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# Role of TLR gene expression and cytokine profiling in the immunopathogenesis of viral hepatitis E

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ABSTRACT

*Background:* The clinical manifestations of Hepatitis E virus (HEV) range from self-limiting acute viral hepatitis (AVH) to acute liver failure (ALF). The varied clinical course is thought to be immune-mediated. Toll-like receptors (TLRs) play a central role in sensing and initiating innate antiviral-response and downstream signaling of TLRs modulates cytokine production, thereby playing an important role in determining the disease course.
*Objectives:* The present study was designed to elucidate the role of TLRs and cytokine production in the immunopathogenesis of HEV.
*Study design:* Peripheral blood mono-nuclear cells were separated from 50 AVH-HEV, 30 ALF-HEV patients and 50 healthy-controls. One-part of the PBMC was processed for RNA-extraction another pulsed with HEV-ORF2-peptide. Gene-expression levels of TLR (2–4, 7, and 8) were checked using semi-quantitative Real-time-PCR. Cytokine levels were analyzed using Cytokine-Bead-Array. TLR3-silencing experiments were performed and post-silencing cytokine levels were estimated.
*Results:* TLR3 gene-expression in AVH was significantly higher than ALF ($202.4 \pm 36.36$ Vs $13.71 \pm 5.01$; $p < 0.0001$). Higher amount of both anti-and pro-inflammatory cytokines; IFNγ, TNF-α, IL10 and TGF-β were detected in the PBMC culture-supernatant of AVH Vs ALF ($p < 0.0001$, $p = 0.0008$, $p = 0.0002$, $p < 0.0001$ respectively). Post-silencing TLR3, significant decrease in IFNγ level was observed in the PBMC culture-supernatant ($4.08 \pm 1.06$ Vs $23.20 \pm 12.51$; $p$ =N0.0213).
*Conclusions:* TLR3 and IFNγ were found to play an important role in HEV disease pathogenesis. Patients capable of expressing high levels of TLR 3 and robust IFNγ response are able to limit the disease and recover uneventfully; while the patients with lower expression of TLR3 and IFNγ progress to ALF.


## 1. Background

Cloning and sequencing of the novel agent of enterically transmitted viral hepatitis has revolutionized the understanding of Hepatitis E virus (HEV) infection [1–3]. Annually 3.3 million cases of acute hepatitis E with 56,600 deaths have been reported by World health organization (WHO) [4].The disease ranges from asymptomatic infection to self-limiting acute viral hepatitis (AVH) and acute liver failure (ALF). In India nearly 30–70% of sporadic hepatitis cases are due to Hepatitis E virus [5,6]. Further, evidence of HEV infection has been detected in 30–45% of ALF cases [7]. Detection of anti-HEV IgM remains the conventional method of diagnosis [8]. In contrast, information on cell mediated immune response against HEV is limited. Studies have reported that AVH patients can mount T-cell mediated interferon-γ (IFN-γ) and tumor-necrosis factor-α (TNF-α) production [9,10], whereas, significantly lower lymphocyte proliferation index has been documented in ALF patients on stimulation with HEV specific immunogenic peptides [11].

Pathogens have unique signature molecules (PAMPs) that are recognized by Toll like receptors (TLRs) which results in either activation of antigen presenting cells and/or co-stimulation of T-cells, inducing both innate and adaptive immunity [12,13]. The expression level of TLRs can have direct effects on T cells, for example, expression of TLR2 will enhance T cell proliferation and will gener-

*Abbreviations:* AVH, acute viral hepatitis; ALF, acute liver failure; CTLs, cytotoxic T lymphocytes; DCs, dendritic cells; HEV, hepatitis E virus; MODS, multiple organ dysfunction syndromes; ODN, oligodeoxynucleotide; ORF2, open reading frame 2; PBMCs, peripheral blood mononuclear cells; PRRs, pattern-recognition receptors; PAMPs, pathogen associated molecular patterns; SIRS, systemic inflammatory response syndrome; TLRs, toll like receptors; WHO, World health organization.

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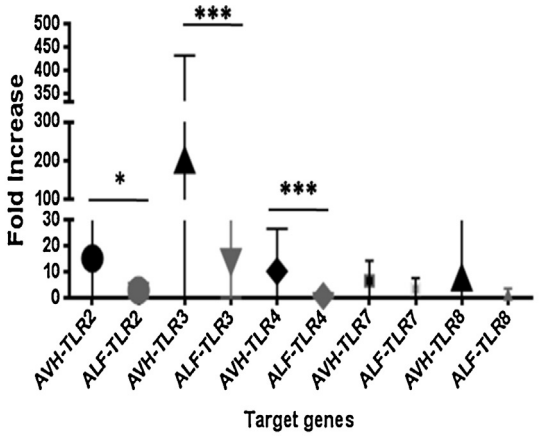


| | TLR-2 | TLR-3 | TLR-4 | TLR-7 | TLR-8 |
|---|---|---|---|---|---|
| **AVH (Un-stimulated)** | 15.26±4.39 | 202.4±36.36 | 10.25±2.58 | 6.52±1.23 | 7.99±4.07 |
| **ALF (Un-stimulated)** | 2.84±0.79 | 13.71±5.01 | 0.69±0.51 | 3.27±1.22 | 2.29±0.66 |
| **AVH Vs ALF P-value; Mann-whitney** | **0.022** | **<0.0001** | **<0.0001** | 0.428 | 0.089 |

**Fig. 1.** Representing gene-expression profile of TLRs in HEV patient's PBMCs, clinically presented with features of AVH or ALF in un-stimulated condition as compared to the expression in healthy controls. The gene-expression of TLRs in AVH patients was higher than ALF patients. TLR2, 3 and 4 were significantly up regulated in AVH as compared to ALF in un-stimulated condition and marked bold faced.

ate efficient memory T cells [14]. TLR3 mediates cellular response to double stranded RNA (dsRNA) and promote activated $CD4^+$ T-cell survival [15]. Further,TLRs can shape the outcome of viral infections via producing an array of cytokines [16]. TLR signaling has been found to play a key role in HBV and HCV infections [17,18].

## 2. Objectives

The current study was undertaken to determine the gene expression profile of TLR 2–4, 7 and 8 in HEV patients clinically presenting with AVH or ALF. Further, the cytokine response on stimulation of patient PBMCs with HEV-ORF2 immunogenic peptide (452–617a.a.) and the effect of in-vitro silencing TLR3 was studied to understand the roles played by TLRs in HEV pathogenesis.

## 3. Study design

The patients with clinical presentation of AVH or ALF with suspected viral etiology were recruited in this study during April 2009–June 2012. They were tested for the presence of viral hepatitis markers i.e., hepatitis B surface antigen (HBsAg; J Mitra, India), anti-hepatitis C antibodies (anti-HCV; J Mitra, India), anti-hepatitis A IgM (anti-HAV IgM; Orgenics, Israel) and anti-hepatitis E IgM (anti-HEV IgM; ImmunoVision, USA) using commercially available Enzyme linked immunosorbant assay (ELISA) kit, as per the manufacturer's instructions. The patients who were positive for anti-HEV IgM and negative for anti-HAV IgM, HBsAg and anti-HCV antibodies by ELISA, were enrolled for the study proper.Age and sex matched, apparently healthy individuals were additionally tested for anti-HEV IgG (DSI,Italy), and those found negative for HBsAg, anti-HCV, anti-HAV IgM, anti-HEV IgM and IgG were recruited in the healthy control (HC) group.The study group comprised of 50 AVH, 30 ALF and 50 healthy controls. Chronic alcoholics, patients with history of hepatotropic drug use, immunosuppressant medication, chronic liver disease, hepatitis due to non-infectious or known bacterial cause were excluded from the study. The study was commenced following the approval of the institute ethics committee as per the National guidelines.

### 3.1. Definitions

Self-limiting Acute viral hepatitis (AVH) due to HEV was defined as patients positive for anti-HEV IgM, with serum aspartate aminotransferase elevation of at least five-fold or clinical jaundice or both. ALF was considered when the patient develop evidence of coagulation abnormality with an INR $\geq$1.5 and develop encephalopathy with an illness of <26 weeks duration, without any history of pre-existing liver disease [19].

**Table 1**
Serum biochemical parameters of AVH and ALF patients included in this study.

| Variables | AVH ($n$ = 50) (Mean ± SD) | (95% CI) | ALF ($n$ = 30) (Mean ± SD) | (95% CI) | $p$-Value |
|---|---|---|---|---|---|
| Bilirubin mg/dl | **13.21 ± 7.74** | **(10.67–15.76)** | **18.85 ± 9.66** | **(15.49–22.22)** | **0.0054** |
| ALT IU/ml | 764.2 ± 703.2 | (496.7–1032) | 710.4 ± 1064 | (350.4–1070) | 0.2376 |
| AST IU/ml | 779.4 ± 833.1 | (468.3–1091) | 326.4 ± 325 | (213.0–439.9) | 0.0570 |
| ALP IU/ml | 388.9 ± 261.9 | (287.4–490.5) | 256.4 ± 144.6 | (211.3–301.4) | 0.0607 |
| PT in seconds | **16.09 ± 8.47** | **(11.88–20.31)** | **33.66 ± 15.88** | **(28.12–39.20)** | ***p*** < 0.0001 |
| PTI | **78.55 ± 24.78** | **(66.95–90.14)** | **47.00 ± 20.91** | **(39.33–54.67)** | ***p*** < 0.0001 |

Results are expressed as mean ± standard deviation. $p < 0.05$ was considered significant. Values which significantly differed in the two groups were marked in bold. ALT, alanine transferase; AST, aspartate transferase; ALP, alkaline phosphatase; PT, prothrombine time; PTI,prothrombine time index; IU/ml, international unit/ml. AVH and ALF patients were infected with HEV genotype 1.