

Prevalence of hepatitis E virus infection in West Bengal, India: a hospital-based study

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India is an endemic zone for hepatitis E virus (HEV), which is associated with both epidemic and sporadic infections. In West Bengal, only two hepatitis E outbreaks have been studied to date. However, sporadic cases of HEV infection also occur during inter-epidemic periods. The aim of this hospital-based study was to detect the prevalence of HEV infection in patients with acute sporadic hepatitis in West Bengal, India. Blood samples and clinical information were collected from 285 patients of both sexes and different ages with acute viral hepatitis (AVH) at Calcutta Medical College, Kolkata, a tertiary-care centre. Samples were tested for hepatitis B virus (HBV) surface antigen, anti-hepatitis C virus antibodies, anti-hepatitis A virus IgM and anti-HEV antibodies (IgM and IgG) by ELISA. Only those patients with AVH who were in their first week of illness and negative for all hepatotropic viral antibodies were tested for HEV RNA by reverse transcriptase nested PCR. HEV was identified as the most common cause of AVH (41.8% of patients), followed by HBV (21.4%), hepatitis A virus (17.2%) and hepatitis C virus (4.6%). Co-infections with more than one virus were found in 22 patients, with HBV–HEV the most common co-infection (3.8%). Only 14.7% of patients had no viral marker. To the best of our knowledge, this is the first documented epidemiological study of acute sporadic hepatitis with HEV in the state of West Bengal, India, indicating that this state is an endemic zone for HEV infection.

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BACKGROUND

Hepatitis E virus (HEV) causes a self-limiting viral infection that is transmitted by the faeco-oral route, primarily through the consumption of contaminated food and water. It can occur as both epidemic (Arankalle *et al.*, 1994) and sporadic cases (Arankalle *et al.*, 1993) in developing countries, with sporadic HEV infection occurring with increased frequency in both developing and developed countries (Aggarwal, 2011). Interestingly, this virus results in 20–30% mortality among pregnant women (Kumar *et al.*, 2004) and has been implicated as an important aetiological agent for sporadic fulminant hepatic failure in developing countries (Nanda *et al.*, 1994).

To the best of our knowledge, most documented studies on the incidence of acute sporadic HEV infection to date have been from the northern, western and south central parts of India (Amarapurkar *et al.*, 2008; Chadha *et al.*, 2003; Chandra *et al.*, 2012b; Das *et al.*, 2000; Jain *et al.*, 2013; Kaur *et al.*, 2003; Khuroo *et al.*, 1983; Kumar *et al.*, 2007; Madan *et al.*, 1998; Radhakrishnan *et al.*, 2000). Only one study has been reported from the eastern Indian city of Patna,

with fewer acute sporadic hepatitis patients (Fig. 1) (Bansal *et al.*, 1998). To date, only two hepatitis E outbreaks from West Bengal have been studied (Das *et al.*, 2007; Neogi *et al.*, 1995), although neither of those outbreaks was thoroughly documented or characterized. However, sporadic cases of HEV infection also occur in endemic areas, usually during periods between outbreaks. Therefore, the present study investigates the incidence of HEV infection in patients with acute sporadic hepatitis from West Bengal, India.

METHODS

Patients and samples. This study was carried out from 1 August 2012 to 31 October 2013 in Calcutta Medical College, Kolkata. This tertiary-care centre in West Bengal treats patients from Kolkata, neighbouring areas and cities, and the states of Bihar, Chhattisgarh, Jharkhand and Orissa.

Serum samples from 285 consecutive individuals with acute hepatitis of less than 42 days' duration were collected and stored at -80°C for further analysis. An acute viral hepatitis (AVH) case was defined as a person having an acute illness with a discrete onset of any sign or symptom (e.g. fever, headache, malaise, anorexia, nausea, vomiting, diarrhoea, abdominal pain) and either jaundice or elevated serum alanine aminotransferase levels higher than 100 IU l^{-1} on at least two occasions during a week without any history of pre-existing liver disease (United States Centers for Disease Control and Prevention,

Abbreviations: AVH, acute viral hepatitis; HAV, hepatitis A virus; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus; ORF, open reading frame.



Fig. 1. Prevalence of acute sporadic HEV infection in different parts of India.

2012). Patients who developed encephalopathy after the onset of icterus were considered to have acute liver failure (Acharya *et al.*, 2002). Information on each patient's age, sex and other related data was retrieved from patients' medical records. Patients with a previous history of liver disease, congestive heart failure, metastatic cancer, sexually transmitted disease or other infection within 6 months of presentation were excluded from the study. A total of 100 healthy persons who visited the centre for routine health examinations were included as a control group.

All serum samples were tested for anti-hepatitis A virus (HAV), hepatitis B virus (HBV) surface antigen (HBsAg) and anti-hepatitis C virus (HCV) antibodies, along with anti-HEV IgG and IgM assays for

the diagnosis of acute hepatitis E (AHE). Samples were also tested for the presence of HEV RNA.

Immunoassay. The samples were screened using commercially available micro-ELISA kits for HAV (DSI), HBV (HBsAg; DSI), anti-hepatitis B core antigen IgM in HBsAg-positive cases (Abbott Laboratories), HCV (J. Mitra, India) and HEV (DSI). ELISAs were performed as per manufacturers' protocols.

RNA extraction and reverse transcriptase PCR. HEV RNA detection was performed using serum samples from seven patients

within 7 days of illness whose samples were negative for anti-HEV antibodies and other virological markers. RNA was extracted using the GITC chloroform phenol method with minor modifications (Chomczynski & Sacchi, 1987) and subjected to cDNA synthesis. The cDNA synthesis was carried out using the MuLV reverse transcriptase enzyme, reverse primer (20 pmol ml⁻¹), RNaseOUT (20 U µl⁻¹; Gibco), 0.1 M DTT and 5 µl templates at 42 °C for 1 h. Following cDNA synthesis, PCR amplification was carried out using the specific primers selected from non-structural open reading frame (ORF)1 region (Gene Bank accession no. M-32400) (Jameel *et al.*, 1992). The primers used were external sense: 5'-CCGGATCCACACACATCT-GAGCTACATTCGTGAGCT-3'; external anti-sense: 5'-CCGAATTC-AAAGGCATCCATGGTGTGTTGAGAATGAC-3'; internal sense: 5'-GGAATTCGACTCCACCCAGAATTACTT-3'; and internal anti-sense 5'-GGAATTCACAGCCGGCGATCAGGACAG-3'. The thermal cycling conditions were initial denaturation 94 °C for 5 min, followed by 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 59 °C and extension for 30 s at 72 °C, as well as a final extension for 7 min at 72 °C. The final PCR products were analysed on 2% gel electrophoresis stained with ethidium bromide (10 mg ml⁻¹) using UV Transilluminator (Chandra *et al.*, 2012a).

Statistical analysis. Statistical analyses were performed using SPSS 19.0. The χ^2 or Fisher's exact tests were used to analyse categorical variables and ANOVA was used for continuous variables. Continuous variables are expressed as mean \pm SD and categorical variables are expressed as percentage (number). Results with *P* values <0.05 were considered significant.

Ethics. This study was approved by the institutional ethics committee. Participants were informed about the project and written consent was obtained from all participants before the use of their samples and clinical records. All data were securely stored in the study database.

RESULTS

A total of 285 patients who presented with acute hepatitis were included and screened for hepatotropic viral markers. All of the patients had abnormal liver function tests suggestive of acute hepatitis, and were sporadic cases with no apparent links between patients. Although patients were identified in every month throughout the year, the incidence of HEV infection was highest from March to May (Fig. 2).

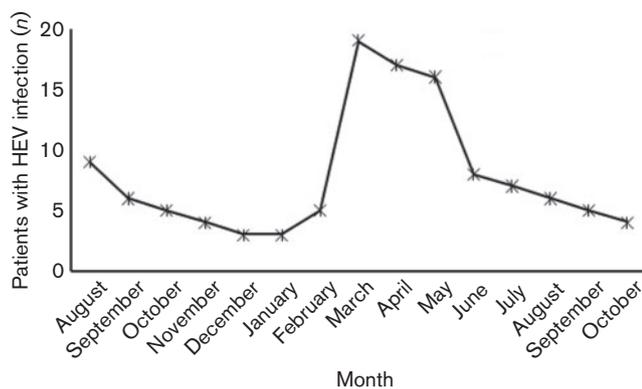


Fig. 2. Numbers of hospitalized patients with HEV infection from August 2012 to October 2013.

Table 1. Prevalence of causative agents of AVH

Testing for HEV RNA was performed in seven out of 42 patients within 7 days of illness; two of these seven patients (28.6%) were positive for HEV RNA.

Viral aetiology	Patients, n (%)
HAV	49 (17.2)
HBV	61 (21.4)
HCV	13 (4.6)
HEV	98 (34.4)
HEV + HBV	11 (3.8)
HEV + HAV	6 (2.1)
HEV + HCV	1 (0.35)
HAV + HBV	2 (0.70)
HBV + HCV	1 (0.35)
No viral marker	42 (14.7)

There was no obvious variation in the incidence of HEV cases in other months.

HEV was the most common cause of AVH, followed by HBV, HAV and HCV (Table 1). Of the 285 patients with acute hepatitis, 117 (41.05%) were positive for HEV either alone (*n*=98) or in combination with another hepatotropic virus (*n*=18) by serology (Table 1). All of the 117 patients with HEV infection were icteric, but otherwise presented with different clinical signs and symptoms at the time of enrolment into the study (Table 2). Among these 117 AVH patients, 70 had acute hepatitis and 47 developed acute liver failure during the course of infection. There was no mortality among the 70 AVH patients, while seven patients (14.9%) with acute liver failure died.

Of the 117 patients with HEV infection, 87 (74.4%) were male and 30 (25.6%) were female. The patients were aged 13–67 years (mean \pm SD 32.4 \pm 11.2 years). The age distributions of males and females were similar. The largest number of patients with HEV infection was seen in the age

Table 2. Characteristics and clinical features of patients with acute sporadic HEV infection

Data are expressed as mean \pm SD (range) or percentage.

Parameter	Value (<i>n</i> =117)
Age (years)	32.4 \pm 11.2 (13–67)
Height (cm)	151.4 \pm 5.06 (145–162)
Weight (kg)	51.05 \pm 5.76 (43–63)
Fever	82.8
Jaundice	100
Anorexia	85.1
Pale-coloured urine	93.7
Nausea	76.2
Vomiting	70
Abdominal discomfort	62
Hepatomegaly	21.4

group 21–40 years (Fig. 3). Out of 30 consecutively enrolled females, seven (23.3%) were pregnant. Two of these seven women (28.6%) died, compared with no deaths among non-pregnant women.

Infection with more than one virus was detected in 22 patients. The most common co-infection was HBV–HEV, which was seen in 11 patients (3.8%); three of these patients presented with AVH alone, while eight patients developed acute hepatic failure. Co-infection of HBV with another hepatotropic virus was found in three patients (one with HCV, two with HAV).

The 100 healthy individuals (75 males and 25 females) included in the study had a mean age of 29 ± 10.5 years (range 17–42 years). All sera were negative for HAV antibodies, HBsAg and anti-HCV used for the study of anti-HEV IgG and IgM assays in the diagnosis of AHE. These sera, when further tested for HEV RNA, were all found to be negative. Thus, none of the healthy individuals had anti-HEV IgM, while 23% were positive for anti-HEV IgG.

Among the 117 patients with acute HEV infection E, 41 were positive for anti-HEV IgM, 51 were positive for anti-HEV IgG and 25 were positive for both. When these three populations were compared, we observed that anti-HEV IgM positivity was significantly associated with higher levels of serum alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and total bilirubin (Table 3).

Serum samples from seven of 42 patients (16.7%) with acute sporadic hepatitis (within 7 days of illness and negative for all serological markers) were investigated for the presence of HEV RNA. Two patients (28.6%) were found to be positive for HEV RNA but, overall, 119 of 285 patients (41.8%) were found to be HEV positive by either ELISA or PCR.

DISCUSSION

HEV is the most common cause of AVH in developing countries, including India. HEV infection occurs in epidemics as well as sporadically, with periodic resurgences accounting for 30–70% of cases of acute sporadic hepatitis,

regarded as a major cause of acute liver failure (Acharya *et al.*, 2000). Several studies of AVH from India have documented varying prevalences of HEV in sporadic cases (Amarapurkar *et al.*, 2008; Bansal *et al.*, 1998; Chadha *et al.*, 2003; Chandra *et al.*, 2012b; Das *et al.*, 2000; Jain *et al.*, 2013; Kaur *et al.*, 2003; Khuroo *et al.*, 1983; Kumar *et al.*, 2007; Madan *et al.*, 1998; Radhakrishnan *et al.*, 2000), with results in accordance with those of the present study.

The present study found that HEV primarily affects young adults between the ages of 21–40 years, and is also reported in endemic regions (Arankalle *et al.*, 1995). Children younger than 10 years were excluded from the study, because anicteric hepatitis or subclinical infection is common in children younger than 9 years of age in cases of endemic hepatitis (Uchida *et al.*, 1992). An alternative explanation could be that HEV is maintained in the community as a sporadic infection; thus, HEV is acquired early in life, making infants and children immune to another attack (Chau *et al.*, 2006; Datta *et al.*, 1987).

In this study, 119 out of 285 ACV patients were diagnosed as having HEV infection. Among these patients, 117 were diagnosed by ELISA and two by PCR. It has previously been reported that PCR might be a better indicator than ELISA of acute HEV infection during the first week of illness (Clayson *et al.*, 1995). Hence, we tested seven of 42 acute sporadic hepatitis patients (within 7 days of illness and negative for all serological markers) for the presence of HEV RNA, and found that 28.6% were positive for HEV RNA. Based on our results and earlier reports, it appears that serological and molecular analyses should be combined for the diagnosis of viral infections, especially in endemic areas.

We also observed that HEV infection occurred throughout the year although seasonal variations in incidence were evident, with more patients with HEV infection from March to May than in any other month. This pattern is distinct from the epidemiological pattern in northern India reported by Aggarwal (2011), suggesting that seasonal patterns can differ by geographical area.

Furthermore, we compared the magnitudes and temporal relationships of anti-HEV IgM and IgG responses. In this study, the higher per cent positivity of anti-HEV IgG than IgM could be due to three possible reasons. First, delayed sampling might account for negative anti-HEV IgM results in some patients. Although HEV viraemia is short-lived in most patients (Chauhan *et al.*, 1993), serum anti-HEV IgM becomes detectable days before the onset of symptoms and disappears by 4–6 months (Favorov *et al.*, 1992; Singh *et al.*, 2008), whereas anti-HEV IgG appears soon after the IgM response and can persist for up to 12 years after infection (Chadha *et al.*, 1999). The second possible explanation is sequence variation among different genotypes. It has previously been reported that anti-HEV IgMs were undetectable in patients infected with HEV strain US-1 using an assay based on Burmese and Mexican strains (Schlauder *et al.*, 1998). Wide variations in the sensitivity

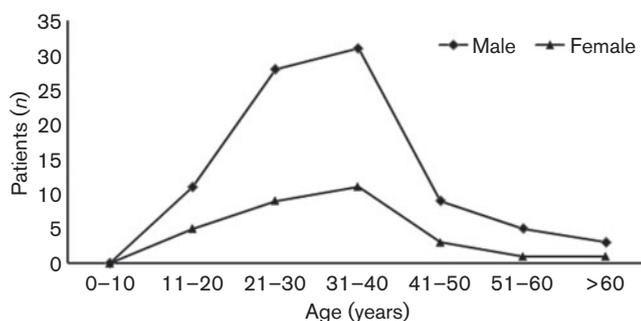


Fig. 3. Age distribution of the study population.

Table 3. Comparison of the clinical spectrum of patients seropositive for HEVData are expressed as mean \pm SD.

Marker	Anti-HEV IgM positive (n=41)	Anti-HEV IgG positive (n=51)	Anti-HEV IgM and IgG positive (n=25)	P value*
ALT (U l ⁻¹)	877.1 \pm 600.4	326.2 \pm 388.5	594.7 \pm 582.2	0.001
AST (U l ⁻¹)	1051.7 \pm 660.7	449.7 \pm 484.4	745.1 \pm 670.4	0.001
TBil (mg dl ⁻¹)	9.2 \pm 5.0	5.7 \pm 4.0	6.7 \pm 4.2	0.018
ALP (KAU l ⁻¹)	1223.3 \pm 706.9	646 \pm 535.7	844 \pm 688.1	0.003

*P<0.05 was considered statistically significant. ALT, Alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; TBil, total bilirubin. P value, compared by ANOVA test.

(17–100%) of ELISA kits have also been reported, depending on the recombinant HEV antigens or synthetic peptides used, which correspond to HEV epitopes. Interestingly, kits using the ORF2 sequence have shown little variation in geographically diverse strains compared with kits using ORF3 (Favorov *et al.*, 1992; Lin *et al.*, 2000; Longer *et al.*, 1993; Mast *et al.*, 1998; Ticehurst, 1999). Thirdly, poor host immune responses to HEV infection might account for undetectable anti-HEV IgM in patients with acute hepatitis. Therefore, anti-HEV IgM alone may not be informative for the diagnosis of acute sporadic HEV infection. In the present study, 21.4% of patients were positive for both anti-HEV IgM and IgG, which represents a transition phase during the course of infection.

In endemic areas, infection with HEV is known to be associated with other hepatotropic viruses such as HAV, HBV and HCV. In our study, dual infection with HBV, HAV and HCV in acute HEV patients was observed in 3.8, 2.1 and 0.35% of patients, respectively, without any squeal. Dual HEV–HBV infection could be related to reactivation of latent HBV as a result of clinical HEV infection. Other studies have also described the possibility of co-infection or superinfection with HEV and HBV, HAV or HCV (Arora *et al.*, 1996; Chandra *et al.*, 2012b; Kumar *et al.*, 2007; Mohanavalli *et al.*, 1996).

In conclusion, our findings indicate that West Bengal is an endemic zone for HEV infection, with detectable anti-HEV IgG antibody in 41.8% of AVH patients and 23% of healthy individuals. The high prevalence of anti-HEV IgG in healthy controls indicates the existence of subclinical infection. We also found that 14.7% of patients were negative for common hepatotropic viral markers. However, additional serological and molecular tests are required to identify other viruses and variants of known pathogens that may circulate in this part of India. To the best of our knowledge, this is the first documented study indicating the epidemiology of acute sporadic hepatitis with HEV in West Bengal.

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