INTRODUCTION

Viral hepatitis is a major public health problem in developing countries including India. Hepatitis E virus (HEV), a single-stranded positive-sense RNA virus, is the major cause of enterically transmitted non-A non-B hepatitis, particularly in developing countries (Emerson & Purcell, 2003). Transmission of the virus occurs primarily through the faecal–oral route by contaminated drinking water (Sarin & Kumar, 2006; Arankalle et al., 2000). Although young adults are mainly affected by the disease (Khuroo et al., 2004), the severity of hepatitis E is particularly high with a high rate of mortality in the third trimester of pregnancy (Khuroo et al., 1981). Transplacental transmission of HEV in the third trimester of pregnancy has also been associated with high perinatal mortality (Khuroo et al., 1999). Preterm delivery has been recorded in two-thirds of women infected with HEV. Vertical transmission of HEV has also been described in 33.3% of cases (Kumar et al., 2004). Studies carried out in Iran, Africa, Middle East and Asian countries have found high mortality due to acute liver failure (ALF) during pregnancy in women with HEV infection (Balayan, 1997; Dilawari et al., 1994; Arankalle et al., 1993; Tsega et al., 1993) and factors such as viral load, immunity, hormones (Jilani et al., 2007; Pal et al., 2005) and some signalling molecules (Prusty et al., 2007) have been strongly implicated.

Although HEV is transmitted by the faecal–oral route, it is not clear how the virus reaches the liver, the only site in humans where HEV is known to replicate. It is possible that the virus replicates at other sites in the body besides the liver; there is evidence of extrahepatic replication of HEV in animal models. Billam et al. (2008) identified sites of extrahepatic HEV replication in chickens following experimental infection with an avian HEV strain. Detection of positive-strand HEV RNA in a variety of tissues after clearance of viraemia, and the occurrence of a higher titre of HEV genome in faeces than in bile in human-HEV-infected pigs, suggests that HEV may replicate in sites other than hepatocytes (Williams et al., 2001). Since HEV is a positive-strand RNA virus, HEV replication produces a negative-strand RNA. Negative-strand-specific reverse transcriptase (RT-)PCR assay identified replicative viral RNA in gastrointestinal, colorectal, caecal, jejunal, ileal, duodenal and caecal tonsil tissues suggesting that after oral ingestion, HEV replicates in the gastrointestinal tract before it reaches the liver. When pigs were infected with swine HEV, replicative, negative-strand viral RNA was detected in the liver, lymph nodes, colon, small intestines and spleen (Williams et al., 2001). Similarly, for pigs inoculated with human HEV,
negative-strand HEV RNA was detected in the liver, lymph nodes, colon, small intestines, stomach, spleen, kidneys, tonsils and salivary glands.

In humans, extrahepatic sites for HEV replication have not been reported to date. However, observations of intratissue HEV infection and transplacental transmission of HEV with high fetal mortality are suggestive of possible extrahepatic replication of HEV in the placenta.

**RESULTS**

The mean ± SD age of HEV-infected ALF and acute viral hepatitis (AVH) pregnant subjects was 24.4 ± 3.3 years (n=22) and 22.9 ± 2.5 years (n=68), respectively. The mean period of pregnancy of ALF patients (28 ± 2 weeks) was shorter than for AVH patients (32.2 ± 2.4 weeks). Preterm delivery was more frequent in the ALF (18/22, 81.8%) than AVH group (36/68, 52.9%). Fetal loss due to intrauterine death (IUD) or stillbirth was 27.9% (19/68) in AVH and 86.4% (19/22) in ALF cases.

**Detection of HEV RNA in serum and tissue**

Serum HEV RNA was detected in 64.7% (44/68) pregnant AVH patients (Fig. 1a, b). Fourteen cases were also positive for HEV RNA in their placenta. Eight cases showed the presence of HEV RNA only in placenta but not in blood (Table 1).

In the ALF group, 19 out of 22 patients (86.4%) were positive for serum HEV RNA; five of these were also positive for tissue HEV RNA (Table 1, Fig. 1a, b). Three cases were HEV RNA negative both in placenta and blood but showed 100% fetal and patient mortality.

**Detection of replicative HEV RNA in placental tissue**

HEV-RNA-positive placental tissues of 22 AVH patients and five ALF patients tested positive for replicative negative-strand HEV RNA (Fig. 2a, b). All these patients were negative for replicative negative-strand HEV RNA in their blood cells confirming that the replicative HEV RNA in placental tissue was not derived from circulating blood and that the blood cells are not the site of HEV replication.

**Association of viral replication in placenta with pregnancy outcome**

Fetal loss due to IUD or stillbirth was 50% (7/14) in AVH patients positive for HEV RNA both in serum and placenta. Fetal loss was lower in AVH patients positive for serum HEV RNA and negative for tissue HEV RNA, (8/30; 26.7%). In patients negative for HEV RNA in both serum and placenta, no fetal loss was observed. None of the AVH cases was associated with patient mortality. Fetal loss and patient mortality was 100% (5/5) in ALF patients positive for both serum and tissue HEV RNA. ALF patients positive for serum HEV RNA but negative for tissue HEV RNA also showed high patient mortality (11/14; 78.5%) and IUD of the fetus (14/14; 100%).

**Estimation of viral load in serum and placenta**

There was no significant difference in serum and tissue viral load between AVH patients experiencing fetal loss and those who did not. The mean ± SD viral load in patients with fetal loss (n=19) was 8.1 × 10^2 ± 4.1 × 10^3 copies ml^-1 while that in the patients without fetal loss (n=49) was 8.7 × 10^3 ± 3.5 × 10^3 copies ml^-1 in serum (P=0.61), and in tissue was 5.5 × 10^2 ± 1.6 × 10^2 vs 6.5 × 10^2 ± 9.9 × 10^2 copies ml^-1 (P=0.07), respectively. In ALF cases where both patient and fetal death occurred (n=19) serum viral load was much higher than in the group where the patient survived but the baby died (n=3), (1.7 × 10^3 ± 1.5 × 10^3 vs 2.1 × 10^3 ± 5.7 × 10^2 copies ml^-1) (P<0.001). There was no detectable viral load present in the placental tissue of ALF cases where the patient survived with fetal loss. However, when both mother and baby died, viral load in placenta was 6.3 × 10^2 ± 2.6 × 10^1 copies ml^-1. Irrespective of the outcome of the pregnancy, the serum viral load of ALF patients was significantly higher than in AVH patients, whereas the tissue viral load in AVH and ALF groups was not significantly different (except for the ALF group where the mother survived but fetal death occurred, in which no tissue viral load was detected).

**Immunohistochemical detection of HEV in human placental tissue**

Positive staining of placental tissue sections with HEV antibody against the viral structural protein ORF3(pORF3) was found in all four representative AVH and five ALF.

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cases; there was no staining in the controls and negative controls. Fig. 3 shows representative images of tissue from four ALF and one AVH case. pORF3 is present on the surface of HEV particles released from infected cells. pORF3 is also a viral regulatory protein involved in mitogenic signalling pathways, virion morphogenesis and viral pathogenesis. Thus, the presence of pORF3 is indicative of an infectious or replicative viral state. These findings substantiate and confirm the presence of replicating HEV in the placenta, in agreement with the negative-strand-specific RT-PCR results, and represent the first identification of an extrahepatic site of HEV replication in humans.

**DISCUSSION**

It is known that during pregnancy, especially in late pregnancy, when levels of sex steroid hormones are high (Ponta et al., 1985), viruses are more likely to replicate and spread rapidly, attaining high titres in tissues and producing more severe disease. Haematogenous spread of viral infection from the mother to the placenta occurs early in the course of infection and virus replicates are found in the placenta in cases of human cytomegalovirus (Gabrielli et al., 2001). Placenta is known to be a site for viral replication in pregnant women with lassa fever; a
A high titre of lassa virus was found in placenta (McCormick et al., 1986). Although extrahepatic replication of viruses, particularly of HEV, has been shown in animal models (Williams et al., 2001), to date no HEV replication has been reported in human tissues other than hepatocytes. Although liver is the primary site for HEV replication, this virus can also infect the placenta and in the present study we demonstrate for the first time the extrahepatic replication of HEV in the human placenta.

In this study, replicative HEV RNA in placental tissue was detected using negative strand-specific RT-PCR (Fig. 2a). Because HEV is a single-strand positive-sense RNA virus, it produces an intermediate negative-strand viral RNA during replication. Therefore, detection of negative-strand viral RNA in tissues indicates active virus replication. In order to confirm that the viral RNA detected in placental tissue is not due to contamination from viral RNA in circulating blood and that the virus is replicating in the placenta, negative-strand-specific RT-PCR was also performed on RNA extracted from both blood and placental tissue. Most interestingly, HEV did not replicate in the blood cells as shown in Fig. 2(b). Immunohistochemical detection of pORF3 in placental tissue, indicative of an infectious state of the virus, confirmed these findings.

A higher frequency of preterm delivery was found in the ALF (18/22, 81.8%) compared with the AVH group (36/68, 52.9%). This finding is similar to that observed by Kumar et al. (2004) who reported that 14/21 women with HEV infection (66.6%) had preterm deliveries. It has been suggested that a significantly higher proportion of preterm deliveries occur in HEV-infected women; full-term deliveries were more common in non-HEV infected women (Patra et al., 2007). Immune-mediated damage to the placenta due to HEV infection could be a possible explanation; it was hypothesized that liver damage induced by HEV infection may be caused by immune responses to the invading virus rather than a direct result of viral replication in hepatocytes (Purcell, 1996). Pregnancy-related hormones and reduced cell-mediated immunity are established factors associated with hepatitis E severity and complications in pregnant women (Jilani et al., 2007). Exploring the placenta as an extrahepatic site of HEV replication is important because in placenta, the endocrine and immune system work together to protect the fetus by exerting an anti-abortive effect. During pregnancy, placental cells upregulate the expression of progesterone and its receptors; this plays a key role in retention of the fetal allograft in the mother’s body by suppressing cell-mediated immunity and creating a T-helper cell type 2 bias (Druckmann & Druckmann, 2005). Therefore, if damage occurs in the placenta due to HEV replication, maintenance of immune status might be hampered and IUD of the fetus might occur.

In the AVH group, the viral titre in serum and placenta did not differ between patients with and without fetal mortality. In the ALF group, the serum viral load was comparable between patients who survived or died. This suggests that there is no association between HEV viral load and fetal or maternal outcome of hepatitis E for AVH or ALF cases, respectively.

Interestingly, although serum viral load was much higher in ALF compared with AVH patients, tissue viral load was comparable between these two groups indicating that high
viral load in serum but not placenta is associated with the severity of the disease in HEV-infected pregnant women. Alternatively, it may not be the viral load per se but presence of replicating HEV in placenta in conjunction with high levels of sex steroid hormones that contributes to the severity of the disease.

Abortions and IUDs are common in pregnant women with hepatitis E. It has been reported that intrauterine infection and vertical transmission of HEV can lead to a high rate of abortions and IUDs in fulminant and even in non-fulminant AVH (12.4%) patients (Khuroo et al., 1995). In the AVH group of the present study, 22 out of 68 (32.4%) patients had evidence of HEV replication in their placenta. Fetal mortality was seen in 50% of patients positive for tissue HEV RNA; a lower level of fetal mortality (26.7%) was found in patients who were negative for tissue HEV RNA suggesting that HEV replication in placenta may be associated with fetal mortality. The presence of the most virulent genotype of HEV (genotype 1) in patients increases the severity of the disease (Purcell & Emerson, 2008).

The placenta may serve as a virus reservoir. Virus may be present in the placenta for weeks after clearance from blood and replicate in placental tissues (Pereira et al., 2005). Therefore identification of the placenta as an extrahepatic site of HEV replication is important and could stimulate the development of antiviral drugs that reduce infection at the uterine-placental interface.

METHODS

Study population. The present study included 90 HEV infected pregnant women. Of these, 68 patients exhibited acute viral hepatitis (AVH) and 22 acute liver failure (ALF).

Sample collection. The study period was May 2008 to September 2011. Blood samples were collected from patients admitted to the wards of Medicine and Gynaecology in Lok Nayak Jay Prakash (LNJP) hospital, Maulana Azad Medical College (MAMC), New Delhi. All patients were in labour and with symptoms of clinical jaundice. Patients were included on the basis of history, physical examination and liver function tests. Serological evaluation included hepatitis B surface antigen (HBsAg), IgM anti-HEV, IgM anti-hepatitis B core antigen (HBC), IgM anti-hepatitis A virus (HAV) and IgM anti-hepatitis C virus (HCV) using commercially available ELISA kits. The course of pregnancy was monitored under the supervision of a gynaecologist. Informed consent was obtained from all patients and/or from their close relatives. Serum was separated from the blood and stored at −70 °C until further processing. Placental tissues were collected from the patients during delivery, washed several times with PBS to remove adhering blood and stored in RNAlater (Qiagen). RNA later-stored placental tissues (30 mg) were homogenized in ice-cold PBS (600 μl) and the tissue homogenates were centrifuged at 3000 r.p.m. for 15 min at 4 °C to obtain a clear supernatant. Viral RNA was extracted from these supernatants using a QIAamp Viral RNA kit. Five microlitres RNA were subjected to cDNA synthesis at 37 °C for 1 h using random hexamer as primer and HEV RNA was amplified by nested PCR using primers for ORF1 (Jameel et al., 1992; Reyes et al., 1990). Outer primers: 5′-CCGGATCCACACACATC-TGAGCTACATTGTTGAGCT-3′ (sense primer), 5′-CCGAAATTCA-AAGGCATCATGGTTGTTGTAGATGC-3′ (antisense primer); inner primers: 5′-GGAATTCCACTCACCAGCAGTAC-3′ (sense primer), 5′-GGAATTCCACGCGCGCATTCA-3′ (antisense primer).

Detection of replicative HEV RNA in placental tissue. To detect HEV replication in placenta, negative-strand-specific reverse transcription PCR (RT-PCR) was performed. Viral RNA was subjected to cDNA synthesis using sense primer (5′-CCGGATCCACACACATC-TGAGCTACATTGTTGAGCT-3′) instead of random hexamer. To rule out the possibility of contamination of replicating tissue HEV RNA with that in the circulating blood, a test was performed to investigate replication of HEV RNA in blood cells. White blood cells were separated from 1 ml whole blood using erythrocyte lysis buffer containing 155 mM NH₄Cl, 10 mM, KHCO₃, and 0.1 mM EDTA. Cells were homogenized in ice-cold PBS (600 μl) and the homogenized lysate was centrifuged at 3000 r.p.m. (Biofuge Primo R, Heraeus) for 15 min at 4 °C to obtain a clear supernatant. Viral RNA was extracted from these supernatants using a QIAamp Viral RNA kit. To detect the presence of replicative negative-strand HEV RNA, cDNA synthesis was performed using the sense primer as described above.

Estimation of viral load by real-time PCR. Hepatitis E viral load was estimated in serum and tissue by real-time PCR (Rotor-Gene 3000, Corbett Research) using a reporter fluorochrome [6-carboxy-fluorescein (6-FAM)] system according to the manufacturer’s instruction (Genome Diagnostics, India), in a total reaction volume of 50 μl with 15 μl primers and probes mix, 5 μl MgCl₂ solution and 30 μl extracted sample or standard. The master mix contained reagents and enzymes for the specific amplification of HEV and for the direct detection of specific amplificons in the fluorescence channels 6-carboxyfluorescein (FAM) and 4,5-dichloro-dimethoxy-fluorescein (JOE) of the Rotor-Gene 3000. External positive standards (HEV S1–S5) were used to generate a standard curve allowing the determination of the viral load of unknown samples in subsequent runs. Calibration was performed at 55 °C FAM.

Immunohistochemistry. Placental tissues from five representative cases of ALF and four of AVH as well as two controls without any disease were subjected to immunohistochemical detection of HEV using anti-ORF3 polyclonal antibodies (Abbiotech). An equal number of cases were subjected to immunohistochemical detection without the primary antibody, serving as negative controls. This HEV antibody recognizes several putative HEV proteins including ORF3 (pORF3), a highly immunogenic protein from the viral capsid and structural proteins.

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REFERENCES


