 4%) group(Romano *et al.,* 2007). By measuring their levels the exact role of PR receptor and progesterone itself can be determined more easily but in our study only levels of progesterone are determined.

In case of second trimester, IgM positive patients showed abnormally decrease hormonal level as compared to normal level, which means in HEV presence the levels are reduced or they might be some other factors involved in the reduction of levels such as nutritional deficiency, superinfection, folate deficiency and low immunity etc. In three HEV-IgG positive samples, which were also positive for HCV showed

abnormally low level of progesterone, indicating that in case of super infection, the hormonal levels are greatly altered.

In some of the IgG positive patients of third trimester the hormonal levels were very low as compared to normal range of hormone levels. The reason behind this could be due to poor nutrition, climate, emotional or physical stress and emotional stress causing a progesterone deficiency as the patients were of low socioeconomic status women (Jukes, 1983) or the possible role of HEV infection in this.

HEV recent infection (IgM +ve) and previous infection (IgG +ve) showed different levels of hormones. In case of recent infection the level of progesterone were high in all the fifteen samples as compared to levels of previous infection of HEV. This can be compared to study in india (Nargis et al., 2007) in which FHF and AVH patient were taken and it was seen that although levels were high in AVH patients but in case of FHF patients the hormone levels were abnormally high.

Pregnancy is an immune compromised state in which hormonal as well as immunological changes occur. During pregnancy any infection can occur as the cell mediated immunity is reduced to maintain the antigenic fetus and underline mechanisms of the disease severity cannot be attributed to only a single factor. Further investigations like determination of levels of other pregnancy related hormones e.g. estrogen n B-HCG, similarly levels of IL-10 and IL-12 and their ratio in HEV positive patients (as in them it is Th-1 biased and results in high fetal mortality as compared to normal pregnancy in which it is Th-2 biased) can help to understand the possible mechanism. RT-PCR for the confirmation of presence of HEV in IgG positive as well

as in IgM positive patients is necessary in order to find out the exact role of HEV in alteration of progesterone levels. Liver function tests and ALT and AST levels must be determined in order to find out the liver damage and to find out the AVH or FHF in case of IgM positive patients. Further more local genotypic variations, environmental conditions and immunity could also contribute (Nishat et al. 2007).

From this study we can conclude that:

- Comparison of results of all the three trimesters of HEV IgG +ve and HEV IgG –ve patients showed low levels of hormone.
- The Socio-economic status appeared to be the risk factor for low progesterone levels in pregnant women.
- Poor nutritional conditions, climate, physical and emotional stress, immune status and prevalent genotype of this region might contribute to these low levels of progesterone as well as high prevalence of HEV in these patients.