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Novel molecular alterations in the ORF 2 capsid gene of hepatitis E virus in patients with acute liver failure in North India

Jayanta Borkakoti • Giasuddin Ahmed • Syed Akhtar Hussain • Arvind Rai • Premashis Kar

Abstract Hepatitis E virus (HEV) is evolving as a major global threat to public health, including in developed countries. We partially sequenced the ORF 2 capsid protein genes of HEV genomes from patients with acute liver failure, including pregnant women in the northern part of India. Five unique synonymous substitutions and one non-synonymous substitution, along with a novel mutation, P299S, in the capsid gene, were identified that might be associated with the poor outcome in the patients. Phylogenetic analysis revealed that the isolates belonged to genotype 1 with subtype 1a. The significance of these findings for disease pathogenicity needs to be investigated further.

Abbreviations

ORF 2  Open reading frame 2
P         Proline
S         Serine

Hepatitis E virus (HEV; genus Hepevirus, family Hepeviridae), which occurs in liver transplant patients in non-endemic countries and, importantly, in pregnant women, causing 25% maternal mortality in South Asian countries, has changed radically in the past decade, proving to be a threat to global health, including in developed countries [1, 2]. HEV possesses a single-stranded positive-sense RNA genome of 7.2 kb with three forward open reading frames (ORF1, ORF2, and ORF3) encoding three different proteins [3, 4]. Previous studies have shown that genotype 4 of HEV is more closely associated with acute liver failure (ALF) than genotype 3 [5]. The genotype of HEV influences the severity of hepatitis E, which can cause 95% mortality in pregnant women, especially in the third trimester [6]. It has also been observed that men and non-pregnant women are also predisposed to acute liver failure to varying degrees [7]. ORF2 encodes the viral capsid protein, which contains a signal peptide (aa 1–22) that is involved in the translocation of the protein from the endoplasmic reticulum [3, 8]. Three structural domains are present within the C-terminus of the HEV capsid protein: S (residues 118–313), P1 (residues 314–453) and P2 (residues 454–606), which are responsible for forming the capsid shell, binding of the virus to host-cell receptors, and antigenicity, respectively [9, 10]. The HEV capsid protein has also been shown to be a key antigen that stimulates the host immune response, and six antigenic domains have been identified [11]. Preclinical studies of an insect-cell-expressed protein spanning aa 112–607 of the ORF2 protein of a genotype 1 virus showed it to be a promising candidate vaccine [12]. This protein contains three putative N-glycosylation sites [8], but the biological significance of such potential modifications is unclear [13, 14].

Partial nucleotide sequences of ORF2 have been predicted to be well suited for phylogenetic classification of
HEV [15], and previous studies with different viruses have shown that single or multiple variations in the amino acid sequence of the capsid or envelope protein can result in an attenuated viral phenotype [16, 17]. Recently, Córdoba et al. [18] demonstrated that mutations in the PI domain were associated with attenuation. Hence, the present study was aimed at identifying the molecular alterations in the ORF 2 region of the HEV genome in cases of acute liver failure associated with HEV infection, including those in pregnant women from the northern part of India, that might be associated with disease severity.

Serum samples were collected from 12 patients with acute liver failure and 85 patients with acute viral hepatitis (AVH) with HEV infection attending the outpatient department of the Department of Medicine at Lok Nayak Hospital, New Delhi, during the period from June 2012 to July 2013, within one week of the onset of clinical symptoms. A total of 31 of the 97 patients involved in the study were pregnant (31.95 %), of which six had acute liver failure and 25 had acute viral hepatitis. The samples were immediately stored at -70 °C to keep the viral RNA intact. Patients with acute liver failure were diagnosed on the basis of development of encephalopathy within eight weeks of the onset of jaundice without any past history of chronic liver disease [19]. Acute viral hepatitis was diagnosed as a self-limiting disease with at least a fivefold elevation of serum aspartate aminotransferase levels or clinical jaundice, or both [20]. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional ethical committee of Maulana Azad Medical College, New Delhi. The patients were evaluated on the basis of history, physical examination, and liver function tests.

HEV RNA was isolated from blood serum using a Viral RNA Extraction Kit (QIAGEN, Heidelberg, Germany). The HEV viral load (copies/ml) was determined by real-time PCR (Rotor-Gene b RG-2000, Corbett Research) using Geno Sen's HEV Real Time PCR Reagents Kit, India. The real-time PCR reaction was carried out as follows: 50 °C for 15 min (cDNA synthesis) and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s, 55 °C for 20 s and 72 °C for 15 s. cDNA preparation was done using a cDNA extraction kit (Thermo Fischer Scientific, Massachusetts, USA). A portion of ORF 2 of the HEV genome, with a length of 506 bp, was amplified by reverse transcription PCR (RT PCR), using the following primers, which were designed using Primer 3 software [21]: Forward, 5'AGGCCATCTCCATCTCTACCCG3'; Reverse, 5'GAACAGGGTGAGGGCTATC-3'. The PCR was performed using 2 μl of cDNA as a template, 10 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 400 μM dNTPs, 0.4 μM each primer and 1.2 U of Taq polymerase in a 25-μl reaction mixture. The temperature profile used was as follows: initial denaturation at 94 °C for 1 min, followed by 35 cycles of DNA denaturation carried out at 94 °C for 45 s, annealing at 53 °C for 45 s, and nucleotide extension at 72 °C for 45 s, followed by a final elongation at 72 °C for 7 min. The representative amplicons were sequenced directly and were deposited in the GenBank database under accession numbers KF777253-KF777257. The isolates JBDEL1, JBDEL2, JBDEL3 were from pregnant patients who died due to acute liver failure. For each of these patients, the clinical profile and mutational features are presented in Table 1. Amino acid changes in the HEV sequences derived from patients with acute liver failure or acute viral hepatitis were compared using Fischer's exact (two-tailed) test. The mean age of the patients with acute viral hepatitis (AVH) and acute liver failure (ALF) was 23.85 ± 3.68 years and 24.6 ± 3.32 years, respectively. Viral RNA was detected in 43 patients with acute viral hepatitis (50.58 %) and nine patients with acute liver failure (75 %) respectively. Nucleotide sequences of HEV isolates were retrieved from Gen Bank and were aligned using Clustal W (Version 1.81) software. Considering AF459438 (North Indian Strain of HEV) as the prototype, 6098 (G6098A), 6104 (C6104T), 6014 (T6014C), 6032 (C6032T), 5927 (C5927T), 5933 (C5933T) nucleotide substitutions were identified, five of which resulted in amino acid changes (Table 1), that differed significantly from the other reference sequences derived from patients with acute viral hepatitis. This is the first report describing molecular alterations in pregnant patients with acute liver failure due to HEV. Among the 33 patients from whom representative sequences were obtained, five out of five patients (100 %) with acute liver failure had a novel significant mutation at amino acid position 259 (P259S) that was absent in all 28 patients with acute viral hepatitis (p < 0.0001). The mutation T6209C in the isolate JBDEL3 caused a change from phenylalanine 356 to leucine (F356L), which was absent in the other patients. In the same isolate, another novel mutation (K350E) was observed. However, the F356L and K350E mutations were each observed in only one patient with acute liver failure and were therefore not statistically significant (p = 0.15). Even though the present study reflected interesting findings, it was done on a limited number of patients, and hence the above conclusions can only be confirmed by larger studies. These novel mutations were also relevant due to their absence in patients with acute viral hepatitis. Phylogenetic analysis revealed that the isolates belonged to genotype 1, subtype 1a, and clustered separately from genotypes 2, 3, 4 and viruses from ferrets (Fig. 1). The isolates also shared 98 % homology with the Yam-67 isolate from North India [22].
Table 1 Profiles of five patients with acute liver failure who were infected with hepatitis E virus genotype 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Isolate name</th>
<th>GenBank accession number</th>
<th>Date of isolation</th>
<th>Age, sex</th>
<th>Status</th>
<th>Laboratory profile</th>
<th>Viral load (copies /ml)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>JBDEL1</td>
<td>KF777253</td>
<td>2012, Jan 10</td>
<td>24/F</td>
<td>Pregnant</td>
<td>7.3</td>
<td>42000</td>
<td>Died</td>
</tr>
<tr>
<td>2</td>
<td>JBDEL2</td>
<td>KF777254</td>
<td>2012, Jan 16</td>
<td>30/F</td>
<td>Pregnant</td>
<td>10.4</td>
<td>121000</td>
<td>Died</td>
</tr>
<tr>
<td>3</td>
<td>JBDEL3</td>
<td>KF777255</td>
<td>2012, Jan 31</td>
<td>28/F</td>
<td>Pregnant</td>
<td>8.3</td>
<td>13000</td>
<td>Survived</td>
</tr>
<tr>
<td>4</td>
<td>JBDEL4</td>
<td>KF777256</td>
<td>2012, Feb 4</td>
<td>30/M</td>
<td>—</td>
<td>11.5</td>
<td>80</td>
<td>Survived</td>
</tr>
<tr>
<td>5</td>
<td>JBDEL5</td>
<td>KF777257</td>
<td>2012, Mar 23</td>
<td>25/M</td>
<td>—</td>
<td>9.2</td>
<td>680</td>
<td>Survived</td>
</tr>
</tbody>
</table>

**Nucleotide substitutions**

- C5933T*
- T6014C*
- C6032T*
- G6098A*
- C6104T*
- T6209del

**Significant substitutions and mutations are highlighted in bold**

**ALF**, acute liver failure; **AST**, aspartate aminotransferase; **ALT**, alanine transaminase; *, significant substitutions and mutations; +, mutation present; −, mutation absent

**Fig. 1** Phylogenetic tree constructed on the basis of 305 nucleotides from ORF2, based on genomic sequences from human, rabbit, swine and ferret HEV strains. Sequence alignment was performed by using ClustalX in the MEGA4.0 software package (http://www.megasoftware.net), and the trees were constructed using the neighbor-joining method with p-distance (gap/missing data treatment; pairwise deletion) and 1,000 bootstrap replicates. The scale bar indicates nucleotide substitutions per site. The isolates in the present study are indicated with circles

It has been reported earlier that silent substitutions at C5907, located in the capsid gene, are associated with high viral load and may be favourable for the viral translation machinery and for disease severity in patients with genotype 3 [23]. Takahashi et al. [24] identified certain amino acid mutations in viruses from eight patients infected with
genotype-3 HEV with self-limiting disease. Recent data from western India showed that the V11201 amino acid substitution in the helicase domain might play a significant role in determining the outcome of HEV infection [25].

Previous studies identified mutations that were associated with significantly reduced viremia and the viral loads in the liver [18], but none of them included pregnant women. This is a pioneering study reporting substitutions in viruses isolated from pregnant women with HEV infection. Although the novel mutation P259S was present in all of the patients with acute liver failure, the significance of this mutation in terms of pathogenicity needs to be determined. In conclusion, the present study identified the novel ORF 2 substitutions P259S and K350E, which may play a crucial role in influencing HEV replication by interacting with other proteins, thus leading to increased disease severity and poor outcome in patients with acute liver failure. Further studies are warranted to investigate these mutations.

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Conflict of interest The authors declare no conflict of interest.

References


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J. Borkakoti et al.
Does High Viral Load of Hepatitis E Virus Influence the Severity and Prognosis of Acute Liver Failure During Pregnancy?

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The incidence and mortality in pregnant women with acute liver failure caused by hepatitis E virus (HEV) is high. Data on the viral load of HEV during pregnancy are limited. The study was designed to determine the viral load of HEV and its association with the disease severity in patients with acute liver failure. A total of HEV related 163 patients with acute liver failure which included 105 pregnant, 46 non-pregnant women and girls and 12 men and 730 patients with acute viral hepatitis which comprised of 220 pregnant women; 282 non-pregnant women and girls and 228 men were included. Viral load was measured by real-time PCR. Comparison was made between the pregnant and non-pregnant women. HEV RNA was detectable in 265 patients (142 pregnant; 75 non-pregnant women and girls and 48 men) and 104 patients with acute liver failure (64 pregnant, 34 non-pregnant and 6 men). The viral load of HEV in pregnant women with acute liver failure and acute viral hepatitis was significantly higher 129,984.0 ± 103,104.17 and 768.92 ± 1,105.40 copies/ml, respectively compared to the non-pregnant women which was 189.2 ± 225 and 12.73 ± 7.8 copies/ml (P< 0.0001). The viral load of HEV was also significantly higher in the pregnant patients with acute liver failure compared to the pregnant woman with acute viral hepatitis and also men (P< 0.0001). High viral load of HEV during pregnancy could be one of the factors responsible for the severity of the infection during pregnancy.

INTRODUCTION

Hepatitis E virus (HEV) is an important cause of epidemic and sporadic acute viral hepatitis in many developing countries, including India [Teshale et al., 2010]. Hepatitis E, a positive-sense single-stranded RNA virus approximately 7.2 kb in length had been classified provisionally into the Caliciviridae family from 1988 to 1998 but HEV is currently placed in the genus Hepaeroirus and is the only member of the family Hepaeriformidae [Emerson and Purcell, 2003; Graff et al., 2006]. Pregnant women with jaundice and acute viral hepatitis caused by HEV infection have worse fetal and obstetric outcomes and higher maternal mortality compared to other types of viral hepatitis [Tsega et al., 1993]. Studies from various developing countries have shown that the incidence of HEV infection in pregnancy is high and a significant proportion of pregnant women can progress to fulminant hepatitis with a mortality rate varying from 30% to 100% [Medhat et al., 1993; Khuroo and Kamili, 2003; Singh et al., 2003; Strand et al., 2003; Patra et al., 2007]. The incidence of HBV related acute liver failure is known widely in comparison to HCV infection in which acute liver failure is rare. But the severe course of HEV infection causing acute liver failure during pregnancy is unique to this virus with chronicity occurring in recipients of solid organ transplants.

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Various factors have been suggested to be associated with the mortality rate of the HEV in pregnant women along with the abortion of the fetus. Steroid hormones play a significant role in the viral replication through their effects on viral regulatory elements [Hussaini et al., 1997]. The NF-kB signaling pathway regulating at the transcriptional level through p50 subunits has been suggested to correlate with the severe liver damage, leading to multiple organ failure and the death of both the mother and the fetus [Prusty et al., 2007]. Pregnant women in Asia suffer from folate deficiency reducing the immunocompetence to greater risk of multiple viral infections and higher viral load [Chandra and Chandra, 1986]. The viral load of HEV was found to be significantly higher \((P < 0.05)\) in pregnant patients compared to the non-pregnant and the viral copies of HEV in FHF pregnant women were comparatively higher when compared to the pregnant women with acute viral hepatitis, which may be related to the severity of the disease in these patients [Kar et al., 2008]. Besides, reduced expression of progesterone and progesterone-induced-blocking factor and the high viral load of HEV have been regarded as a cause of poor pregnancy outcome in hepatitis E infection [Bose et al., 2011].

Vertical transmission of the HEV infection has been reported [Kheroo et al., 1995]. There are published reports of abortion, death of the fetus in utero, premature delivery or death of the baby soon after birth in patients with icteric hepatitis or with acute liver failure caused by HEV [Jaiswal et al., 2001; Emerson and Purcell, 2003]. However, studies in Europe and United States have shown the course of viral hepatitis during pregnancy resembling with the non-pregnant women [Cahill, 1962; Adams and Combes, 1965]. In contrast, various reports carried out in India, Iran, Africa, and Middle East have reported the incidence of acute liver failure to be higher during pregnancy [Arankalle et al., 1993; Balyean, 1997]. Thus based on the experience from a previous study [Kar et al., 2008], the present study was designed to substantiate further the role of hepatitis E viral load in acute liver failure with pregnancy in a large population pool and to assess the underlying severity in both the maternal and neonatal sphere. Further, a brief comparison of the viral load was also evaluated among the men and boys for the estimation of any significant alterations in the outcome of the disease with respect to the male patients with acute viral hepatitis and acute liver failure.

**MATERIALS AND METHODS**

**Enrollment of the Patients**

The study group comprised of patients attending the outpatient department of the Medicine and Obstetrics and Gynaecology in the Lok Nayak Hospital, New Delhi during the period from January 1, 2006 to December 21, 2011. Since the present study was conducted with a different objective as compared to the previous study [Kar et al., 2008] and in order to avoid biasness, the data of all those patients reported earlier were also included in the current study. The patients were categorized as acute viral hepatitis and acute liver failure which included pregnant women, non-pregnant women and girls and men and boys. The patients of acute liver failure were diagnosed on the basis of development of encephalopathy within 8 weeks of the onset of jaundice without any past history of chronic liver disease [Trey and Davidson, 1970]. Acute viral hepatitis was diagnosed as a self-limiting disease and a serum aspartate aminotransferase elevation of at least fivefold or clinical jaundice and both [Smedile et al., 1982]. The pregnant patients were observed until delivery for the development of any obstetric complications. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional ethical committee of Maulana Azad Medical College, New Delhi. The patients were evaluated on the basis of history, physical examination, and liver function tests. The serum samples were collected during the first week of the onset of clinical symptoms in the patients with acute liver failure whereas in the patients with acute viral hepatitis the samples were collected within 15 ± 3.2 days. Written consent was obtained from the patients. However, in the study only those patients were recruited who were positive serologically for HEV IgM alone. Serum samples were preserved separately in 1.5 ml Eppendorf tubes at −70°C to prevent frequent thawing of the samples and keep the viral RNA intact. Liver function tests were conducted. The final outcome of the pregnant women with acute viral hepatitis and acute liver failure was assessed with a close vigilance on the successful completion of pregnancy, maternal death, fetal loss, preterm delivery, and intrauterine death.

**Serological Analysis**

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 Eliscan micro ELISA strips (Ranbaxy Diagnostics, Gurgaon, India), IgM anti-HBc using anticorease 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). Samples were only examined when positive for HEV IgM. HEV positive cases were correlated clinically and grouped as acute liver failure and acute viral hepatitis patients.

**Extraction and Detection of Viral RNA**

Viral RNA was extracted from 140 μl serum of the samples using viral RNA extraction kit (QIAamp J. Med. Virol. DOI 10.1002/jmv
Viral RNA Mini Kit 52904) according to the manufacturer’s instructions. Extracted RNA was eluted in 50 μl elution buffer and stored at −70°C in small aliquots. HEV RNA was detected in the serum by reverse transcriptase PCR (RT-PCR) which was carried out using extracted RNA and HEV primers selected from its non-structural region (ORF-1) [Jilani et al., 2007]. Outer primers #3043 (+): 5'-CCGGATCCACACATCTGAGCTACATTCGCT 3' #3044 (−): 5'-CCGAAATCAGGATCATGTTGGAATGA C-3'; inner primers #HEV-1 (+): 5'-GGAATT CATCACATCTGAGCTACATTCGCT-3' #HEV-3 (−): 5'-GGAATT CACAGCCGGCGATCAGGACAG-3'. Both positive and negative controls were included in the amplification step.

Quantitation of HEV Using Real-Time PCR

HEV-RNA load (copies/ml) was determined for those acute viral hepatitis and acute liver failure samples which were positive for HEV-RNA using real-time PCR machine (Rotor-Gene b RG-2000, Corbett Research, Australia) using Geno Sen’s HEV Real-Time PCR Reagents kit, India. The Specific master mix contained reagents and enzymes for the specific amplification of HEV and for the direct detection of the specific amplicons in the fluoroscence channels Cycling A.FAM of the Rotor-Gene 2000 and the Reference gene on Cycling A. Joe. External positive standards (HEV S1-S5) with the specified concentrations were used to generate the standard curve, which allowed the determination of the viral load of unknown samples in subsequent runs. The reaction was carried out in a total volume of 50 μl with 15 μl primers and probes mix, 5 μl Mg2+ solution and 30 μl extracted sample or standard. Calibration was done at 56°C FAM.

Genotypic Characterization of HEV

The amplified products were purified using the gel extraction kit (Qiagen, Germany) using manufacturer’s instructions, sequenced commercially and compared with the HEV sequences in the Gen Bank using the CLUSTAL W analysis software and phylogenetic tree was constructed using the PHYLIP package software using the neighbor-joining method based on the partial nucleotide sequence of the ORF1 region.

Statistical Analysis

Data were analyzed using EPI Info Software version 3.5.1 (CDC Atlanta, GA). Data were expressed as means standard deviations (SD). The Fischer’s exact test was used for univariate analysis and the t-test was used to compare continuous variables between two groups, \( P < 0.05 \) was considered significant. Mann–Whitney U-test non-parametric test was used to analyze the viral load since the samples were selected randomly.

RESULTS

Demographic Profile of the Study Population

During the time span of 6 years from 2006 to 2011, serum samples were collected from 2,364 female and male patients who were diagnosed with acute viral hepatitis and acute liver failure. Men and boys were recruited only if found to be positive serologically for HEV IgM. The patients were further categorized as pregnant women (425 acute viral hepatitis and 160 acute liver failure); non-pregnant women and girls (1,381 acute viral hepatitis and 158 acute liver failure) and men and boys (228 acute viral hepatitis and 12 acute liver failure). HEV IgM alone was exclusively associated with 893 patients, which included 730 non-pregnant women and girls and 228 men and boys) and 163 acute liver failure (105 pregnant women; 46 non-pregnant women and girls and 12 men and boys). The average age of the pregnant patients with acute liver failure was 24.6 ± 3.78 years and that of the non-pregnant patients with acute liver failures was 26.5 ± 4.78 years. The average age of the pregnant and the non-pregnant patients with acute viral hepatitis was 24.21 ± 3.8 and 29.45 ± 8.7 years, respectively (Table I). The average age of the men and boys with acute liver failure was 26.75 ± 15.39 (age range: 11-48) years while that of the girls and women with acute liver failure were 26.5 ± 4.78 (age range: 8-40) years, respectively.

Estimation of HEV Infection in Pregnant Women, Non-Pregnant Women and Girls; Men and Boys

Amongst the overall pregnant patients with acute liver failure which constituted 160 cases, HEV was solely responsible for 105 (65.8%) cases whereas in

**TABLE I. Clinical and Demographic Profile of the HEV Positive Patients**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Acute viral hepatitis</th>
<th>Acute liver failure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pregnant</td>
<td>Non-pregnant women and Girls</td>
</tr>
<tr>
<td>Number (N)</td>
<td>220</td>
<td>282</td>
</tr>
<tr>
<td>Age (Mean ± SD)</td>
<td>24.21 ± 3.8</td>
<td>29.45 ± 8.7</td>
</tr>
<tr>
<td>Mortality (%)</td>
<td>2 (0.9%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

*J. Med. Virol. DOI 10.1002/jmv*
the pregnant women with acute viral hepatitis, HEV infection was detected in 220 out of 425 (51.7%) cases. In the non-pregnant women and girls presenting with acute liver failure HEV was present in 58 out of 170 (34.1%) cases. In the non-pregnant patients with acute viral hepatitis, HEV was alone detected in 282 out of 1,381 (20.4%) cases. The status of the other viral markers, as well as the other co-infections are depicted in Table II. All the co-infected cases were excluded from the study to inhibit any ambiguity of the data. High frequency of HEV infection was found in the pregnant patients with acute liver failure (135/160, 84.3%) when compared with those of non-pregnant patients with acute liver failure (76/158, 48.1%), \( P < 0.0001 \).

Of the male patients, 228 patients with acute viral hepatitis and 12 patients with acute liver failure were all found to be positive serologically for HEV IgM. All the male patients positive for other viral markers including the HEV dual infections were excluded from the study.

**HEV Viral Load of the Assorted Groups**

Detection of HEV RNA and viral load was done in the patients who were positive exclusively for HEV IgM. HEV RNA was detectable in was 41.3% (369/893) patients, which included 265 patients with acute viral hepatitis (142 pregnant and 75 non-pregnant, 48 men and boys) and 104 patients with acute liver failure (64 pregnant, 34 non-pregnant females and 6 males).

The viral load of HEV in the pregnant women with acute liver failure was found to be 129,984 ± 103,104.17 copies/ml as compared to the pregnant women with acute viral hepatitis 768.92 ± 1,105.40 copies/ml whereas in the non-pregnant category, it was 189.2 ± 225 and 12.73 ± 7.8 copies/ml in the women with acute liver failure and acute viral hepatitis, respectively (Table III). In comparison, the viral load of the patients with acute viral hepatitis and acute liver failure had a significant difference \( P < 0.0001 \) between both the pregnant and non-pregnant groups, respectively.

The male patients with acute viral hepatitis and acute liver failure were tested for their viral loads but no significant difference was obtained amongst them. But the viral load of the pregnant women with acute liver failure was significantly higher \( P < 0.0001 \) than their male counterparts as well as the men and boys with acute viral hepatitis \( P < 0.0001 \).

**Outcomes of the Study Patients**

With respect to the patients with acute viral hepatitis and acute liver failure during pregnancy, both the maternal as well as the fetal outcomes was found to be worst in the patients with acute liver failure. In pregnant patients with acute liver failure the fetal outcome was severe, where intrauterine death was

### Table II. Aetiologial Distribution of the Various Agents of Viral Hepatitis in our Center (2006–2011)

<table>
<thead>
<tr>
<th>Viral markers</th>
<th>Pregnant (N = 425)</th>
<th>ALF (N = 160)</th>
<th>Non-pregnant (N = 1,381)</th>
<th>ALF (N = 158)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEV</td>
<td>220 (51.76%)</td>
<td>105 (65.62%)</td>
<td>282 (20.41%)</td>
<td>46 (29.11%)</td>
</tr>
<tr>
<td>HAV</td>
<td>5 (1.17)</td>
<td>3 (1.87)</td>
<td>140 (9.44%)</td>
<td>0</td>
</tr>
<tr>
<td>HBV</td>
<td>12 (2.82)</td>
<td>2 (1.25%)</td>
<td>200 (13.48%)</td>
<td>28 (16.47%)</td>
</tr>
<tr>
<td>HCV</td>
<td>9 (2.11)</td>
<td>0</td>
<td>48 (3.39%)</td>
<td>3 (1.76%)</td>
</tr>
<tr>
<td>HAV + HEV</td>
<td>30 (13.63)</td>
<td>15 (9.37%)</td>
<td>54 (3.64%)</td>
<td>5 (2.94%)</td>
</tr>
<tr>
<td>HEV + HBsAg</td>
<td>0</td>
<td>3 (1.87%)</td>
<td>30 (2.02%)</td>
<td>0</td>
</tr>
<tr>
<td>HEV + HBcIgM</td>
<td>0</td>
<td>2 (0.47)</td>
<td>18 (1.21%)</td>
<td>17 (10%)</td>
</tr>
<tr>
<td>Non A-E</td>
<td>108 (25.41)</td>
<td>20 (12.5%)</td>
<td>525 (35.40%)</td>
<td>51 (30%)</td>
</tr>
<tr>
<td>Total</td>
<td>425</td>
<td>160</td>
<td>1,381</td>
<td>158</td>
</tr>
</tbody>
</table>

*HBsAg, Hepatitis B surface antigen.

### Table III. Viral Load in Pregnant, Non-Pregnant Women and Girls and Men With Acute Liver Failure (ALF) Patients and Acute Viral Hepatitis (AVH)

<table>
<thead>
<tr>
<th>Group</th>
<th>Status</th>
<th>Viral load (copies/ml) means ± standard deviation</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVH (N = 130)</td>
<td>Pregnant</td>
<td>768.92 ± 1,105.40</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AVH (N = 50)</td>
<td>Non-pregnant</td>
<td>12.73 ± 7.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ALF (N = 158)</td>
<td>Pregnant</td>
<td>129,984 ± 103,104.17</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ALF (N = 28)</td>
<td>Non-pregnant</td>
<td>189.2 ± 225</td>
<td>&gt;0.05, non-significant</td>
</tr>
<tr>
<td>AVH (N = 20)</td>
<td>Men and boys</td>
<td>15.08 ± 3.4</td>
<td>&gt;0.05, non-significant</td>
</tr>
<tr>
<td>ALF (N = 5)</td>
<td>Men and boys</td>
<td>19.4 ± 20</td>
<td>&gt;0.05, non-significant</td>
</tr>
</tbody>
</table>
found in 57% (55/70) of the cases and preterm delivery in 65.7% (46/70) of the cases. In comparison, preterm delivery was found in 41% (90/220) and intrauterine death in 34% (74/220) of the cases of acute viral hepatitis and the difference was found to be statistically significant (Table IV). Maternal death was found in 59% (59/105) of the cases in the pregnant acute liver failure patients whereas only 0.9% (2/220) of the acute viral hepatitis cases had the same (Table IV). Amongst the non-pregnant women, 26.1% (12/46) succumbed to death among the patients with acute liver failure category whereas no casualty was observed in the acute viral hepatitis group.

**Genotypic Characterization of HEV**

The phylogenetic analysis of the representative (42) samples showed that all the samples belonged to genotype 1.

**DISCUSSION**

The viral load in infection with hepatitis A, B, C, and E has been a subject of speculation in the severity of the disease outcome. In the present study HEV viremia was detected in 369/893 (41.3%) cases; HEV RNA could not be detected in all the patients positive for HEV IgM since the viremia decreases considerably with the course of the disease. Therefore, viral load was examined in only for those samples which were positive for HEV RNA. Saravanabalaji et al. [2009] reported the absence of the HEV viremia in majority of the acute liver failure patients (1/15 cases of acute liver failure) but in the current study HEV RNA was detected in 63.8% (104/163) of the patients with a high viral load. The presence of viremia in the pregnant patients with acute liver failure is expected but, many times the absence of viremia in the samples could be attributed to the improper storage or handling of the samples. The precise underlying mechanism of acute liver failure caused by HEV infection in pregnancy still remains to be explored. Significant findings in the current study include the high rate of HEV infection in the pregnant patients with acute liver failure which was 105/160 (65.6%) compared to non-pregnant patients with acute liver failure 46/158 (29.1%). In the present study a significantly higher viral load was observed in pregnant patients with acute liver failure compared to the pregnant patients with acute viral hepatitis (P < 0.0001) and also when compared to their non-pregnant counterparts (P < 0.0001). Similar studies with respect to viral load have been reported; a significant correlation between high viral load of hepatitis A and fulminant hepatitis was established by means of real-time PCR [Fujitawa et al., 2011]. However, lack of correlation between viral load and disease severity had been reported by many authors in HCV [Hollingsworth et al., 1996; Pereira et al., 2002]. On the other hand, high viral loads have been claimed to influence the therapeutic outcome in HCV [Martinot-Peignoux et al., 1998; Berg et al., 2003].

Although HEV infection is self-limiting, recent reports suggest chronicity of the disease in solid organ transplant recipients and immunocompromised patients. HEV infection has been reported in 60% of the patients with solid organ transplants [Kamar et al., 2011]. Recent reports indicate that in immunocompromised patients, about 50% of the cases of acute HEV infection progress to chronic hepatitis with rapid progression to cirrhosis [Niet de et al., 2012].

Pregnant women tend to show a higher severity of acute liver failure in comparison to the non-pregnant group. In a contrasting study in the United States, there was no significant difference between the severity of pregnant and non-pregnant women. Bhatia et al. [2008] concluded that the mortality rate in the pregnant women with HEV was similar in comparison with the non-pregnant women but in the current study the mortality rate in the pregnant patients with HEV infection with acute liver failure (59/105; 56.1%) was significantly higher relative to the non-pregnant patients with HEV infection with acute liver failure, (11/58; 18.96%) P < 0.001 whereas no mortality was observed in the non-pregnant women and girls and among men and boys.

The risk of maternal-to-fetal transmission of HEV infection from infected mothers to their babies has been documented in some studies with significant perinatal morbidity and mortality [Khuroo et al., 1995, 2009; Kumar et al., 2004]. But, whether the viral load is responsible for the transmission remains questionable. In a prospective study, it was reported about the absence of prolonged viremia in the surviving babies of infected mothers with HEV infection [Khuroo et al., 2009]. Whereas, in hepatitis B virus infection, perinatal transmission of the infection was mainly seen in infants born to HBsAg-positive mothers with high levels of viral replication [Soderstrom et al., 2003; Wang et al., 2008]. Intrauterine transmission of HBV is positively associated with maternal HBsAg-positivity and high maternal viral load as well as with placental HBV infection [Wang et al., 2003]. Among the HEV related pregnancy cases enrolled in our study and followed up until outcome, preterm

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Acute Liver Failure During Pregnancy

birth was observed in 41% (90/220) and 65.7% (46/70) of the cases with acute viral hepatitis and acute liver failure respectively, which was much higher than normal pregnancy preterm birth rate. Another important observation was the high rate of cases with intrauterine death in our study population which was 34% (74/220) and 78.6% (55/70) in cases with acute viral hepatitis and acute liver failure, respectively.

Viral infection itself could be a major determinant of disease severity. Interestingly, the viral load of the pregnant women with acute liver failure obtained was also significantly higher than their male counterparts both in the acute liver failure and acute viral hepatitis category (P < 0.0001). However, the male patients infected with HEV were very few that developed to acute liver failure and subsequently recovered with time without no mortality. This is the first time in India where a large series of patients with acute liver failure have been reported whose viral load has been analyzed. The estimation of the viral load of the male patients with both acute viral hepatitis and acute liver failure has been studied but they showed no significant variation among them except when compared with the pregnant women with acute liver failure. The aspartate transaminase levels were found to be raised in the pregnant acute liver failure group compared to the pregnant acute viral hepatitis group. It is also possible that the high viral load may be the result of intense viral replication or may be due to the massive release of viral particles from infected hepatocytes due to the vigorous response against infected hepatocytes which is not clear and needs to be clarified in future studies. The study suggests that high viral load is one of the prognostic factors that influences severely the liver disease during pregnancy and could also be one of the predictors of the final outcome of pregnancy associated with acute liver failure.

ACKNOWLEDGMENTS

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REFERENCES


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Topic: 1 Acute Liver Failure
Absno: 22

Acute liver failure in Sapporo, Japan: clinical features according to the aetiologies

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Background/aims: As for acute liver failure (ALF), not all patients need liver transplantation (LT), because clinical outcome is different by every etiology. Artificial liver support (ALS) including hemodiafiltration has started to treat ALF with encephalopathy in 2000. Our study was aimed to clarify the clinical impact of etiology for ALF.

Methods: A total of 102 ALF patients (52 females, median age 48 years) referred from all Hokkaido areas in Japan during 1997 through June 2013 were enrolled, and etiological assessment underwent. ALF was defined to be 1,5 or more in INR. When hepatic coma was diagnosed, intensive care including ALS started using antivirals and/or corticosteroid, if necessary. LT underwent for the patients showed refractory.

Results: The distribution of etiologies for ALF was viral infection 48, circulatory disturbance 12, autoimmune hepatitis 9, drug-induced liver injury 7, metabolic diseases 4, infiltration of liver by malignant cells 2 and miscellaneous 20. Among 48 cases of viral infection, hepatitis B virus (HBV), HEV, HAV or others was causative in 26, 15, 5, and 2, respectively. Out of 53 patients presented hepatic encephalopathy, 25 (47.2 %) had viral infection, 6 (11.3 %) AIH 5 (9.4 %) drug-induced, 6 (11.3 %) others, and 11 (20.7 %) miscellaneous. Non-comatose 46 and comatose 29 including 8 undergone LT survived. 37 of 48 patients with viral infection were saved by antivirals and/or ALS, and 11 of 12 caused by circulatory disturbance survived after the treatment for underlying disorders, whereas all 4 with HBV reactivation and 2 caused by malignancy were died, despite of intensive therapy.

Conclusions: In Japan, infections of hepatotropic viruses have significant impact for the etiologies of ALF. According to the different etiologies, the optimal treatments should be prepared, and ALS is one of useful arms against hepatic encephalopathy in Japan where chronic donor shortage for LT has persisted.

Keywords: Acute liver failure, Etiology, Clinical feature

Topic: 1 Acute Liver Failure
Absno: 35

Adherence to surveillance poor in chronic liver disease presenting as acute on chronic liver failure: single centre experience

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Introduction: Acute on chronic liver failure is increasingly being recognised as a condition which results in acute deterioration of patients leading to liver transplant or mortality. The causes for this deterioration have been variable and in spite of varied etiology. Early recognition of this condition has resulted in early intervention and GSF has been shown to improve survival in acute on chronic liver failure (ACLF). Prevalence and demographics for such group of patients is not known in Singapore.

Aim: This retrospective study in prospective was done to assess the disease burden, know the demographics and the triggering event for ACLF.

Method: 1835 discharge summaries of liver related condition was scrutinised to assess of the patients fulfilled the APASL diagnostic criteria for ACLF i.e.

Observation: 55 patients were identified who fulfilled the above criteria. Records of 53 patients was available at the time of reporting.

Mean age of presentation was 55.89 ± 1.46 years with 73.5 % being male. 74.5 % were Chinese, 20 % Indians and 1.8 % others. The common cause of underlying chronic liver disease was Hepatitis B in 63.6 % of patients and 23.6 % had alcoholic liver disease. The most common cause of Acute deterioration was spontaneous hepatitis B flare seen in 24.5 % of patients, while Alcoholic hepatitis was seen on 18.9 %. Cirrhosis was seen in 66 % of patients with ACLF. Non adherence to regular follow-up was seen in 69.8 % of patients. All the
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Introduction: D-galactosamine (D-GalN)-induced liver injury is a well-known experimental model used to develop acute hepatic failure (AHF) as a result of massive hepatocyte death.

Materials and methods: In the present study, for induction of AHF, 25 rats were intraperitoneally injected with D-GalN (1,400 mg/kg), and every 3 rats were sacrificed at 12, 24, 36, 48 and 72 h respectively, while 5 healthy rats served as controls. Ultra-performance liquid chromatography–mass spectrometry (UPLC/MS) based metabolomic analysis of livers was used to investigate the metabolites changes in the process of progression and regeneration of D-GalN-induced rat model of AHF. Characteristic metabolites were screened and identified to clarify the possible mechanism of AHF and liver regeneration on metabolic level. Liver tissues were extracted for histopathological and metabolomics analysis, and serum samples were used in biochemical determination.

Results: Metabolomic analysis of liver tissues was consistent with conventional biochemical and histopathological results, and meanwhile, a number of metabolites involved in biochemical pathways such as energy metabolism, lipid biosynthesis and others, were valuable in monitoring the process of progression and regeneration of a D-GalN-induced rat model of AHF.

Conclusions: UPLC-MS-based metabolic profiling provides new insight into the mechanism of liver injury and regeneration, and suggests putative metabolic biomarkers for the monitoring of process of progression and regeneration of AHF.

Topic: 1 Acute Liver Failure

Absno: 760

A 5 year single centre experience of hepatitis E virus infection during pregnancy

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Objective: Viral hepatitis in pregnancy has been a subject of controversy. Reports from Europe and United States suggest that the disease in pregnant women is of no great severity and outcome of acute liver failure during pregnancy is in no way different from non-pregnant women. The study was designed to examine the hypothesis whether pregnancy influences the course and severity of the disease in acute viral hepatitis (AVH) and acute liver failure (ALF).

Method: 768 AVH and 320 cases of ALF patients were analysed. This included 411 AVH and 139 ALF cases associated with pregnancy.

Results: The mortality in pregnancy associated acute liver failure cases was 83.45 % (116/139) pregnancy associated AVH cases was 3.16 % (13/411), while the mortality observed in non pregnant group was 16.6 % in ALF cases and no mortality in AVH cases (p < 0.001). Hepatitis E Virus was the most common etiological agent in both the groups.

Conclusion: Mortality in pregnant women was found to be significantly higher than non-pregnant cohort. Hepatitis E has a high incidence and severe course in pregnancy. This study further substantiates the observation that mortality in ALF and AVH with HEV infection during pregnancy is higher compared to cases without pregnancy.

Topic: 1 Acute Liver Failure

Absno: 880

Do nucleotide substitutions in the hepatitis E Virus genome play a key agent in acute liver failure?

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Introduction: Preliminary studies suggest that the genotype of Hepatitis E virus influence the severity of hepatitis E. Viral capsid proteins play widespread role in interacting cellular proteins during capsid assembly and virus entry. Studies at the molecular level of the capsid protein of the HEV genome are limited. We aimed to study the molecular alterations in the HEV genome in patients with acute liver failure and acute viral hepatitis.

Materials and methods: A total of 32 patients with acute liver failure and 155 patients with acute viral hepatitis were screened for the study during the years 2011–2013. HEV IgM was detected by anti HEV IgM ELISA. HEV RNA was detected using Viral RNA extraction kit. The Open Reading Frame 2 (ORF 2) region of the HEV genome was amplified using Reverse Transcriptase PCR. Representative samples were directly sequenced. Full length nucleotide sequences of HEV isolates were retrieved from the Gen Bank /EMBL/DDBJ databases and compared with the strains. Sequences were aligned by CLUSTAL W software.

Results: The mean age of the AVH and ALF patients were 23.85 ± 3.68 years and 24.6 ± 3.32 years respectively. HEV RNA was detected in 84 (54.19 %) AVH and 24 (75 %) ALF patients respectively. A total of 15 nucleotide substitutions at various positions of the ORF 2 were observed after aligning the obtained sequence of 5 ALF patients with the other reference sequences. The nucleotide substitutions obtained were mainly silent substitutions with some conserved substitutions, one deletion and only a single amino acid change from lysine to cysteine in all the patients.

Conclusion: Substitutions encompassing these regions may play a crucial role in enhancing HEV replication through interactions with other proteins thus leading to disease severity. The single amino acid substitution and the silent substitutions may be associated with the poor outcome in ALF patients.

Topic: 1 Acute Liver Failure

Absno: 880

Ultra-deep sequencing analysis of the HAV 5' untranslated region among HAV-outbreak patients associated with a revolving sushi bar

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Chiba University, Graduate School of Medicine, Chiba, Japan

Aim: Hepatitis A virus (HAV) is a causative agent of acute viral hepatitis for which an effective vaccine has been developed. Here we describe ultra-deep pyrosequences (UDPS) of HAV 5'-untranslated region (5'UTR) among cases of the same outbreak, which arose from a single source, associated with a revolving sushi bar.
CONFERENCES ATTENDED
This document certifies that
Jayanta Borkakoti

was awarded a
National Scholar Award

at UEG Week Amsterdam 2012 for their abstract

VIRAL LOAD OF HEPATITIS E VIRUS: A KEY AGENT IN ACUTE LIVER FAILURE DURING PREGNANCY (OP153)

on Tuesday, October 23, 2012

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12 - 15 March 2014

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