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Does Hepatitis E Viral Load and Genotypes Influence the Final Outcome of Acute Liver Failure During Pregnancy?

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BACKGROUND: Hepatitis E is a major health problem in developing countries including India. The incidence and mortality rate in pregnant women with fulminant hepatic failure (FHF) due to hepatitis E virus (HEV) has been reported to be significantly higher, specifically in Asian women. Pregnancy is usually associated with an altered status of sex steroid hormones and immunity. Steroid hormones directly influence the replication through their effects on viral regulatory elements. Moreover, pregnant women in Asia generally suffer from folate deficiency, which is known to cause reduced immunocompetence leading to greater risk of multiple viral infections and higher viral load.

OBJECTIVES: To correlate and analyze the viral load and genotypes of HEV in acute liver failure with that of acute viral hepatitis among pregnant and nonpregnant women.

MATERIALS AND METHODS: A total of 100 FHF and 150 acute viral hepatitis (AVH) patients (50, 75 pregnant and 50, 75 nonpregnant, respectively), were included in the study. These cases were evaluated on the basis of history, clinical examination, liver function profile, and serological test of hepatitis A, B, C, and E using commercially available ELISA kits. Quantification of HEV RNA-positive samples was carried out.

RESULTS: Out of 100 FHF and 150 acute viral hepatitis (AVH) patients, 28 (56%) and 22 (29.3%) pregnant and 7 (14%) and 8 (16%) nonpregnant, respectively, were HEV RNA-positive. HEV viral load in FHF pregnant women was $5.87 \times 10^4 \pm 1.5 \times 10^5$ μL/mL as compared to AVH pregnant women 343.29 ± 216.44 μL/mL and FHF and AVH nonpregnant 199.2 ± 225.5 μL/mL and 13.83 ± 7.8 μL/mL, respectively. Sequencing data of all the positive samples of FHF and AVH pregnant and nonpregnant women showed genotype 1.

CONCLUSION: HEV viral load was found to be significantly higher ($P < 0.05$) in pregnant patients compared to the nonpregnant. Pregnancy appears to be a risk factor for viral replication. The viral copies of HEV in FHF pregnant women were comparatively higher when compared to AVH pregnant women, which may be related to the severity of the disease in these patients. We could detect only one genotype (genotype 1) in our study population. Thus in the absence of other genotypes in this population, the impact of genotype could not be adequately assessed in this study.

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INTRODUCTION

Hepatitis E is an important public health concern in many developing countries particularly in Asia, Africa, and Latin America. It is responsible for sporadic cases as well as large water-borne epidemic related to poor hygiene and sanitation. Recent studies documented that hepatitis E virus (HEV)-associated hepatitis also occurs among individuals in industrialized countries with no history of travel to areas endemic for HEV (1–3). HEV genome consists of a linear, single stranded, positive sense RNA of approximately 7.5 Kb containing 3'poly (A) tail and 3'noncoding (NC) regions, and contains three overlapping open reading frames (ORFs). All three coding frames are used to express different proteins (4). At present, the virus is classified into a separate family Hepeviridae and genus *Hepevirus*. Phylogenetic analyses with complete genomes indicate that HEV can be separated into at least four major groups.

HEV infection is the most common cause of viral hepatitis in India in the general population. Among pregnant female, the incidence is much higher particularly during second and third trimesters of pregnancy than in first trimester or in nonpregnant women or men (5). The mortality rate in case of pregnant women with fulminant hepatic failure has been

reported to be significantly higher specifically in Asian women, with maximum severity in third trimester (5–9). Studies carried out in Iran, Africa, and Middle East have also found the incidence of fulminant hepatitis to be significantly higher during pregnancy (6, 10, 11). The hepatitis E-induced fatality rate during pregnancy ranges from 11.4 to 21% (5, 10, 12, 13). Thus, hepatitis E during pregnancy is a serious health risk since pregnant women are more likely to be infected by the virus, more likely to develop FHF, and more likely to die with concomitant loss of the fetus. Reports indicate that abortion, death of the fetus in utero, premature deliver, or death of the baby soon after birth are seen in women with icteric hepatitis or with FHF induced by hepatitis E (5, 10). In contrast, reports from Europe and the United States have shown that the course and severity of viral hepatitis during pregnancy is no way different from that in nonpregnant women (14, 15).

Pregnancy in women is generally associated with an altered status of sex steroid hormones and immunity. These hormones play a pivotal role during the course of viral hepatitis (5, 7, 16) and other viral infections (17–19) during pregnancy. Steroid hormones directly influence the viral replication through their effects on viral regulatory elements (20). Malnutrition and reduced immune response have also been postulated to be responsible for the greater severity of viral hepatitis in pregnant women (5, 17). Furthermore, pregnant women in Asia generally suffer from folate deficiency, which is known to cause reduced immunocompetence leading to greater risk of multiple viral infections and higher viral load (21). Although almost all studies to date demonstrate high mortality rate associated with HEV infection during pregnancy, nothing is clear about the exact cause or mechanism(s) for its occurrence. It is also quite possible that HEV-infected pregnant FHF patients have higher viral load than the HEV-infected pregnant or nonpregnant AVH patients or nonpregnant FHF patients causing higher mortality in these patients. Keeping the above hypotheses in mind, we proposed to correlate and analyze the viral load and genotypes of HEV in acute liver failure with that of acute viral hepatitis among pregnant and nonpregnant women and to finally state whether hepatitis E viral load and genotypes influence the final outcome of acute liver failure during pregnancy.

MATERIALS AND METHOD

The present study included a total of 250 subjects. A total of 150 cases of acute viral hepatitis (75 with pregnancy and 75 without pregnancy) as control and 100 cases of fulminant hepatic failure (50 with pregnancy and 50 without pregnancy) constituted the patient group. The impact of HEV viral load and genotype were carried out on a cohort consisting solely of consecutive pregnant patients with HEV-related fulminant hepatic failure, whereas the control group consisted of consecutive pregnant patients with uncomplicated AVH. All the cases were recruited from the patients reporting to the outpatient services (OPD) and from those admitted in the medical, antenatal, and emergency wards of Lok Nayak Hospital, New Delhi.

The criteria for diagnosis of acute viral hepatitis were defined as those cases that had an acute self-limiting disease and a serum aspartate transaminase elevation of at least five-fold or jaundice or both (22). The fulminant hepatic failure was diagnosed when after a typical acute onset, patient become deeply jaundiced and went into hepatic encephalopathy within 8 wk of onset of disease without any past history of chronic liver disease (23). The pregnant women were examined for a palpable uterus, also all the patients were subjected to urine pregnancy test to confirm pregnancy. The duration of pregnancy was related to the date of their last menstrual period. A detailed history followed by a complete physical examination was carried out as per the standardized routine performa.

Serological tests were performed using commercially available ELISA kits according to the instructions given in the manufacturer's manual. The various serological tests performed in all the study samples were: IgM anti-HAV using HAV AB EIA test kit (Abbott Laboratories, Abbott Park, IL), HBsAg using Eliscaan micro ELISA strips (Ranbaxy Diagnostics, Gurgaon, India), IgM anti-HBc using anticoarse MB-96 (TMB) kit (General Biologicals, Hsin-Chu Taiwan), anti-HCV using Innotest HCV Ab III (Innogenetics N.V., Ghent, Belgium), IgM anti-HEV using IgM anti-HEV ELISA kit (Genelabs Diagnostics, Singapore).

RNA was extracted by Quiagen kit according to manufacturer's protocol. To determine the viral load of HEV-positive samples, real-time PCR was carried out using FAM system in the real-time PCR machine (Corbett Research, Sydney, Australia). The real-time PCR was carried out in a total reaction volume of 50 μL with 15 μL primers and probes mix (Reagent 1) each reaction, 5 μL Mg solution (Reagent 2) each reaction, and 30 μL extracted sample or standard. Calibration was done at 55°C FAM.

Nested RT-PCR was performed using ORF1 primers and the purified PCR product were subjected to cycle sequencing using 8 μL of dye terminator from a DNA sequencing kit (Big Dye Terminator V3.1 Cycle Sequencing Kit, Applied Biosystems, Foster City, CA) and 2 μL (20 pmole/μL) of specific primer (in a final reaction of 20 μL). For PGEM, 8 μL Reaction Mix, 4 μL PGEM DNA, and 8 μL of M13 Primer (in a final reaction of 20 μL) were subjected to PCR in a thermocycler (9600 Perkin Elmer Cetus, Norwalk, CT).

The samples were subjected to sequence analysis by the ABI Prism 310 Genetic Analyser (ABI, Foster City, CA). Every third sample (HEV RNA-positive) from the AVH group (pregnant and nonpregnant) and every second sample (HEV RNA positive) from the FHF group (pregnant and nonpregnant) were taken for viral load estimation as well as sequencing, following systemic random sampling. The sequences were compared with the HEV sequences in the Gen Bank using the CLUSTALW program. Phylogenetic analysis of the regions sequenced was performed by the maximum likelihood method using the PHYLIP

package and the graphical output was created with the TREEVIEW 1.5.

RESULT

The study comprised a total of 100 FHF patients (50 pregnant and 50 nonpregnant). The average age of the pregnant FHF patients was 24.8 ± 4.2 yr and that of nonpregnant women was 26.7 ± 4.9 yr. Mean period of pregnancy was 31.2 ± 4.5 wk.

Both serological tests as well as PCR methods were employed to detect HEV and other hepatitis viruses (HAV, HBV, and HCV) in all 100 patients. HEV infection was found to be significantly higher ($P < 0.001$) in pregnant FHF women (38/50, 76%) than that in nonpregnant FHF patients (15/50, 35%).

In the pregnant FHF group, 29 (58%) were serologically positive exclusively for HEV infection, 1 (2%) was positive for HAV infection alone, whereas none showed exclusively positive for HBV or HCV infection. Five (10%) were positive for HEV and HAV co-infection, 1 (2%) showed co-infection for HEV and HCV, 1 (2%) for HEV and HBcIgM, whereas 2 (4%) for HEV and HBsAg co-infection. One (2%) was positive for HBsAg alone showing carrier state. Ten (20%) patients showed negativity for all viral markers (HAV, HBV, HCV, and HEV). Out of total 38 HEV-positive samples, 28 (56%) were positive by RT-PCR for HEV RNA.

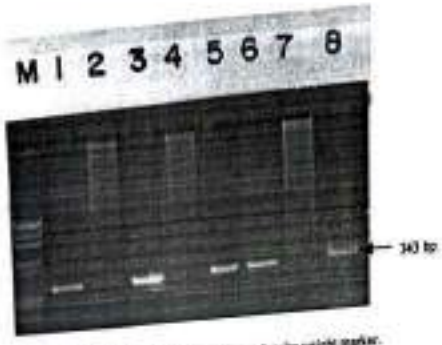
Among the 50 nonpregnant FHF patients, 11 (22%) were serologically positive for HEV infection alone, 8 (16%) were positive for HBV infection alone, 1 (2%) for HCV infection, whereas none showed positivity for HAV infection. Four (8%) showed co-infection of HEV along with HBsAg, whereas 2 (4%) were positive only for HBsAg showing carrier state and 2 (4%) were positive exclusively for HBcIgM. Twenty-two (44%) patients were negative for all the viral markers. Out of 11 serologically positive HEV infection alone, 7 (14%) were positive by RT-PCR as well.

Out of the 28 cases of HEV RNA-positive in FHF pregnant women, 20 expired (20/28; 71.4%). Six of the eight HEV RNA-positive patients who survived had preterm delivery (stillbirth) and the other two cases became HEV RNA-negative during the course of the disease and had term labor and their babies survived (Fig. 1).

In nonpregnant FHF group out of the 7 HEV RNA-positive case, 3 (42.8%) expired, and 4 patients survived HEV RNA-negative during the course of the disease. One patient also expired who was only HEV IgM-positive.

The viral load of the randomly selected 14 pregnant FHF women and 3 nonpregnant FHF women showed an average of $5.87 \times 10^6 \pm 1.5 \times 10^5$ IU/mL and 199.2 ± 225.5 IU/mL, respectively. The genotype of all 17 cases showed the same genotype, type I (Table 1).

In the control group of 150 AVH patients (75 pregnant and 75 nonpregnant), the average age of pregnant AVH patients was 24.28 ± 3.9 yr and that of the nonpregnant women was 29.57 ± 9.9 yr. Mean period of pregnancy in AVH group was 26.7 ± 7.1 wk.



Lane 1 : Φ X174 Hae III digestive DNA molecular weight marker.
Lane 2 : Positive Control
Lane 3 : Negative Control
Lanes 4-9 : Test Samples showing positivity for samples in lanes 4,6,7 & 8

Figure 1. Ethidium promide stained agarose gel electrophoresis of RT-PCR product of HEV-RNA showing desired amplicon of 343 bp.

Both serological tests as well as PCR methods were employed to detect HEV and other hepatitis viruses (HAV, HBV, and HCV). HEV infection was found to be slightly higher ($P < 0.06$) in pregnant AVH patients (36/75; 48%) than that in nonpregnant AVH patients (23/75; 30.7%).

Of the 75 pregnant AVH patients, 31 (41.3%) were serologically positive exclusively for HEV infection, 1 (1.33%) was positive for HAV infection, whereas none was found positive for HBV or HCV infection. Two (2.7%) had co-infection of HEV along with HAV, 3 (4%) had HEV and HBV (HBsAg + HBcIgM) infection. Thirty-eight (50.6%) cases were serologically negative for all the markers HAV, HBV, HCV, and HEV. Out of the total HEV-positive patients, 22 were positive by serology as well as by RT-PCR.

Forty percent of the patients did not have etiology of any known virus, which could not be detected by 3rd generation ELISA kit. However, when the samples were subjected to PCR for HBV and HCV, there was evidence of occult HBV and HCV infection in each one of the samples, 2/106 (1.9%).

Among the 75 nonpregnant patients, 21 (28%) were serologically positive exclusively for HEV infection, 1 (1.33%) was positive only for HAV, and 10 (13.3) were positive only for HBV infection, whereas none had HCV infection. Co-infection of HEV and HBV was found in 2 (2.7%), 6 (4%) were only HBsAg-positive indicating HBsAg carrier state,

Table 1. Viral Load Quantification and Genotype in AVH and FHF Patients With and Without Pregnancy

Group	Viral Load IU/mL	Genotype
AVH PREG (N = 7)	343.29 ± 216.44	1
AVH NON-PREG (N = 6)	13.83 ± 7.8*	1
FHF PREG (N = 14)	$5.87 \times 10^6 \pm 1.5 \times 10^5$	1
FHF NON-PREG (N = 3)	199.2 ± 225.5*	1

*$P < 0.05$ (significant). Statistical analysis by Mann-Whitney U test.

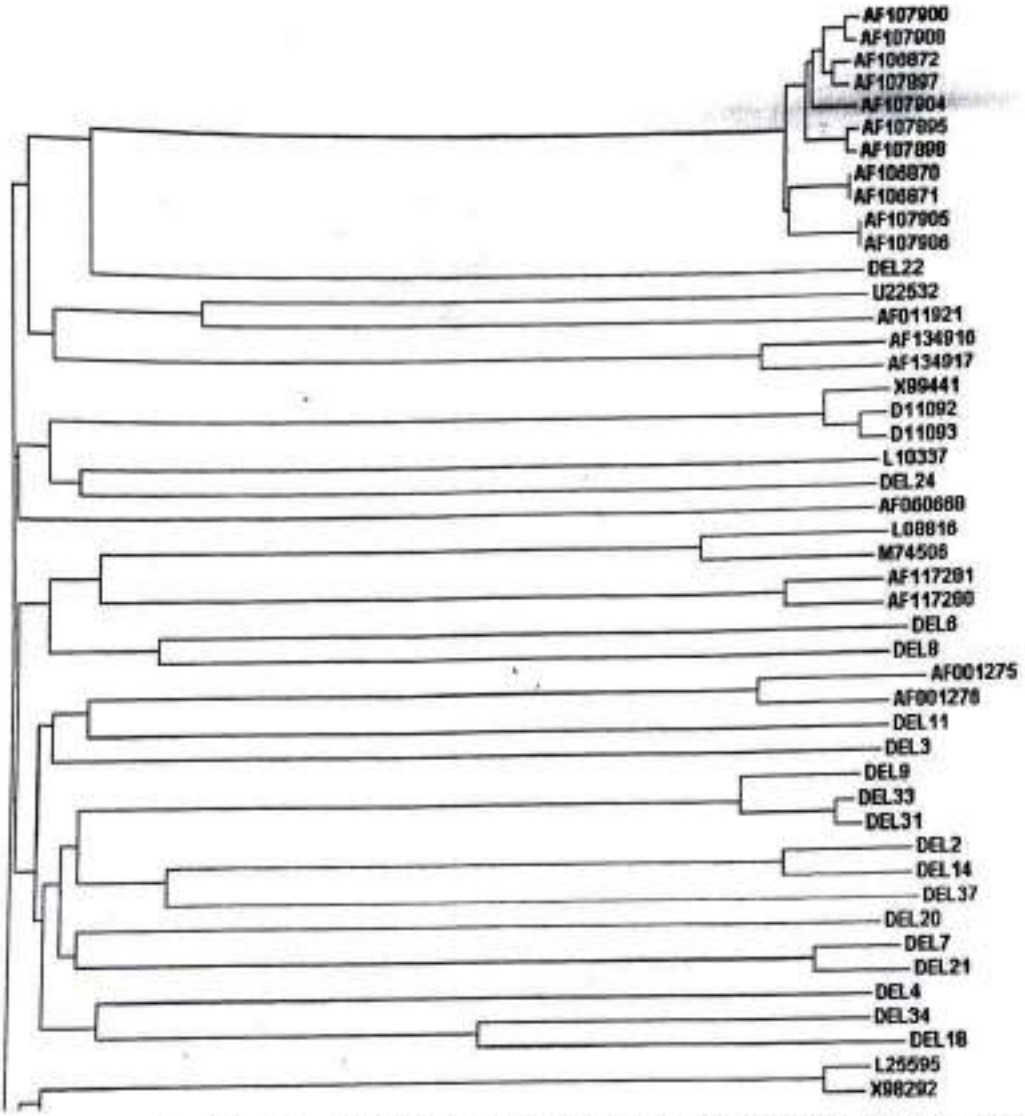


Figure 2. Phylogenetic tree for isolates of hepatitis E virus based on the sequencing of the ORFI region of the HEV genome. All of the isolates (DEL 1 to DEL 30) belonged to genotype 1 (Gene Bank accession number dq318819 to dq318848, respectively).

whereas 35 (46.66%) were serologically negative for all markers. Out of total 23 HEV-positive patients, 12 were positive by serology as well as by RT-PCR and 11 were positive by serology alone.

Out of the 22 HEV RNA-positive cases, 12 (54.6%) recovered by the time of delivery and delivered at term. But the other 10 (45.4%) patients who were in their third trimester showed preterm labor. There was no maternal mortality. Four babies (18.1%) were stillborn.

The viral load of randomly selected 7 AVH patients and 6 nonpregnant showed 343.29 ± 216.44 IU/mL and 13.83 ± 7.8 IU/mL, respectively. The genotype of all the 13 samples was genotype 1 (Fig. 2). Log transformation was done to normalize the data.

DISCUSSION

Viral hepatitis constitutes a major public health problem in developing countries including India. It is responsible for sporadic cases as well as large water-borne epidemics related to poor hygiene and sanitation (1, 2). The majority of the cases are due to enterically transmitted HEV. In the present study, we have detected HEV infection in 45.2% (113/250) of total patients and 53% (53/100) of patients in the FHF group. Our observations are similar to those of other authors (21, 24) and support the hypothesis that HEV is the major etiological agent of sporadic FHF in India. The most interesting finding is the very high rate (38/50, 76%) of HEV infection in the pregnant FHF patients when compared with those of nonpregnant FHF

patients (17/50, 34%; $P < 0.05$). These findings are consistent with several other reports from India, Africa, and Middle East countries (6, 21, 25). In contrast, this was not the situation reported from western countries (26, 27).

It is known that viremia in acute HEV infection lasts for a short period and disappears soon after the onset of symptoms (19). Thus in acute cases, if the samples are taken at a later date after the development of symptoms, only antibodies might be detected, but RNA PCR may become negative. In our study, out of 113 patients who were positive for HEV infection by serology, 69 showed positivity by RT-PCR also. The samples from 43 patients who were negative for HEV by RT-PCR may have been collected after the development of symptoms since these patients were presented late to the hospital. In India, most of the patients report to the hospital much later after the onset of the symptoms especially in a mild form of disease. It is also quite likely that those pregnant women are brought to the hospital quite early compared to nonpregnants and that may be the cause of low levels viral load in the latter group. Interestingly, in 106 (106/205; 42.4%) cases, no known viral agent could be detected by ELISA. However, one sample showed positivity for HBV DNA and one for HCV RNA by PCR (2/106; 1.9%). It is possible that the disease in these 104 cases may have been caused by some yet uncharacterized hepatotropic viruses or HSV II, which was not tested by us.

Another interesting finding was the significantly higher mortality rate in HEV-infected pregnant women (25/38; 65.8%) than in HEV-infected nonpregnant ones (4/17; 23.5%) ($P < 0.0\%$). Similar high mortality rate (55.5%) in HEV-positive pregnant women has been reported earlier (7).

Studies so far on viral hepatitis in pregnant Asian women clearly suggest that hepatitis E causes serious liver disease during late pregnancy in women (6–10, 20, 25). However, the exact mechanism(s) and the effect of the virus specifically during the late stage of pregnancy only in Asian women are not clearly understood. It is known that the level of sex steroid hormones, particularly progesterone and estrogens that increase during the latter half of pregnancy, can directly influence viral replication through their effects on viral regulatory elements (9, 28). It is possible that these hormones may enhance viral replication/expression leading to severity of disease in pregnancy and subsequent death. Till now there is no report on the influence of viral load in FHF due to HEV in pregnant women. Our results show that the viral load in pregnant AVH or FHF patients was higher than their nonpregnant counterparts (343.29 ± 216.44 IU/mL as against 13.83 ± 7.8 IU/mL for AVH patients; $P < 0.05$ and $5.87 \times 10^4 \pm 1.5 \times 10^5$ IU/mL vs 199.2 ± 225.5 IU/mL for FHF patients; $P < 0.05$) being highest in pregnant FHF patients. However, lack of correlation between viral load and disease severity had been reported by many authors in HCV (29–31). On the other hand, high viral loads have been claimed to influence the therapeutic outcome in HCV (32, 33). From the present study, we can say that the viral load was directly proportional to the severity of the disease (in terms of mortality) in cases of HEV infection. Hence according to our study pregnancy appears to be risk factor for viral replication, which would be the reason for higher morbidity and mortality in pregnant patients, especially FHF.

Although HEV strains belong to a single serotype, they play considerable genetic diversity according to the time and place of isolation (34). Initial studies of the prototype Burmese strain of HEV (26, 35) and the highly divergent Mexican strain (36) demonstrated wide genetic variation, and the recent reports of many equally divergent HEV strains provide a basis for classification of strains into different genotypes with around 75% nucleotide homology between genotypes. The majority of HEV infections in developing countries are caused by genotype 1 and only isolated cases of infection with HEV of genotype 3 or 4 have been described in industrialized nations (3).

Although the prevalence of HEV infection has been reported in many developing and industrialized countries, the genetic changes that occur in HEV strains in a community over time are poorly understood. In contrast to several other RNA viruses, HEV does not seem to mutate rapidly. Our study involved 30 HEV isolates that were sequenced in the ORFI region of the HEV genome. All of the isolates belonged to genotype 1 which is the most prevalent genotype present in India. The sequence analysis of the isolates from these patients of acute viral hepatitis showed 95–99% homology with the Indian strain (37–39).

The strains of HEV isolated from the FHF cases (pregnant and nonpregnant) were highly identical to the strains isolated from the AVH cases. They showed similar comparison analysis when compared to the different isolates. The isolate from both the groups showed a 94% similarity to the isolate reported by Donatti *et al.* (40) (D10330).

We could detect only one genotype (genotype 1) in our study population. Thus in the absence of other genotypes in this population, the impact of genotype could not be adequately assessed in this study. In short, our study presents that pregnancy appears to be a risk factor for viral replication, which could be the reason for higher morbidity and mortality in these patients.

STUDY HIGHLIGHTS

What Is Current Knowledge

- Hepatitis E virus (HEV) genotypes and subtype analysis lead to better understanding of the epidemiological spread of the disease.

What Is New Here

- This study highlights the association of HEV genotype in patients of acute liver failure during pregnancy.
- Higher HEV viral load in acute liver failure during pregnancy leads to severe liver disease implicating pregnancy as a risk factor for viral replication.

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CONFLICT OF INTEREST

Guarantor of the article: Premashis Kar, M.D., D.M.

Specific author contributions: Premashis Kar raised the original idea and design of the study and prepared the first draft of the manuscript. Nishat Jilani participated in its design and carried out the various tests under the supervision of Bhudev C. Das and Syed A. Hussain. S.T. Pasha, R. Anand, and A. Rai carried out the HEV viral load and genotyping data. Bhudev C. Das revised the draft manuscript. All authors have read and approved the manuscript.

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Research Article

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High viral load and deregulation of the progesterone receptor signaling pathway: Association with Hepatitis E-related poor pregnancy outcome

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Background & Aims: Hepatitis E virus (HEV) infection is associated with high maternal and fetal mortalities. A prospective study was undertaken to evaluate the role of viral and host factors in HEV related pregnancy outcomes.

Methods: The study included HEV infected pregnancy cases; acute viral hepatitis (AVH), n = 100 and fulminant hepatic failure (FHF), n = 43, and healthy pregnancy cases, n = 50. HEV genotypes and viremia were studied by nucleotide sequencing and real time PCR, respectively. Progesterone receptor (PR) gene mutations (PROGINS) were studied by PCR. PR expression at the mRNA and protein levels in the placenta were studied by semi-quantitative RT-PCR and immunohistochemistry, respectively. Progesterone induced blocking factor (PIBF) expression was studied by RT-PCR in blood. Serum interleukin-10 (IL-10) and interleukin-12 (IL-12) levels were assayed by ELISA.

Results: HEV viral load was significantly higher in FHF than AVH ($p < 0.001$) and in cases with fetal mortality in AVH ($p = 0.001$) and FHF ($p = 0.018$). PROGINS were predominant in FHF compared to AVH ($p = 0.26$) and showed reduced mRNA and protein expression. The risk of fetal mortality in AVH was two times higher (OR, 2.190; CI, 0.303–15.85) and maternal and fetal mortalities in FHF were 4-fold (OR, 4.0; CI, 0.363–44.113) increased in PROGINS carriers. PR and PIBF expression was lower in AVH and even lower in FHF compared to healthy controls. The higher IL-12/IL-10 ratio observed in FHF compared to other groups correlated with fetal mortality in AVH and FHF ($p < 0.001$).

Conclusions: In conclusion, reduced expression of PR and PIBF, a higher IL-12/IL-10 ratio, and a high viral load results in poor pregnancy outcome in Hepatitis E.

Keywords: Hepatitis E virus; Pregnancy; Viral load; Progesterone receptor; PIBF; Cytokines.
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Introduction

Hepatitis E virus (HEV), a member of the genus *Hepevirus* in the family Hepeviridae [1], is a major cause of enterically transmitted non-A and non-B hepatitis in many developing countries with large epidemics already reported in Asia, Africa, and Latin America [2]. Most importantly, high mortality rates have been reported for HEV-related infection during pregnancy [3,4]. The disease may range in severity from sub-clinical to acute viral hepatitis (AVH) or fulminant hepatic failure (FHF), the most severe form of acute hepatitis, during pregnancy where the death rate approaches 15–20%. Common complications during pregnancy may include death of the mother and fetus, abortion, premature delivery, or death of a live-born baby soon after birth [5]. FHF during pregnancy is associated with higher viral load of HEV compared to AVH [6].

Incidence of FHF with high mortality in Hepatitis E is high in pregnancy when the steroid hormone level is altered [7]. Progesterone is critical for the establishment and the maintenance of pregnancy, both for its endocrine and immunological effects. The outcome of the immunological recognition of pregnancy is an upregulation 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 [8].

Immunomodulatory effects of progesterone are mediated by PIBF. Lymphocytes of women with threatened abortion fail to produce this factor. PIBF stabilizes the mRNA of interleukin-10 (IL-10) [9]. In mice PIBF is anti-abortive, which is partly due to induction of a Th2 biased cytokine production and blocking of natural killer cell (NK) activity [9,10].

Neutralization of the biological effects of PIBF in vivo results in a Th1 shift [11]. Normal human pregnancy is associated with a Th2 biased peripheral cytokine profile. It has been previously reported that there are significantly higher levels of the type 1 and lower levels of the type 2 cytokines in supernatants of activated peripheral lymphocytes from women undergoing preterm delivery [12], than in those from healthy pregnant women.

Furthermore work from research groups have shown that abortion-prone women who proceeded to have successful pregnancy have more Th2 bias than abortion prone women who aborted [13]. Moreover, PIBF expression shows an inverse correlation with NK activity and a positive relationship with the outcome of pregnancy [14,15].

Worse maternal and fetal outcomes of Hepatitis E compared to other types of viral hepatitis have been observed in pregnant women with HEV infection [16]. However, the mechanism of severe liver injury in pregnant women leading to maternal and fetal deaths with Hepatitis E still remains a mystery. The presently available research data and literature support the need for a study to elucidate the role of the PR and PIBF mediated pathway in the maintenance of pregnancy under the influence of HEV infection. We hypothesize that the normal outcome of pregnancy mediated by progesterone receptors and associated proteins are affected by HEV infection via the modulation of the host immune system, which in turn leads to pregnancy-related mortalities in patients. The present study was undertaken with the following objectives: [1] screen the important mutation (PROGINS) and differential expression of the progesterone receptor (PR) gene in Hepatitis E infected pregnant women with AVH and FHF, [2] differential expression of PIBF in HEV infected pregnant women with AVH and FHF, [3] influence of the HEV viral load in the severity of the disease, [4] affect of the above three on the alteration of the cytokine profiles of the patients (Th1/Th2 ratio in terms of IL-12/IL-10) and disease outcomes.

Materials and methods

Patient enrollment

Consecutive cases (n = 143) of Hepatitis E related AVH (n = 100) and fulminant hepatic failure (n = 43) with pregnancy admitted in the wards of Lok Nayak Jai Prakash Hospital, New Delhi were enrolled in the study with informed consent. Blood was collected in heparinized (2 ml) and plain vials (4 ml). Whole blood and serum (4 ml) were stored at 4 and −70 °C, respectively. The patients were followed up till delivery or disease outcome. Placental tissue samples (1 cm^3) were collected from a representative number of AVH (n = 35) and FHF (n = 14) cases during delivery and stored in RNA later (Sigma) at −70 °C. Healthy pregnancy cases (n = 50) were also enrolled as a control group and blood and placental sections were also collected with informed consent. Healthy, age matched non-pregnant volunteer's (n = 30) were also enrolled in the study as comparative controls for the Th1/Th2 analysis. The study was conducted with the permission of the institutional ethical committee of MAMC, New Delhi, and followed all the guidelines recommended by the Indian Council of Medical Research.

Diagnosis

Viral markers for Hepatitis A–E (IgM anti-HAV; IgM anti-HEV, HBsAg and anti-HCV) were determined by ELISA with commercial kits (RADIM, Italy). Cases serologically positive for IgM anti-HEV only served as the selection criteria for further analysis. HEV positive cases were clinically correlated and grouped either as AVH or FHF cases. The criteria for diagnosis of acute viral hepatitis were defined as those cases that had an acute self-limiting disease and a serum aspartate transaminase elevation of at least fivefold or jaundice or both [17]. The fulminant hepatic failure was diagnosed when after a typical acute onset, the patient became deeply jaundiced and went into hepatic encephalopathy within 8 weeks of onset of the disease without any past history of chronic liver disease [18].

HEV factor analysis

HEV RNA was isolated from the serum of IgM anti-HEV positive cases using a standard viral RNA extraction kit (Qiagen) according to the manufacturer's protocol. Isolated viral RNAs were subject to RT-PCR analysis using primers [19,20] based on the ORF1 region of HEV. The amplified products were purified by a gel extraction kit (Qiagen) and sequenced. HEV genotyping was performed by sequencing analysis of the amplicon and comparing with the standard HEV genotype sequences from GenBank using editable softwares. HEV viremia (viral load) in the respective HEV positive AVH and FHF samples were evaluated by real time PCR using the FAM system in the real-time PCR machine (Rotor-Gene RG-3000, Corbett Research) using Genie Sen's HEV Real Time PCR Reagents kit, India.

Host factor analysis

Genomic DNA extraction was performed by a standard phenol–chloroform method using 200 µl of whole blood from pregnant patients with a different severity of HEV infection; as well as healthy controls. Total RNA was extracted from whole blood and placental tissues of AVH, FHF, and healthy pregnancy cases by the standard Trizol method (Qiazol). Five micrograms of the extracted quantified RNA was converted to cDNA by reverse transcription at 37 °C using a random hexamer as a primer and stored at −20 °C.

Mutation analysis of the progesterone receptor (PR) gene or PROGINS detection

PROGINS, a haplotype of the progesterone receptor consisting of a 320-bp insertion in intron G together with point mutations in exons 4 and 5 is associated with reduced amounts of gene transcript and lower response to progesterone. Since these three mutations always occur together and are in complete linkage disequilibrium [21], we detected only the 320 bp insertion in intron G to identify PROGINS. PCR was carried out using the primers designed by Agoulnik et al. [21] to amplify intron G of the PR gene. A mutation in intron G was detected by the presence of 494 bp fragments while the presence of only 174 bp fragments denoted the wild-type allele.

PR and PIBF expression analysis

Expression Studies of Progesterone Receptor (PR) and progesterone induced blocking factor (PIBF) were performed by semiquantitative RT-PCR analysis using the cDNA prepared from total RNA extracted from placental tissue and whole blood, respectively, as template. Beta actin (β-actin) was used as an internal control. Differential expression analysis of PR at the protein level was performed on the 5 µm sections of formalin fixed paraffin embedded placental tissue of AVH, FHF, and healthy pregnancy cases. Immunohistochemistry was performed following the regular ABC method using PR antibody (Sc-539, Santacruz Biotechnologies) and LSAB plus detection kit (DAKO). The expression profile was analyzed and graded as high (+++), moderate (++), faint (+), and no expression (−) by senior pathologists from the Department of Pathology, G.B. Pant Hospital, New Delhi. The pathologists were double blinded for the analysis.

Th1/Th2 cytokine profile analysis

Several studies investigating the ratio of Th2 to Th1 in terms of circulatory levels of serum cytokines reveal a Th-2 bias in normal pregnancy and a Th-1 bias in cases of recurrent miscarriages. IL-12 (Th1 response) and IL-10 (Th2 response) expression analyses were studied in HEV related pregnancy cases compared to healthy pregnant and non-pregnant control cases by ELISA method using standard kits (RayBio ELISA kit, human IL-10, and human IL-12p70).

Statistical analysis

The differences in viral and host factors between the AVH and FHF groups were analyzed using χ^2 and Mann–Whitney non-parametric tests. The odds ratios (ORs) and confidence intervals (CIs) at 95% confidence levels were obtained using unconditional logistic regression analysis. All analysis was performed by the statistical package for social science, version 10 (SPSS, Chicago, IL).

Results

Overall 143 HEV related pregnancy cases (AVH 100, FHF 43) along with 50 healthy pregnant and 30 non-pregnant control cases were studied. The mean age of AVH patients was 24 ± 3.52 years, FHF patients 25 ± 3.91 years, healthy pregnant

Table 1. Details of HEV related pregnancy cases (n = 143) enrolled in the study.

Parameters	Pregnancy cases			
	Healthy	AVH	FHF	
Number	50	100	43	
ALT [Mean ± SD]	31.6 ± 18.24	471.20 ± 669.85	808.31 ± 841.91	
AST [Mean ± SD]	28.83 ± 13.50	557.83 ± 721.03	853.18 ± 851.54	
IUD [N, % age]	0, 0	25, 25%	26*, 86.67;	p <0.001
Maternal mortality [N, % age]	0, 0	0, 0%	25, 58.14;	p <0.001
Preterm delivery [N, % age]	0, 0	42, 42%	22*, 73.33;	p <0.05

p value indicated for FHF compared to AVH and control.
* Considered only for the 30 patients who delivered.

controls 26 ± 5.42 years, and 21 ± 3.2 years for non-pregnant volunteers. Among the HEV infected AVH patients, 91 (91%) were at the 3rd, 6 (6%) were at the 2nd, and 3 (3%) were at the 1st trimester of their pregnancy. Among the FHF patients, 35 (81.4%), 4 (9.3%), and 4 (9.3%) were at their 3rd, 2nd, and 1st trimesters of pregnancy, respectively. Maternal mortality was significantly higher (p <0.001) in FHF (25/43, 58.14%) (13 patients died undelivered, 12 died after delivery) while in AVH all patients survived. Intrauterine death (IUD) and still birth of the baby were found in 26/30 (86.67%) in delivered cases of FHF compared to 25/100 (25%) in AVH, which was highly significant (p <0.001). Patients of FHF mostly had preterm delivery (22/30, 73.33%), compared to AVH (42/100, 42%); p <0.05. In the healthy pregnant group, there was no maternal or fetal mortality and all the patients had term labor. The details of the cases are tabulated in Table 1.

HEV factors (genotype and viral load) in pregnancy outcome

HEV genotype

HEV RNA was detectable in 62/100 (62%) of AVH cases and 29/43 (67.44%) of FHF cases. The amplified products were sequenced and compared with standard GenBank sequences for genotypes 1–4 using Clustal W analysis software and phylogenetic tree analysis. No other genotype except genotype 1 could be detected in the cases.

HEV viral load

The mean HEV viral load in 29 HEV RNA positive FHF patients was found to be higher, 139,994.0 ± 103,104.17 copies/ml compared to 62 HEV RNA positive AVH patients, 768.92 ± 1105.40 copies/ml, which was statistically significant (p = 0.046). Higher viral load was observed in patients with fetal death compared to patients without fetal death in both AVH (p = 0.001) and FHF (p = 0.018).

PR gene mutation (PROGINS) analysis

PROGINS haplotype results in reduced response to progesterone hormone which is a prerequisite for PIBF formation. Detection of PROGINS haplotype was done by PCR method followed by agarose gel electrophoresis (Fig. 1). PROGINS distribution was higher in FHF cases (10/43, 23.26%) compared to AVH (14/100, 14%) but their prevalence between the groups was found to be statistically insignificant (OR, 1.86; CI, (0.69–502); p = 0.26). PROGINS was

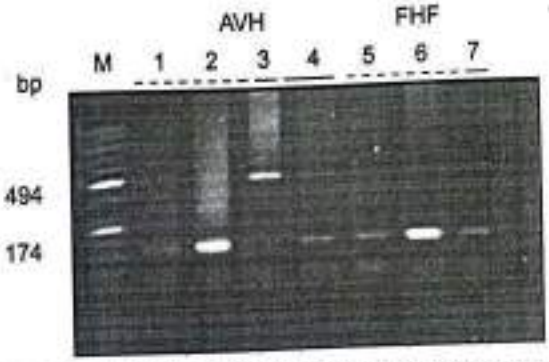

Fig. 1. Detection of PROGINS. M, 50 bp marker; lane 1, 2, and 5, homozygous wild-type (174 bp); lane 3, homozygous PROGINS (494 bp); lane 4, 6, and 7, heterozygous PROGINS (174, 494 bp).

detectable in 4% (2/50) of the healthy pregnancy cases. The frequency of PROGINS was non-significantly higher in AVH (OR, 1.872; CI, (0.58–6.16); p = 0.288) but significantly higher in FHF (OR, 3.485; CI, (1.00–12.075); p = 0.041) compared to healthy pregnant women.

PR and PIBF expression analysis

PR expression analysis at the mRNA level in different study groups was done by semiquantitative RT-PCR analysis using cDNA specific primers for PR and β-actin (as an internal control). The mRNA based expression analysis for progesterone receptor showed downregulated expression in HEV related FHF (p <0.001) and AVH (p = 0.016) pregnancy cases compared to healthy pregnant cases. Moreover, PR mRNA expression was significantly higher (p <0.001) in AVH than FHF cases. PROGINS carriers showed lower expression of PR at the mRNA level in FHF cases compared to non-PROGINS (wild-type) in FHF, as well as PROGINS carriers in AVH cases (Fig. 2).

The results of the mRNA level expression analysis for PR were correlated with PR protein expression analysis by IHC on placental tissue. The IHC results were comparable and consistent with the mRNA expression results. There was noticeably (+++) higher expression of PR in healthy placental cases compared to AVH or FHF cases. PROGINS carrier cases showed downregulated PR

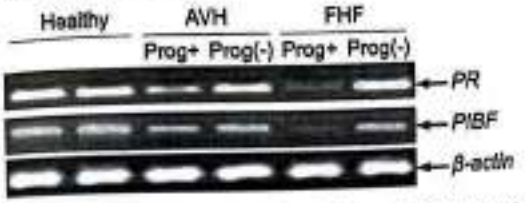

Fig. 2. Semiquantitative RT-PCR analysis showing down-regulation of PR and PIBF expression in AVH and FHF cases compared to healthy pregnant cases. Also, PROGINS carriers (Prog+) in AVH and FHF cases showed lower expression of PR and PIBF compared to non-PROGINS (Prog−). β-Actin was internal control.

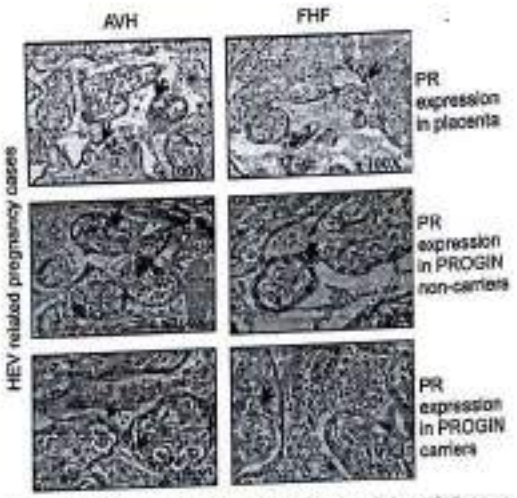

Fig. 3. Panel of photographs representing the down-regulation of PR expression in FHF compared to AVH in the placenta of the HEV infected pregnancy cases. Also notice the significant down-regulation of PR expression (no or very faint expression) in PROGINS carriers of the FHF group of HEV infected pregnancy cases.

expression compared to the non-PROGINS cases. Furthermore, PR expression was reduced significantly in FHF (especially in PROGINS positive cases which showed no or very faint expression) compared to AVH (Fig. 3).

When considered among all the cases, the Wilcoxon signed rank test for correlation showed that high viral load was significantly associated with lower PR mRNA expression ($p = 0.005$). But when the AVH and FHF case groups were analyzed separately, only the AVH cases showed significant association between high viral load and lower PR mRNA expression ($p = 0.018$).

Progesterone induced blocking factor (PIBF) expression was analyzed at the mRNA level using the total RNA isolated from whole blood and placental tissues by semiquantitative RT-PCR analysis. PIBF expression was consistent with the PR expression pattern; PIBF expression was found significantly downregulated in AVH ($p < 0.001$) and FHF ($p < 0.001$) cases compared to healthy pregnancy cases. Interestingly, the difference in PIBF expression was more significant and more prominent in FHF cases between PROGINS carriers and non-carriers than AVH where the differential expression was comparable (Fig. 2).

Th1/Th2 cytokine profile analysis

IL-12 (Th1 response) and IL-10 (Th2 response) expression analyses were studied in HEV related pregnancy cases compared to healthy pregnant and non-pregnant control cases. The average IL-12 expression in FHF cases was much higher than any other comparative group. IL-10 concentration was also found to be slightly higher in FHF cases compared to other groups. The difference in Th1/Th2 ratio (IL-12/IL-10) was not statistically significant ($p = 0.932$) either between PROGINS carriers and non-carriers in HEV related pregnancy cases, or within the AVH ($p = 0.604$) and FHF ($p = 0.457$) groups; as well as between the AVH and FHF groups ($p = 0.576$). The higher IL-12/IL-10 ratio was found to be statistically significant with regard to fetal mortality in AVH ($p < 0.001$) as well as FHF ($p < 0.001$). The IL-12/IL-10 ratio for AVH and FHF patients together was found to be significantly higher in cases with fetal death compared to cases where fetal survival occurred ($p < 0.001$) (Fig. 4A). Although statistically insignificant, a higher Th1/Th2 kind of response in terms of average IL-12 and IL-10 expression ratio was observed in FHF cases compared to AVH, healthy pregnant, and even healthy non-pregnant patients (Fig. 4B).

Factors affecting pregnancy outcome

The factors analyzed in the current study perpetuate the fate of a pregnancy event. The cumulative results show that fetal and maternal mortality are statistically significant in FHF cases ($p < 0.001$) in which the HEV viral load is also significantly higher ($p = 0.018$) with higher encephalopathy grade. Also in AVH, viral load was significantly higher in cases with fetal mortality ($p = 0.001$). As only HEV genotype 1 was predominantly present in our enrolled cases, the effect of HEV genotype on pregnancy outcome could not be delineated. Overall, PROGINS haplotype in HEV related pregnancy cases was found to be significantly associated with fetal death ($p < 0.001$) but insignificantly associated in maternal mortality ($p < 0.593$); the odds ratio showed higher chances of prevalence of PROGINS in FHF (1.438 (0.689–2.999) at a 95% confidence level) compared to AVH cases ($p = 0.26$). The risk of fetal mortality in AVH was found to be double in PROGINS carriers (2.190 (0.303–15.851) at a 95% confidence level). The risk of maternal mortality in FHF was found to be increased fourfold in PROGINS carriers (4.000 (0.363–44.113) at a 95% CI level) which also showed 100% fetal mortality. In the HEV infected pregnancy cases enrolled and evaluated in the current study, the PROGINS carriers showed reduced PR and PIBF expression, and therefore are critically associated with pregnancy complications, and fetal and maternal mortality.

Th2 biased immunological state (higher IL-10/IL-12 ratio) is a prerequisite for successful pregnancy maintenance, which was altered maximally toward the Th1 biased state in HEV related FHF pregnancy cases which showed high fetal and maternal mortality, compared to healthy or HEV related AVH pregnancy cases. The higher IL-12/IL-10 ratio was found to be statistically significant with regards to fetal mortality in FHF ($p < 0.001$) as well as AVH ($p < 0.001$) cases, suggesting a role for immune alteration in poor maternal and fetal outcomes in HEV infected pregnancy cases.

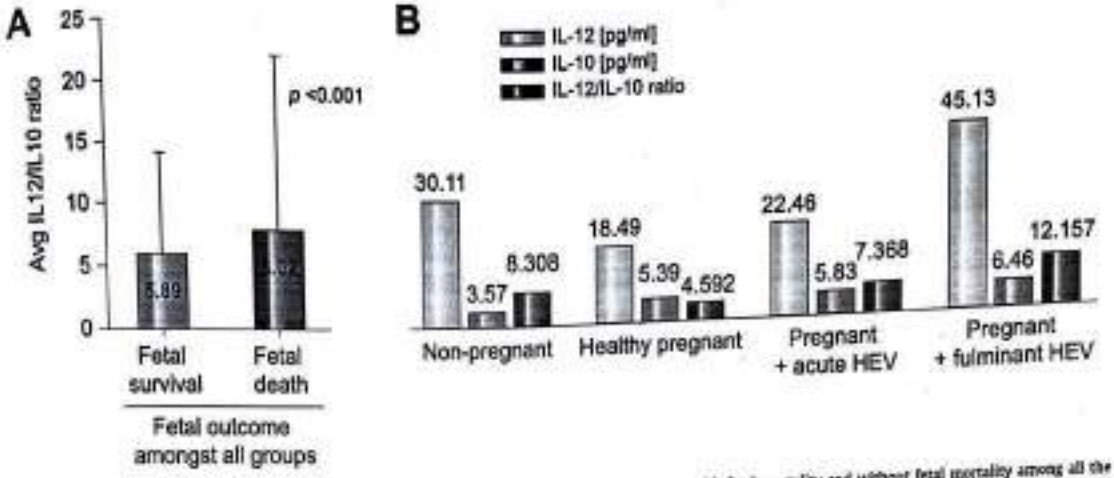

Fig. 4. Th1/Th2 cytokine profile analysis. (A) Graph representing the average IL-12/IL-10 ratio in cases with fetal mortality and without fetal mortality among all the groups. Cases with fetal mortality showed higher IL-12/IL-10 ratio. (B) Graph representing the average IL-12 and IL-10 concentration in different cases and controls, and the IL-12/IL-10 ratio. The increased IL-12/IL-10 ratio in case of FHF clearly indicates the Th1 bias resulting in fetal mortality in the majority of cases.

Discussion

Hepatitis E virus (HEV) infection during pregnancy leads to severe complications which may result in fetal and/or maternal mortality, abortion, premature delivery, or death of a live-born baby soon after birth depending on the severity of the infection which is stratified as AVH or FHF (most severe form of AVH). HEV infection is one of the predominant causes of pregnancy related complications in developing countries including India [3,4]. A successful outcome of pregnancy is coordinated in such a way that timely birth is achieved which involves the activities of multiple effector pathways whose alterations may lead to IUD or preterm birth. Compelling evidence indicates the steroid receptors for progesterone and progesterone induced blocking factors (PIBF) as candidate upstream regulators of labor-associated processes, pregnancy maintenance, and outcome [22,23]. The current prospective study was undertaken to delineate the molecular etiology underlying the HEV-related poor pregnancy outcome by virtue of the modulation of the progesterone receptors and associated proteins finally leading to altered Th1/Th2 bias resulting in high fetal and maternal mortalities in different severity of HEV infection.

Spontaneous preterm birth before 34 weeks of gestation occurs in 3–7% of pregnancies but accounts for around 75% of neonatal mortality and 50% of long-term neurological impairment in children [24]. Among the HEV related pregnancy cases ($n = 143$) enrolled for our study and followed up until pregnancy outcome, preterm birth was observed in 42% and 73.33% of the AVH and FHF cases respectively, which was much higher than normal pregnancy preterm birth rate. Another important observation made in our study was the higher IUD cases which were 18% and 86% in AVH and FHF cases, respectively. This clearly underlines and establishes HEV's role in pregnancy outcomes.

Progesterone receptors have been proposed to play a key role in human gestation, maintenance of human labor, and parturition [25,26]. Progesterone is critical for the establishment and the maintenance of pregnancy, both because of its endocrine and immunological effects. Incidence of FHF with high mortality in Hepatitis E is high in pregnancy when the steroid hormone level is altered [9]. The genomic actions of progesterone are mediated by the intracellular progesterone receptors [27]. The outcome of the immunological recognition of pregnancy is an upregulation of progesterone receptors on natural killer (NK) cells in the decidua or on lymphocytes among placental cells [8]. 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.

PROGINS, a haplotype of progesterone receptor is associated with reduced amounts of gene transcript and a lesser response to progesterone [28]; was more prevalent in FHF (10/43, 23.26%) compared to AVH (14/100, 14%) and the healthy pregnant (2/50, 4%) group. Although the higher frequency of PROGINS in FHF compared to AVH is not statistically significant, a significantly higher frequency of PROGINS in FHF compared to healthy pregnant controls (OR = 3.485, $p = 0.041$) suggests that PROGINS carriers are predisposed to HEV infection with greater severity during pregnancy. PROGINS carriers showed lower expression of PR compared to non-PROGINS. PROGINS haplotype in HEV related pregnancy cases was found to be significantly associated with fetal death ($p < 0.001$) by Wilcoxon signed rank test; twofold increase in fetal mortality in AVH, and fourfold increased risk of maternal mortality in HEV related FHF pregnancy cases. Therefore, PROGINS may be considered as a candidate prognostic marker for HEV related pregnancy complications and outcomes.

In the presence of sufficient progesterone, activated lymphocytes synthesize progesterone induced blocking factor (PIBF), a mediator that exerts substantial anti-abortive activities by inhibiting NK cell activity and influencing the humoral and cellular immune systems [9,22,23].

Here in our study, PIBF expression seems to follow the same pattern with PR expression in both the AVH and FHF groups which supports the observation of a previous study done in an animal model that blockade of the progesterone receptor results in reduced PIBF production [22]. The PIBF expression was substantially reduced in HEV related AVH and FHF (highly reduced) compared to healthy

controls at the mRNA level, more so in PROGINS carrier cases; therefore, playing a pivotal role in HEV related pregnancy loss.

The human placenta is a complex and vital organ that mediates the selective transfer of materials between the mother and fetus. Most importantly, the placenta produces hormones and other factors that support pregnancy and provides a barrier to the maternal immune system. Immunological changes during pregnancy promote the maintenance of the antigenic fetus in the maternal environment by suppression of T-cell-mediated immunity. There is a clear shift in the T-helper type 1 (Th1):Th2 cell paradigm during pregnancy, with a definite skew toward Th2 cells. Th2 cytokines are necessary for the trophoblast to secrete human placental lactogen (hPL) and hCG. In addition, Th2 cytokines down-regulate Th1 type reactivity [29–31]. In a successful pregnancy, the normal profile is Th2 type immunity.

The modulation of cell mediated immunity during gestation occurs to allow fetal allograft retention, but it also alters the immune response mounted against infections [32–35]. The decrease in T cell activity has been suggested to increase susceptibility to viral infections such as hepatitis. Our results highlight the predominance of Th1 type immunity in HEV related FHF pregnancy cases which showed high fetal and maternal mortality, compared to healthy or HEV related AVH pregnancy cases. Although statistically insignificant, this clear shift toward Th1 dominance seems to be responsible for the progression to FHF and fetal death in pregnant women because of immunological injury, as earlier thought of by workers to be associated with unexplained habitual abortion [36]. Since by increasing IL-10 and decreasing IL-12 production, PIBF inhibits NK cell cytotoxicity [23], the downregulated PIBF expression status, therefore, correlated well with PR expression, Th1 biased status, and HEV related fetal outcome of pregnancy especially in FHF.

Recently, in a study done by Saravanabalaji *et al.* [37], it was reported that there was a substantial increase in IL-10 production along with the higher levels of Th1 cytokines in FHF patients indicating the presence of both inflammatory and anti-inflammatory reactions. The present study confirms this observation as in FHF patients enrolled in our study, both Th1 (IL-12) and Th2 (IL-10) levels were raised compared to AVH patients and healthy controls. However, a higher IL-12 level in AVH compared to FHF patients as observed in their study is not concordant with our finding which shows that the IL-12 level in the FHF group is higher than any other comparative group.

Apart from the above discussed host factors, our study shows that viral factors also play a major role in HEV disease severity as reported earlier in the study done by Kar *et al.* [6]. A comparatively higher HEV viral load was observed in FHF patients (139,994.0 ± 103,104.17 copies/ml) than AVH patients (768.92 ± 1105.40 copies/ml). However, HEV genotype could not be correlated with the disease outcome as only a single genotype (genotype 1) was detected in both the disease groups. High fetal mortality has been explained in AVH and FHF cases which showed vertical transmission of HEV from HEV infected mothers to their infants [38]. From the present study, it is evident that in HEV infected pregnant women, high HEV viral load in a patient's serum is associated with fetal mortality in AVH ($p = 0.001$) and FHF ($p = 0.018$).

The present study shows that higher viral load is significantly associated with reduced PR mRNA expression in both AVH and FHF cases ($p = 0.005$) and this indicates that the HEV viral load may play a critical role in regulating PR mRNA expression, but the HEV specificity of PR mRNA regulation during pregnancy needs to be determined by other studies enrolling pregnancy cases with non-E viral hepatitis.

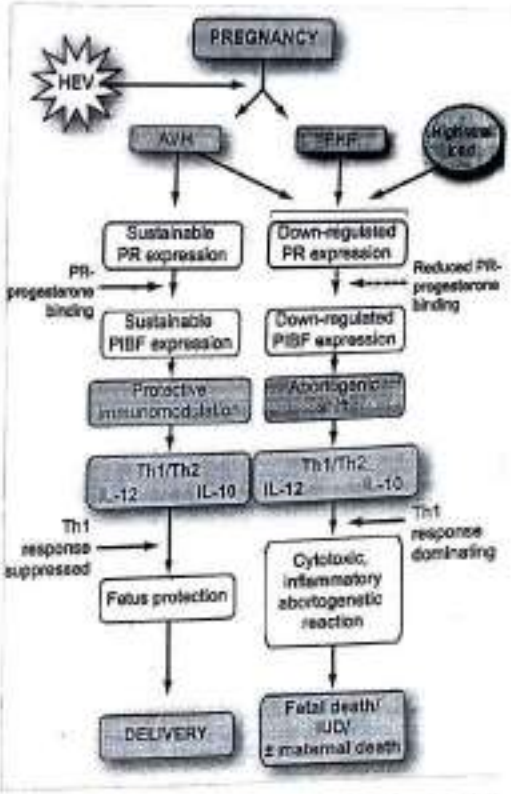

Fig. 5. Model depicting the pathways of HEV modulated pregnancy outcome.

To conclude, PROGINS carriers and lower expression of PR and PIBF, as well as high viral load influences Hepatitis E disease severity and outcomes in pregnancy. Higher IL-12 to IL-10 ratio (Th1 bias) in FHF indicates that after crossing the period when there was a lower IL-12 to IL-10 ratio and after the completion of the HEV incubation period (i.e. 15–64 days), when the virus has started causing damage to the cells, cytotoxic immunity rises (Th1 immunological state). This immunity rises up to a particular level where the body can fight against virally infected cells but during the process, lower PIBF expression and higher NK cell activity results in reduced fetal protection and eventually fetal death occurs because of immunological injury. Based on our novel findings, we propose a model for HEV related pregnancy outcomes (Fig. 5). Our results also provide crucial insights and warrant the development of PIBF based therapeutics for human application for its anti-abortive potential.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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