SUMMARY
VII SUMMARY

Viral hepatitis remains a major public health problem and the most common cause of liver disease worldwide. Hepatitis E virus, the causative agent for hepatitis E (ET-NANABH) is mainly transmitted by feco-oral route and is prevalent nearly in all developing countries, sharing the poor socio-economic and hygienic conditions. Though primarily a self-limiting disease, HEV is reported to be conspicuously associated with a high incidence of fulminant hepatitis. The genome of HEV is approximately 7.5 kb, positive sense, single strand RNA that is capped and polyadenylated. It contains three distinct open reading frames, ORF1 coding for non-structural protein, ORF2 and ORF3 that encode for structural proteins. Immunogenic epitopes have been reported in all ORFs of hepatitis E, however, the significance of host defense and immunopathological reactions in the pathogenesis of infected hepatocytes in the pathogenesis damage is not clear.

The hepatitis viruses are known to be non-cytopathic, the liver damage invariably both in the acute and chronic hepatitis results due to host responses directed against the viral antigens. It is believed that the quality of immune response to one or more viral proteins contributes to liver injury, viral clearance and outcome of the disease. The present study makes an attempt to understand the cellular response and the pathogenesis in hepatitis due to HEV infection in humans. The studies in cellular immune response in hepatitis B and hepatitis C have been well documented in the past but no significant is reported for the host response to hepatitis E virus. Before attempting to unravel the status of cellular immune response in acute patients with and without HEV infection, the study was initiated to understand the role of complement components and nitric oxide by assaying the levels of C4 and C3 component of complement and nitrite ions in the sera samples from prospective and retrospective patients. Further, studies were carried out to understand the T cell mediated cytokines Th-1/ Th-2/ Th-3 type response to potent HEV antigens in patients and healthy controls, since the understanding of cytokine network in infections has dramatically evolved and it is becoming amply clear that a particular pattern of the cytokine release influences the type of immune response and ultimately the outcome of the infection. Studies in the past decade have shown an association between the susceptibility to an infection and polymorphisms in the genes involved in host
defense and immune response. It was also pertinent in the study to explore if the differential expression of cytokines after the stimulation of PBMCs to HEV antigens in-vitro was associated with DNA polymorphism present in the regulatory region of the cytokines. Also, if any polymorphic genotype background of the studied regions of the cytokine genes showed an association with the susceptibility to hepatitis or provided a high risk background for the disease.

Level of C4 and C3 complement components was assessed in the sera samples of prospective and retrospective patients with acute hepatitis and healthy controls to understand their role of complement in the pathogenesis of HEV, if any. The C4 and C3 components were significantly reduced (p<0.05) in the prospective patients with HEV and HEV+HEV coinfection. There was no significant change observed between the retrospective patients with HEV and the healthy controls, which was indicative of a recovery of the C4 and C3 complement component to normal levels over a period of time. The observed pattern suggested a predominant involvement of the classical pathway of complement system in HEV cases, initiating an immune process in the early clinical phase of hepatitis. Such changes in complement are likely to reflect on an immune-complex activity and it is proposed that these complexes may be important in the clearance of viral material. On the basis of our results and the available reports, it is proposed that complement components probably could be used as a valuable parameter for monitoring the course of HEV infection.

Nitric oxide (NO) is known to contribute to the pathological changes featuring in some of the inflammatory diseases, but the role of NO in case of hepatitis E is still unknown. In this study it was observed that the serum nitrite levels in patients with acute hepatitis, HEV infection and HEV+HEV co-infection were significantly increased (p<0.05) in comparison to healthy controls. However, a significant reduction (p<0.05) was observed in retrospective patients in comparison to prospective patients and did not show a significant difference when compared to healthy controls. This indicated a recovery of NO to normal levels in retrospective patients who had suffered with HEV infection or acute hepatitis in the past. The results observed also suggested that the elevation of serum NO levels could occur in patients with acute hepatitis with and without HEV infection, indicating that the NO level probably acts as a general
indicator of liver cell perturbation. Further an increased nitric oxide during the infection could be induced as an outcome of the host immune response and contribute to the viral clearance and liver inflammation. It could also act as predictive factor to assess the inflammatory response in patients. Although, the precise role played by nitric oxide (NO) in hepatic infections remains unclear but, these findings provide an initial evidence with respect to the pathogenesis of HEV infection in hepatitis.

In order to understand the role of viral antigens in the pathogenesis of HEV, PBMCs of retrospective and prospective patients with acute hepatitis and healthy controls were stimulated in-vitro with HEV antigens (baculovirus expressed pORF2, partially deleted from 1-111 and 585-610 and E.coli expressed pORF3) and PHA (non-specific mitogen). The stimulated PBMCs were studied for lymphoproliferative and cytokine (culture supernatants) responses. There was no significant difference observed in stimulation index (SI) in PHA stimulated PBMCs of patients with acute hepatitis, HEV+HEV co-infection and healthy controls. The analysis of cytokines in culture supernatants of PHA stimulated PBMCs revealed a significant decrease (p<0.05) in IFN-γ levels in patients with acute hepatitis and HEV infection when compared to healthy controls. However, no significant difference was observed in IL-4 and TGF-β1 levels in the culture supernatants in PHA stimulated PBMCs when compared to controls.

A low stimulation index (SI) was observed in HEV antigen (pORF2 and pORF3) stimulated PBMCs of patients and controls. There was no significant difference observed in the average stimulation index of pORF2 stimulated PBMCs between the different categories of patients with acute hepatitis and controls. Subsequent analysis of cytokines in culture supernatants of HEV antigen stimulated cells revealed either an absence or a low level of IFN-γ and IL-4 in most of the patients and healthy controls. However, a measurable amount of TGF- β was observed in the pORF2 and pORF3 stimulated cell culture supernatants of different categories of patients and controls. Interestingly, the observed levels of TGF-β1 were significantly reduced (p<0.05) in patients with, acute hepatitis, HEV+HEV co-infection, retrospective HEV+HEV co-infection when compared to healthy controls. Low stimulation index observed in our results was largely attributed to the inhibitory effect of TGF-β on primary T- cell
proliferation probably due to inhibition of interleukin-2 production, a cytokine vital for T cell proliferation. TGF-β has been shown to modulate cytokine production in human PBMCs and inhibit the differentiation of Th-1 and Th-2 cells, supporting the present observation of a low and an undetectable level of IFN-γ and IL-4 in culture supernatants of the cells stimulated with recombinant HEV antigens in patients with acute hepatitis and healthy controls. Although a significant reduction was observed in the levels of TGF-β1 in culture supernatants of the cells stimulated with recombinant HEV antigens, apparently the level of the cytokine available was optimal to inhibit the expression of IFN-γ and IL-4 in different categories of patients and controls. It is suggested that the observed reduction of TGF-β1 in patients with acute hepatitis could have been responsible for a lowering the suppression of the immune response in this study. An increased production of TGF-β in hepatitis B and C has been correlated with a progression to chronicity. It has been proposed that strong cellular immune response has been exist in patients with acute hepatitis whereas, a weak response in chronic cases leads to chronicity. It is proposed that a significant reduction in level of total TGF-β1 in patients in comparison to the level observed in normals could be one of the possible reasons for occurrence of acute hepatitis.

The regulatory regions of IFN-γ, IL-4, IL-6 and TGF-β1 were investigated for polymorphisms, since the polymorphic genetic background of these regions could be a possible reason for their differential expression. The regulatory regions of these cytokines were also studied for the polymorphisms and their association with the susceptibility to Hepatitis E. The analysis revealed a novel polymorphism, -288A>T (as per HUGO nomenclature), in the promoter region of IFN-γ gene. Interestinlgy, the -288A>T polymorphism falls in NF-AT site, which might affect the transcription efficiency of IFN-γ. The study of the status of a highly polymorphic dinucleotide (CA) repeat in the first intron of the IFN-γ gene revealed a significant reduction in the frequency of the homozygotes for (CA)12/(CA)12 in patients with acute hepatitis, HEV+HEV co-infection when compared to healthy controls. The homozygous (CA)12/(CA)12 status has been reported to express high levels of IFN-γ, whereas, the heterozygotes for allele#2 (12 CA repeat) have been shown to produce relatively less amount of IFN-γ. In this study, the heterozygous (CA)12/(CA)14 and (CA)12/(CA)16 status was significantly increased in patients with acute hepatitis and HEV
infection, which supports the observation of a significant reduction in the levels of IFN-γ in the culture supernatants of PHA stimulated cells. The estimated Odds ratio in patients with acute hepatitis, HEV infection, HEV+HEV co-infection indicated a relatively high risk to heterozygous (CA)\textsubscript{12}/(CA)\textsubscript{14/16} profile of CA repeats.

There was no significant difference observed in the genotypic frequencies of -33 C/T and -33T/T in the 5’UTR region of the IL-4 gene between different categories of patients and healthy controls. Whereas, the genotypic frequencies of -236C/C homozygotes in the promoter region of IL-6 gene depicted a moderate risk in patients with HEV, HEV+HEV co-infection. Of the three regions in TGF-β gene, the putative enhancer region- Region I, and the promoter region- Region II showed a polymorphism -800G>A (-1640 G>A as per HUGO nomenclature) and -509 C>T (-1349 C>T as per HUGO nomenclature) respectively, which were not significantly different between different categories of patients and controls. However, the genotypic frequencies of -509 T/T (-1349 T/T as per HUGO nomenclature) homozygotes in the promoter region of Region II of TGF-β1 depicted a moderate risk in patients with HEV, HEV+HEV co-infection. The Region III, which included 5’UTR and part of 1\textsuperscript{st} exon of TGF-β1 gene, showed two known polymorphisms at +29 T>C (Leu\textsubscript{10} Pro, codon 10) and +74 G>C (Arg\textsubscript{25} Pro, codon 25) in patients and controls samples. Mutational screening revealed a novel mutation +29 T>G at codon 10 with a low frequency in patients. The homozygous T/T and G/G status at +29 (codon 10) and +74 (codon25), respectively were significantly reduced (p<0.05) in patients with acute hepatitis, HEV+HEV co-infection in comparison to controls. Very interestingly, a significant increase (p<0.05) of +29 T/C, +29 C/C, +74 G/C genotype profiles was observed in patients with acute hepatitis, HEV, HEV+HEV co-infection when compared to normals. Odds ratio indicated a high risk for +29T/C genotype in patients with acute hepatitis (OR=2.44), HEV (OR=2.022) and HEV+HEV co-infection (OR=1.921). A high risk was also indicated for the genotypic status +29C/C in patients with acute hepatitis (OR=2.307), HEV infection (OR=4.04) and HEV+HEV co-infection (OR=3.306). The estimated Odds for +74 G/C genotype profile in patients with acute hepatitis (OR=3.195), HEV infection (OR=5.471) and HEV+HEV co-infection (OR=4.24) depicted a high risk. The polymorphism at position +29 T>C (Leu 10 Pro) and +74 G>C (Arg 25 Pro) are located in signal peptide sequence region and
have been reported to affect the transportation of TGF-β protein. A further analysis of heterozygous status -509 C/T (-1349 C/T as per HUGO nomenclature) at Region II, in combination with the heterozygous status +29T/C (codon 10 Leu/Pro) at Region III, showed a significant increase in patients (p<0.05) when compared to healthy controls. Similarly, the haplotype frequency of +29T/C;+74G/C of Region III significantly increased in patients. It is evident from the study that a high prevalence of +29 T/C, +29 C/C and +74G/C polymorphisms in patients not only led to the observation of reduced levels of TGF-β1 in HEV antigen stimulated cell cultures but also provided a high risk to acute hepatitis for a susceptibility to acute hepatitis. The study conducted provides the first evidence of production of low levels of total TGF-β1 in response to HEV antigens (pORF2 and pORF3) in culture supernatants in patients with acute hepatitis with a defined genotypic background of cytokines. An assessment of TGF-β1 levels in patients, in future, correlating with the genotypic status would enhance the understanding and suggest if the low production of TGF-β1 could either be mediated through virus or as an outcome of host immune response to the virus. When compared to the levels in healthy controls, reduced levels of TGF-β1, an anti-inflammatory cytokine, obtained in response to HEV antigens result into a functional phenotype of lowered immune suppression. The levels present in uninfected normals could be crucial to combat the virus efficiently without any clinical symptoms or jaundice. It is likely, however, that the reduced level of anti-inflammatory cytokine, TGF-β could lead to increased inflammatory response, which either results into or is as a result of the activation of the classical pathway of complement and increased levels of NO, in prospective acute hepatitis patients, with a particular genetic background. This in turn could be a probable reason for the apparent delay in the viral clearance and resultant inflammation of the liver in acute hepatitis patients. The differential display of cytokine levels in patients and controls in this study could be attributed to the alterations in the regulatory region of the IFN-γ and TGF-β1 gene, too. In conclusion, the present findings provide an insight into the pathogenesis of HEV infection and acute hepatitis and makes a beginning to understand the complex puzzle of genetic and environmental factors involved in infectious diseases.