Expanded Diagnostic Approach to Hepatitis E Virus Detection in Patients with Acute-on-Chronic Liver Failure: A Pilot Study

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Abstract

Introduction: Acute decompensation of pre-existing chronic liver disease (CLD), known as acute-on-chronic liver failure (ACLF), is associated with high mortality. Hepatitis E virus (HEV) as a potential cause was studied. Objectives: The objectives of this study are to evaluate the role of HEV in ACLF patients using an IgM anti-HEV antibody enzyme-linked immunosorbent assay (ELISA), HEV antigen ELISA, and a quantitative HEV polymerase chain reaction (PCR). Materials and Methods: In this prospective cross-sectional study, blood samples were collected from 50 ACLF (cases) as defined by the standard guidelines (APASL, 2014) and 50 patients with stable CLD (controls) from January 2015 to August 2016, after obtaining informed consent. Two IgM ELISAs (MP Diagnostics HEV IgM ELISA 3.0, Singapore and Wantai HEV IgM ELISA, Beijing, China) were compared using plasma from cases and controls. In addition, an HEV antigen detection by ELISA (Wantai, Beijing, China) and a real-time PCR for quantification of HEV RNA in plasma and stool were employed. Results: Alcohol was the leading cause of acute insult in ACLF (54%) cases. HEV infection accounted for 20% of cases. Ten ACLF patients (20%) had 1–3 markers of HEV versus two (4%) among controls ($P = 0.0138$). Among ACLF cases, one had HEV viraemia (403 IU/ml), faecal shedding (2790 IU/ml) and detectable HEV antigenemia. Agreement between the two anti-HEV IgM ELISAs was 0.638 (kappa value). Conclusion: This study shows that alcohol is a major contributing factor for both underlying CLD and ACLF while HEV is the most common infectious cause for ACLF, suggesting a need for a vaccination in such patients, whenever made available.

Keywords: Acute-on-chronic liver failure, antigen detection enzyme-linked immunosorbent assay, hepatitis E virus

Introduction

Hepatitis E virus (HEV) belongs in genus Orthohepevirinae and family Hepeviridae. It is a non-enveloped virus with a positive sense RNA of 7.2 kilobases surrounded by a capsid protein. The genome of HEV comprises three open reading frames (ORFs), which code for non-structural proteins (enzymes required for viral synthesis) and structural proteins (viral capsid). Annually, HEV is estimated to infect 20 million individuals leading to 3.3 million symptomatic cases and 44,000 hepatitis E-related deaths annually. Several HEV outbreaks have been reported from India mostly from a water-borne transmission. India has the highest number of HEV outbreaks reported as per a recent systematic review on the global burden of HEV outbreaks. HEV comprises four genotypes causing infections in both humans (genotypes 1 and 2) and animals (genotypes 3 and 4). HEV is widely distributed among the developing and developed countries of the world. In the developing countries, it is transmitted faeco-orally (genotypes 1 and 2) causing acute illness while in the developed countries, it presents as sporadic cases with a zoonotic mode of transmission (genotypes 3 and 4). Genotype 1 predominates in most of the central and south Asia including India.

Clinical presentation of HEV includes both hepatic and extrahepatic manifestations of the disease. Symptoms vary from asymptomatic illness to fulminant liver failure. Majority of acute HEV cases are asymptomatic and self-limiting while symptomatic infections occur only in a small proportion of...
cases. Studies from India have shown that HEV, especially genotype 1, is a significant cause of fulminant hepatic failure and increased mortality among pregnant women in up to 50% of the cases.\[^9\]\ Genotypes 1 and 2 cause acute infections while chronic HEV has been noted with genotypes 3 and 4. HEV also causes acute decompensation of a chronic liver disease (CLD) leading to liver cell failure and increased mortality,\[^10\]\ a clinical entity named acute-on-chronic liver failure (ACLF).

Laboratory diagnosis of HEV includes direct and indirect methods. Detection and quantification of HEV RNA from clinical specimens and antigen detection of capsid protein (ORF 2 protein) as target using an enzyme-linked immunosorbent assay (ELISA) are direct methods. Antigen detection methods are gaining popularity as a cost-effective alternative to HEV polymerase chain reaction (PCR) in resource-limited settings. Further, capacity to detect HEV antigen in stool renders it a non-invasive approach to detection of HEV infection. Commercially available assays have been validated by the manufacturer using plasma, serum and faecal specimens. HEV RNA detection and quantification and HEV antigen detection are becoming invaluable for the early diagnosis and management of HEV. At present, an established the WHO International Standard containing genotype 3a HEV RNA (code number 6329/10) with 250,000 IU/ml units is commercially available from the Paul Ehrlich Institute\[^11\]\ and the first WHO International Reference Panel for HEV genotypes 1–4 is available.\[^12\]\

Indirect detection methods include antibody detection assays for IgG and IgM. IgG antibody assays are mainly used in seroprevalence studies while IgM antibody assays are used in the diagnosis of acute HEV infection. A four-fold rise in IgG antibodies to HEV can be also used to diagnose recent infection. IgG assays may also be useful in reinfection where IgM may be negative but IgG and PCR are positive.\[^12\]\ Assays that detect early HEV infection are necessary for CLD patients, as they are at risk of decompensation.

Immune response to HEV plays a crucial role in the elimination of the virus as evidenced by spontaneous recovery of HEV among transplant recipients when their level of immunosuppression is reduced.\[^13\]\ Antivirals such as ribavirin and sofosbuvir are being used in the treatment of HEV infection, particularly in patients with ACLF and solid-organ transplants to reduce mortality.\[^13\[^14\]\ The role of direct and indirect markers in cases of ACLF is still not completely understood, and hence, the present study was undertaken to employ a comprehensive approach to the diagnosis of HEV infection in case of ACLF.

**Objectives**

The objectives of this study are to evaluate the role of HEV in ACLF patients using two IgM anti-HEV antibody ELISAs (MP Diagnostics HEV IgM ELISA 3.0, Singapore and Wantai HEV IgM ELISA, Beijing, China), HEV antigen ELISA and a quantitative HEV PCR.

**Materials and Methods**

In this prospective cross-sectional pilot study, blood and stool samples were collected from 50 ACLF (cases) admitted in the ward as defined by the APASL 2014 consensus guidelines and 50 patients with stable CLD (controls), both attending the liver clinic in this tertiary care hospital from January 2015 to August 2016 after obtaining informed consent. This study was approved by the institutional review board.

An ACLF case was defined as an acute hepatic insult manifesting as jaundice (serum bilirubin >5 mg/dl) and coagulopathy (international normalised ratio >1.5 or prothrombin activity <40%), complicated within 4 weeks by clinical ascites and/or encephalopathy in a patient with previously diagnosed or undiagnosed CLD or cirrhosis and associated with a high 28-day mortality.\[^15\]\ Controls were stable CLD patients of any aetiology attending regular checkup in the liver clinic. Patients who are on interferon or ribavirin therapy were excluded from the study.

After getting an informed consent, 7 ml of blood was collected in sterile vacutainer tubes containing K2 EDTA and was transported to the laboratory in cold containers at 4°C. Plasma was separated by centrifugation, aliquoted and stored at −80°C until testing. About 5–10 g of stool was collected in a sterile, wide mouth, screw-capped plastic container. A 20% suspension of the stool was made with 1% phosphate-buffered saline, vortexed thoroughly and centrifuged. The supernatant was aliquoted and stored at −80°C until testing. Necessary clinical information was sourced from the hospital records.

Plasma from cases and controls was subjected to two anti-HEV IgM ELISAs of varying principles (MP Diagnostics HEV IgM ELISA 3.0, Singapore and Wantai HEV IgM ELISA, Beijing, China), i.e., IgM detection and an IgM capture format, respectively, were compared. An HEV antigen ELISA (Wantai, Beijing, China) was also employed for plasma and stool from ACLF cases.

For HEV RNA quantification, RNA was extracted using the QIAamp RNA Mini Kit (QIAGEN, Hilden, Germany). An input volume of 200 µl was used for both plasma and stool supernatant. Quantification of HEV was done using a real-time PCR for plasma and stool (Fast Track Diagnostics, Qui-Si-Sana Seafront, Sliema SLM 3110, Malta) and performed on the Rotor-gene 3000 (Corbett Research, Sydney, Australia). Three quantification standards (QS1 = $3 \times 10^5$ IU/ml, QS2 = $3 \times 10^4$ IU/ml and QS3 = $3 \times 10^3$ IU/ml) provided with the commercial assay were included in each run. An in-house control from an archival stool sample (2982 IU/ml, ct = 29.18) collected during a previous HEV study was also used. The cycling conditions for the real-time PCR were reverse transcription step at 42°C for 15 min, initial denaturation at 94°C for 3 min followed by 40 cycles at 94°C for 8 s and 60°C for 34 s.

HEV RNA was estimated in IU/ml, and the ELISA results were compared using reactivity rate (RR), (RR = optical density of
sample [S]/Cutoff optical density value for that assay [CoV]). The ELISA results were interpreted based on the RR as negative (<1), positive (>1), and borderline (0.9–1.1), respectively.

**Results**

Majority of the cases and controls were between 31 and 60 years of age, males and were from Tamil Nadu. The aetiology for CLD among the cases and controls are shown in Table 1. The acute event most commonly precipitating ACLF was ethanol (54%), followed by HEV (14%) alone. Others were drug-induced (8%) and malignancy-like hepatocellular carcinoma (4%). There was one case each of hepatitis A infection, autoimmune flare up and sepsis leading to decompensation (6%). Dual aetiology due to ethanol and HEV (6%), drug-induced hepatitis in an HBV-related CLD (4%) and in an autoimmune-related CLD (2%). In one patient, the cause of acute injury was unknown (2%).

The median total bilirubin, direct bilirubin, aspartate transaminase and alanine transaminase levels compared among hepatitis E virus positive acute on chronic liver failure cases (Group 1), hepatitis E virus negative ACLF cases (Group 2), and controls (Group 3) are shown in Table 2.

In this study, ten of fifty cases (20%) and two of fifty controls (4%) had IgM antibodies to HEV ($P = 0.0138$). Of the ten cases, only one ACLF case showed HEV viraemia, faecal shedding and antigenaemia. Two IgM ELISAs with different principles were also compared. IgM detection ELISA showed ten cases and two controls while the IgM capture ELISA showed five cases and one control being positive for IgM anti-HEV antibody. Agreement between both these assays was 0.638 (kappa value).

Among these IgM antibody-positive samples, one plasma sample revealed HEV viraemia with a viral load of 403 IU/mL (ct value of 33.23). The same case also demonstrated HEV in the stool sample with a viral load of 2790 IU/mL (ct value of 29.28). All samples from controls were negative for HEV RNA in both plasma and stool.

Antigen was detected in plasma of one ACLF case who showed a positive mean RR of 6.971 as compared to 0.249 (mean + 3SD) of all disease negatives ($n = 99$). Antigen detection ELISA done on stool samples of all the ACLF cases showed a low positive for the same patient with a mean RR of 0.680 compared to the background of 0.149 (mean + 3 SD) of all the cases that were antigen negative ($n = 49$). The same patient was positive for plasma and stool HEV RNA, plasma antigen ELISA and both the antibody assays employed in this study. The RR of IgM detection assay and IgM capture assay of the ten ACLF cases are shown in Figure 1 along with the RR of the one case with HEV antigenaemia in the HEV antigen assay.

The earliest markers for viraemia and antigenaemia were demonstrated in the ACLF case who presented at the day 4 of illness, while all the antibody positives presented around a mean of 6.8 days (2.604–10.996 days). The overall mortality rate among the ACLF cases was 20% as compared to controls where no mortality was seen. HEV contributed to 10% of mortality in this study, only second to ethanol which was the leading cause of mortality among ACLF in this study.

**Discussion**

HEV is a RNA virus with an unique geographical distribution and transmission which varies between the developed and the developing nations around the world. In patients with pre-existing liver disease, HEV is believed to decompensate.
liver function, leading to ACLF resulting in an increased mortality among CLD patients. This study aimed at an expanded diagnostic approach for HEV, targeting different markers using assays with varying principles.

In patients with immunosuppression, where antibody production is impaired, detection of viral RNA/antigen in plasma and/or stool is the most efficient and accurate means of diagnosing HEV infection. Detection of HEV RNA in the stool is a non-invasive alternative and can be detected for at least 1–2 weeks after the vireaemia declines. Protracted faecal shedding of the virus can occur in patients with CLD, which makes stool a useful easy-to-collect specimen for the detection of HEV even if the patient presents after the viraemic phase.

A universally acceptable standard unit (IU/ml) is necessary for establishing treatment cutoffs, to document viral clearance, detect relapse and to recognise treatment failure. A well-standardised quantitative PCR with the ability to detect all HEV genotypes is necessary to understand the dynamics of HEV infection and explore more treatment options. The RNA quantification assay used in this study is capable of detecting all 4 genotypes. In this era where antiviral options are being explored, there is an urgent need of tools for early detection and quantification of HEV.

There is a paucity of studies on HEV causing ACLF from south India. This study was carried out with the aim of finding an appropriate and sensitive diagnostic assay for HEV detection and quantification in patients with ACLF. Studies in the past on HEV-related ACLF have shown a prevalence varying from 8% to 75% (median of 21%).[10] Indian reports studying the impact of HEV on patients with ACLF have showed a wide range of detection rates (4%–66%). An Indian study in 2004 from Lucknow showed a 44% positivity for HEV antibodies using IgM anti-HEV ELISA with a mortality of 14% among ACLF patients (Kumar et al., 2004). Similar studies from the same centre in 2009 showed a 66% prevalence of HEV using IgM HEV ELISA with a mortality of 44% among ACLF.[16] A prospective study in 2007 on cirrhotic patients revealed that 28% of cases had superimposed HEV infection using a nested PCR with a mortality of 43%.[17]

A 20% positivity for HEV markers among ACLF cases in this study was similar to earlier published data (median 21%).[10] HEV accounted for 10% of mortality in this study while other studies from India reported a median short-term mortality of 34%.[10]

This study revealed only one ACLF case with viraemia (403 IU/mL), faecal shedding (2790 IU/mL) and antigenaemia.
This was a cross-sectional study where only one blood and stool sample each was collected from patients for HEV RNA quantification and antigen detection. Being a referral centre, it is possible that ACLF cases may present later in their illness. Longitudinal studies and a larger sample size are needed to understand the duration of HEV viraemia, faecal shedding and antigenaemia to further optimise the diagnostic tools.

The time of presentation plays a vital role in the laboratory diagnosis of HEV. In this study, viraemia, faecal shedding, antigenaemia and IgM positivity were demonstrated in the same case who presented on the day 4 of illness. In previous publications from India, 100% positivity for HEV RNA and antigen was seen in patients presenting in the first 3 days of illness while the rates decreased to 54% for HEV RNA detection and 88% for antigen detection by 7 days of illness, respectively.[18] In this study, there was 100% correlation between the antigen detection ELISA and the HEV RNA positivity in a patient who presented early in illness. Similar findings in an Indian study showed that HEV RNA and antigen detection assays were comparable in detecting early infections. Detection of HEV viraemia and viral shedding requires appropriate sample collected early in illness while IgM antibody detection takes about a week to be positive and can be detected later in illness, as seen in Figure 2. The role of IgM antibody capture assays needs to be further studied to understand its role in eliminating any false positives as compared to ordinary IgM detection assays.

Effective antivirals such as ribavirin, sofosbuvir and pegylated interferon have been used in trials for treatment against HEV infection. In transplant recipients and ACLF patients, 3-month therapy with ribavirin has shown promising results with documented evidence of viral clearance.[14] Thus, with promising antiviral agents in the horizon, it is relevant to document viral loads to demonstrate the actual reduction of viral load/clearance of virus in response to treatment.

Awareness and vaccination against hepatitis A and hepatitis B among CLD patients has decreased the incidence of these infections among them. At present, there is no licensed vaccine or standardised treatment protocols in India. A HEV vaccine recently licensed in China (Hecolin®) has promising long-term efficacy.[19] Such vaccines may be beneficial to patients with CLD in our country who are at a risk of developing ACLF with an increased mortality.

**Conclusion**

This study comprehensively evaluated HEV markers in ACLF patients demonstrating a significant role of HEV in the

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**Figure 2:** Comparison of time of presentation of the 10 hepatitis E virus cases. The percentage positivity for the various assays employed (hepatitis E virus RNA, Antigen detection [Wantai] assay, IgM detection [MP Diagnostics] assay and IgM capture [Wantai] assay) are compared with the days of presentation of the cases.
causation of ACLF. HEV was the most common infectious cause of ACLF with an occurrence of 20% and a mortality of 10% in this study. There is clearly a need to have an expanded diagnostic approach for HEV in such individuals.

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Conflicts of interest
There are no conflicts of interest.

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