SHORT COMMUNICATION

Role of formalin fixed paraffin embedded liver tissues in the diagnosis of viral hepatitis E in patients with undiagnosed acute liver failure

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Abstract Viral hepatitis E is gaining importance as an emerging worldwide disease. Though viral hepatitis E (HEV) has been attributed as an etiology of acute liver failure (ALF), however its actual incidence and the immunopathogenesis are being under explored. The present study is aimed at detecting HEV in post mortem liver tissues of patients with undiagnosed ALF. Twenty six liver tissues of ALF patients died of unknown etiology are subjected to nested reverse transcriptase PCR with HEV *ORF1* gene targeted primers and HEV RNA is detected in 30.4% (7/23) of ALF cases. Out of seven HEV RNA positive cases, three (42.8%) had HEV antigen positivity by immunohistochemistry on liver tissues using HEV ORF2 antibody. Histopathological examination by H&E staining shows multiacinar confluent hepatic necrosis, bile ductular proliferation, bridging hepatic and periportal necrosis in 4, 4, 2 and 1 cases respectively. The isolates were sequenced using *RdRp* gene specific primers and found to HEV genotype-1. Quantitative TaqMan real time PCR estimated the viral load ranged between 7.77×10^3

and 1.44×10^7 viral copies/ μ l. HEV has been associated with 30.4% (7/23) of undiagnosed ALF cases. Immunohistochemistry along with molecular testing in FFPE biopsies might be useful for the detection of HEV in undiagnosed cases.

Keywords Hepatitis E virus · Acute liver failure · Genotype-1 · Formalin-fixed paraffin-embedded · Immunohistochemistry

In recent years, hepatitis E virus (HEV) has been recognized as a major health problem in developing countries [2] and in India has long been known to cause large outbreaks [16]. Though neglected for a long time in developed countries [8], recently, it has been emerging as an important zoonotic disease. The disease ranges from asymptomatic, acute self-limiting viral hepatitis (AVH) to acute liver failure (ALF), with mortality ranging up to 25–30% in pregnant women [1, 10]. Recently, hepatitis E with chronicity has been reported in patients with organ-transplantation. HEV has been evolving as a predominant etiological agent of sporadic hepatitis in India where large outbreaks in the past had been reported [12]. Furthermore, 30–45% of the ALF cases reported from the Indian sub-continent are found to be positive for HEV serological marker. However, there exists very scanty literature on the HEV positivity in liver tissues in unidentified cases of viral hepatitis. Information on the cellular responses, characteristics of intrahepatic immune cells and histopathological alterations in liver in patients with hepatitis E are also sparse [15]. Primarily anti-HEV IgM antibodies or viral RNA in sera remains the mainstay of HEV diagnosis [5]. Due to inherent risk of internal bleeding, liver biopsy is not the preference in cases of acute viral hepatitis, however, in

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acute liver failure cases, biopsies are routinely performed for clinical etiology.

With the hypothesis of sequestration of HEV virus and viral antigen in the liver tissue and due to intrahepatotropic nature of the virus, the present study was designed involving intrahepatic detection of HEV specific viral antigen by immunohistochemistry (IHC), HEV strain characterization by reverse transcriptase PCR, sequencing, viral load by real time PCR and their correlation with histopathological findings as an indicator for disease severity in the formalin-fixed paraffin-embedded (FFPE) liver tissues of undiagnosed ALF cases.

Retrospectively, twenty three FFPE liver tissues from undiagnosed acute liver failure (ALF) and three liver injury cases mediated by drugs (disease control) were enrolled in this study. All the tissues were retrieved from the archives of the Department of Histopathology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. This study was carried out following approval of the Institutional Ethics Committee (IEC).

HEV detection, sequencing, genotyping and phylogenetic analysis were performed. For RNA extraction from FFPE liver tissues, five sections of 10 µm thickness from each FFPE tissue were prepared and processed for nested reverse transcriptase PCR. The tissue sections were incubated for deparaffinization in xylene at 65 °C for 30 min. Samples were lysed in Proteinase K along with tissue lysis buffer (Qiagen, Hilden, Germany) after two washes with ethanol (100%). Subsequently, total RNA was extracted using Pure Link™ FFPE Total RNA Isolation kit (Invitrogen, US). After verifying the RNA purity, RNA (10 µl) was converted into cDNA using cDNA RT kit (Applied Biosystems™, US) and the nested RT PCR was run using specific primers against HEV ORF-1 gene (Table 1) [7] along with recombinant (pIRES-neo-ORF1) plasmid clone

as the positive control (kindly gifted by Dr. S. Jameel, New Delhi). Seven FFPE liver tissues out of 23 cases (30.4%) with acute liver failure of unknown etiology were positive for HEV RNA by conventional RT PCR (Fig. 1). None of the three cases with drug induced liver failure (disease control) had HEV RNA in their liver tissues.

Genetic variability within the 3' end of ORF-1 gene coding for RNA-dependent RNA-polymerase (RdRp) was analyzed according to Majumdar et al. [14]. Specific bands (~ 560 bp) were excised from the gel and were purified by Gel Extraction kit (Qiagen, Hilden, Germany). Sequencing of the purified product was done by BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems™, US) with appropriate sense (RdRp-3: 5'-ACTCACTGCAAAGCACTATCGAAT-3') and antisense primers (RdRp-4: 5'-GACGTGTCCAGGATCACCTTCTTC-3'). Sequence analysis and comparison using BLAST tool was done in ABI 3500 automated DNA sequencer platform. Multiple sequence alignment and phylogenetic tree analysis were done using Clustal X 2.0.11 and MEGA 7 software respectively. Sequence analysis of RdRp region of three representative samples targeting ORF-1 region belonged to genotype-1 with GenBank accession numbers KX870188, KX870189 and KX870190 respectively. These strains showed a genetic relatedness of 93.3% with the HEV strains of Nepal and previously identified sequences from north India (Fig. 2).

For viral load estimation, cloning of 343 bp product was done by pGEM-T Easy cloning vector (Promega, USA). Competent *E. coli* DH5α cells were used for plasmid DNA transformation and recombinant plasmids containing desired gene insert were isolated from white colonies on LB/ampicillin/IPTG/X-gal plates using Invitrogen Pure Link Plasmid Miniprep Kit. Through 10 fold dilution of the plasmid DNA, the limit of detection in Real Time PCR

Table 1 Showing *ORF-1* and *RdRp* gene primers and TaqMan real time PCR primers

Primer	Sequence (5'–3')	Location	Product (bp)
<i>ORF1 gene amplification by nested RT-PCR</i>			
<i>HEV-1</i>	CCACACACATCTGAGCTACATTCGTGAGCT	4957-4928	343
<i>HEV-2</i>	AGGCATCCATGGTGTGTTGAGAATGAC	4382-4407	
<i>HEV-3</i>	CGACTCCACCCAGAATAACTT	4420-4439	
<i>HEV-4</i>	CACAGCCGGCGATCAGGACAG	4747-4727	
<i>RNA dependent RNA polymerase (RdRp gene) nested RT-PCR</i>			
<i>RdRp-1</i>	ACACACATCTGAGCTACATTCGTGAG	4930-4955	603
<i>RdRp-2</i>	ACATTTGAATTAACAGACATTGTGCA	3880-3905	
<i>RdRp-3</i>	ACTCACTGCAAAGCACTATCGAAT	4682-4705	
<i>RdRp-4</i>	GACGTGTCCAGGATCACCTTCTTC	4147-4170	
<i>Taq-Man real-time PCR primer-probe</i>			
<i>HEVNI_F</i>	GGAGTCTCTGCGAGGGTTTTG		
<i>HEVNI_R</i>	CAGCCATATTCCAGACAGTATTCCA		
<i>HEVNI_M</i>	FAMCCCGGCACTCTTCT5NFQ

Fig. 1 Gel electrophoresis image showing amplification in positive samples at 343 bp. **MM**: molecular marker (100 bp); **NC**: negative control; 1–23 (undiagnosed ALF sample); 1–3 samples from drug induced liver injury cases); 3: *P-18822/8; 5: P-26254/15; 10: *S-18309/15; 14: S-25590/14; 15: S-23915/14; 20: P-23059/12; 22:

S-15265/16; **PC**: positive control (recombinant pIRES-neo-ORF1 plasmid clone). *(P-18822/8: P denotes post mortem samples, 18822 is slide no./8 year 2008). *(S-18309/15: S denotes Biopsy, 18309 is slide no./15: year 2015). Details of each sample given in Tables 1 of Electronic Supplementary Material

Fig. 2 Phylogenetic analysis of Hepatitis E virus strains isolated in this study from FFPE liver tissues, (KX870188/PGIMER/FFPE/India/G-1; KX870189/PGIMER/FFPE/India/G-1; KX870190/PGIMER/FFPE/India/G-1) were performed by constructing NJ tree for the conserved RdRp region (ORF-1) of HEV. Avian (*JN597006.1* and *JN997392.1*) and Rat (*GU345042.1*) HEV strains were included as an outlier group. Length of the branches represent distances computed using the p-distance method. Bootstrap value of 1000 replicates was used for statistical verification. Evolutionary analysis was conducted in MEGA 7

assay was evaluated. For viral load quantification, 1 μ l of cDNA was used in Real Time PCR by custom TaqMan Gene Expression assay kit (Table 1) (Applied Biosystem, USA). HEV genome copy number was estimated using

HEV specific TaqMan MGB probes and primers. Viral load in seven HEV RNA positive tissue samples ranged between 1.44×10^7 and 7.77×10^3 genome copies/ μ l with a mean viral load of 4.183×10^6 copies/ μ l.

HEV antigen detection and histopathological characterization was performed by immuno-histochemistry (IHC) and haematoxylin and eosin (H&E) staining. Five μm tissue sections from 26 FFPE liver blocks were prepared on pre-coated pol-L-Lysine slides (Sigma-Aldrich, USA) and processed for the detection of HEV-pORF-2 protein. Briefly, the sections were de-paraffinized in xylene (100%), rehydrated in different decreasing concentrations of ethanol. Citrate buffer (pH 6.0, 10 mM) (Sigma-Aldrich, USA) was used for 1 h for antigen retrieval. The slides were incubated with hydrogen peroxidase (Sigma-Aldrich, USA) and methanol to quench endogenous peroxidase activity in a ratio of 1:9 and finally washed with PBS. Slides were incubated for 1 hour with three different dilutions of primary polyclonal antibody (HEV ORF2 antibody, Biorbyt, UK) targeting the viral protein. Following primary incubation, slides were incubated with peroxidase-conjugated Goat Anti-Rabbit IgG (AffiniPure) for 45 min and detected by diaminobenzidine (DAB; Dako, Agilent Pathology Solutions). Counterstained slides were scanned under upright light microscope (Nikon eclipse TS100) for the presence of viral antigen. For the histopathological examination, H&E staining were performed to check the extent of architectural alterations, inflammation and hepatocyte damage assessed by bridging, peri-portal or multi-acinar necrosis along with features of fibrosis and steatosis. Three (3) of the seven (7) HEV RNA positive FFPE liver tissues and none of the disease control group had characteristic brownish granular stained (Fig. 3a, b) hepatocytes with heterogeneous staining indicate the HEV antigen positivity in tissues. Of the 7 HEV RNA positive tissues, multiacinar confluent hepatic necrosis was found in 4, bile ductular proliferation in 4, bridging hepatic necrosis in 2 and peri-portal necrosis in 1 case on H & E staining. Information from their Medical Records revealed the mean value of liver enzymes 536 U/l (AST), 885.6 U/l (ALT), 331 U/l (ALP) along with PT (55 s), PTI (95%), INR (3.95) and APTT (70 s). Three biopsies had intracytoplasmic and intracanalicular cholestasis. Drug induced ALF disease controls, showed extensive hepatocytic loss with multiacinar confluent hepatic necrosis.

Hepatitis E is a major entity of epidemic in most of the developing countries including Indian subcontinent with an estimated one third of the global population infected with hepatitis E [9]. Although majority of hepatitis E associated AVH is self-limiting, 30–45% of the ALF cases reported from Indian subcontinent have been positive for HEV serological markers [8]. Recently, increase in incidence of viral hepatitis E following organ transplants and blood donors has labeled it as an important pathogen in developed countries. In general population HEV is associated with a mortality as low as 0.2–1% however, an increase upto 20–30% has been observed in pregnant woman

Fig. 3 Immuno-histochemical detection of HEV ORF 2 antigen in HEV infected ALF FFPE liver tissue (a) as compared to a HEV ORF2 antigen negative ALF FFPE liver tissue (b)

[10, 11]. Contamination of drinking water supply with sewage is common mode of viral hepatitis E transmission contributing to endemicity in North India.

HEV antigen in acute phase, served as an early diagnostic marker with a good correlation with HEV viremia and HEV RNA [14]. High levels of circulating HMGB1 protein in ALF patient with a level ≥ 250 ng/ml have invariably been associated with severity and acute liver failure, indicating a bad prognostic marker of liver damage [13].

In conclusion, the present study, autopsied liver tissues (FFPE) of 23 clinically diagnosed acute liver failure of unknown etiology were selected from archives of Histopathology department between years 2004 and 2016. Three cases of drug induced liver hepatotoxicity were included as disease control. Of the seven HEV RNA

positive samples, 3 (42.8%) had HEV antigen in liver tissues by immunohistochemistry. However, HEV antigen (ORF2 and 3) detection by immunohistochemistry (tissues) has been thought to be superior to molecular detection of HEV RNA (serum) in confirmed HEV infected individuals [6]. The low IHC positivity might be attributed to extensive tissue necrosis, and loss of structural integrity of hepatocytes in ALF cases. In addition, TaqMan Real Time PCR detection of viral copies of HEV in the tissues suggested the importance of specific TaqMan real time Primers and probes against HEV. Viral genome copy number in the positive samples ranged between 1.44×10^7 copies/ μ l and 7.77×10^3 copies/ μ l with mean viral load of 4.183×10^6 copies/ μ l. Thus, the in-house standardized qualitative PCR found to be as sensitive as real time PCR in accordance with Bastien et al. [3]. HEV Genotype 1 is the most prevalent genotype in Indian subcontinent which appears to be more virulent than zoonotic strains (Genotype 3 and 4) reported from developed nations like Germany [4]. Though the sample size is small ($n = 23$), it sheds light on the significance of HEV in archival liver tissues to understand the viral etiology and HEV burden through HEV RNA by conventional PCR, HEV antigen by IHC, viral load as well as genotyping followed by sequencing.

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