Materials and Methods
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3.1 Study subject

3.1.1. Selection criteria

The criteria for diagnosis of acute viral hepatitis (AVH) were recent onset of jaundice in the absence of prior history of chronic liver disease, no other cause to account for jaundice (including drug induced hepatitis, severe infections, cholestatic jaundice of pregnancy, eclampsia, hemolysis, elevated liver enzymes and low platelet syndrome, acute fatty liver pregnancy, etc), serum bilirubin given method and values of 2.0 mg/dl or more, with an increase in transaminases, given method and values two and a half times above the normal upper limit [serum alanine aminotransferase (ALT), 3-26 IU/L] (Khuroo and Kamili, 2003).

3.1.2. Diagnosis of Hepatitis E

Diagnosis of hepatitis E was based on the presence of IgM antibodies to hepatitis E virus (IgM-anti-HEV) as detected by ELISA (Arankalle et al, 1994). All the patients were tested for IgM antibodies to hepatitis A virus (IgM-anti-HAV), Hepatitis B surface antigen (HBsAg) and IgM antibodies to hepatitis B core antigen (IgM-anti-HBc) by ELISA according the protocol described by the manufacturers (Abbott, AXSYM System, Germany). Only IgM-anti-HEV positive patients were included in the study. Subclinical Hepatitis E infection was defined by the presence anti-HEV-IgM with or without IgG and no clinical symptoms during the follow up of 2 months. The controls were apparently healthy individuals without any marker of acute or chronic viral hepatitis and negative for IgG-anti-HEV antibodies.

3.2 Study groups

The study comprised 207 subjects categorized as

(I) Healthy controls negative for both IgM and IgG-anti-HEV antibodies (n=60) and

(II) Individuals with recent HEV infection i.e., positive for the IgM-anti-HEV antibodies, identifying during epidemics of Hepatitis E in the state of Maharashtra, India during 2009-2011 (n=157).

These were further subcategorized as follows:
I. Healthy controls
(a) Non pregnant / non-ANC (n=30)
This group included 14 females and 16 males of mean age 24.8±0.6 years.
(b) Pregnant women in different trimesters of pregnancy / ANC (n=30)
This group included, n=10 individuals from each trimester; mean age of 21.9±1.1, 20.8±0.7 and 21.1±0.8 years for 1st, 2nd and 3rd trimesters respectively.

II. Recent HEV infections (n=157):
Of these, 114 were non-ANC and 43 belonged to the ANC category. Individuals presenting with clinical symptoms were grouped on the basis of the post-onset day (POD) of the collection of samples. The subgroups included:
(a) Non-ANC
[i] 46 non-ANC AVH patients (POD=6.9±1.1),
[ii] 05 non-ANC subclinical cases/ non-ANC SC (POD=not applicable)
[iii] 32 early convalescent cases (POD=17.9±0.8) and
[iv] 31 late convalescent cases (POD=38.2±2.3).
(b) ANC (n=43)
[i] 1st trimester subclinical, SC-ANC-1 n=10,
[ii] Subclinical ANC (2nd + 3rd trimester), SC-ANC-2+3, n=20 and
[iii] 13 ANC AVH patients (2nd + 3rd trimester), ANC-patients, (POD=13.5±1.9)

3.2.1 Detection of anti-HEV IgM/IgG by ELISA:
When the test sample was incubated in microwells coated with recombinant HEV antigen, antigen-antibody (recombinant HEV protein-IgM / IgG) complexes form on the microwell surface, if antibody in the specimen is reactive to the HEV antigen. When conjugate (antibody to human IgM/IgG*-peroxidase) was added, HEV anti human IgM/IgG*-peroxidase complexes were formed in the wells. After the substrate addition, the substrates were oxidized, if bound conjugate was present, resulting in a yellow - brown colored end-product.
3.2.1.1 Protocol

1. Sf9 cell (One ml) pellet (with HEV-ORF2 recombinant antigen) was lysed in 100 µl lysis buffer (0.1% NP 40 in 0.01 M PBS PH 7.4) and immediately 3µl of protease inhibitors mixture (PMSF 100 µg/ml, Leupeptin 0.5 µg/ml, Aprotinin 0.5 µg/ml and Pepstatin A 1 µg/ml) was added.

2. Sf9 cell lysate containing rORF2p was diluted at 1: 50 concentration in carbonate-bicarbonate buffer (0.05M, pH 9.5) and used for coating 96-well micro-titer plate (100 µl/well) (Maxisorp, Nunc, Denmark).

3. The plate was incubated at 37°C for 2 hours, thereafter diluted with assay diluents for 30 min at 37°C, post coating [PBS (0.01M pH 7.4) containing 0.5% gelatin, 0.5% Tween 20, 10% donor calf serum (DCS) (Invitrogen, Carlsbad, USA)].

4. The wells were washed 5 times with wash buffer (PBS 0.01M pH 7.4 containing 0.5% Tween 20) using an automated ELISA plate washer (BioTek instruments INC, USA).

5. 100 µl test sample and anti-HEV IgM/IgG positive and negative controls (diluted 1: 100 in assay diluent) were added to different wells and the plate was incubated for 30 minutes at 37°C. The plate was washed 5 times with wash buffer as described in step 4.

6. 100 µl goat anti human IgM/IgG HRP conjugate (Sigma chemicals, St. Louis, MO), diluted 1:10,000 in assay diluent was added in respective wells and incubated at 37°C for 30 minutes. The plate was washed 5 times with wash buffer as described in step 4.

7. 200 µl substrate (20 mg OPD and 10 mg urea peroxidase in 50 ml citrate phosphate buffer 0.15 M, pH 4.5 – 5.1) was added and the plate was kept in dark at room temperature for 30 minutes for color development (golden yellow).

8. The reaction was stopped by adding 100 µl, 4N H₂SO₄ and the absorbance was read at 492nm on an ELISA reader.

3.2.1.2 Cutoff

Three known anti-HEV IgM/IgG negative and two anti-HEV IgM/IgG positive human samples were included as the controls in each test. The cut-off
value was calculated as mean absorbance (A) of negative control x 3. The sample showing absorbance value greater than cut-off were considered positive while those showing absorbance value less than or equal to the cut-off were considered negative. Samples in the grey zone ± 15% of the cut off value were repeated for the detection of borderline reactives.

3.3 Processing of whole blood samples

1. Fresh blood samples were collected from all the study subjects in 10ml EDTA vacutainers (BD Biosciences, USA). An aliquot was used for whole blood staining and rest of the blood was processed for PBMCs isolation.
2. Plasma and PBMCs were separated within 4 hrs of collection by Ficoll-hypaque gradient method (Boyum, 1968).
3. Plasma samples were subjected to serology testing of Hepatitis B virus (HBV), Hepatitis A virus (HAV), Hepatitis E virus (HEV) and stored in aliquots at -80°C for cytokines assay.

3.3.1 Whole blood staining

Anti-human antibodies conjugated with flourochrome for TLR 2, TLR3, TLR4, TLR7 and TLR8 (Imgenex, USA) and other different anti-human CD markers conjugated with flourochrome i.e. PEcy-7-CD4, PEcy-7-CD14, PE-CD28, PE-CD80, FITC-CD86, FITC-CD137, PE-CD152, FITC-CD209, PE-CD278, HLA-DR (BD Biosciences, USA) were used for cell surface or intracellular staining.

3.3.1.1 Surface TLR staining:

For surface staining 100µl of whole blood was processed for staining with anti-human TLR antibodies. Cells were incubated with surface TLR antibodies like TLR2 and TLR4 for 30 min at RT in dark. The concentration of antibodies was used as per manufacturer protocol. RBCs were lysed with 2ml BD FACS lysis solution (BD Biosciences, USA). Cells were washed with 2ml of PBS buffer twice and fixed in 500µl PBS with 1% Paraformaldehyde and used for flow cytometry analysis.

3.3.1.2 Dual staining for Monocytes and T cells staining

For the frequency of monocytes and associated marker, Monocytes (CD14) were stained with either of CD80, CD86, CD209, HLA-DR, TLR2, TLR4
markers and for T cells and associated marker, T cells were stained with CD4 and either of CD28, CD137, CD152, CD278 markers. The concentration of antibodies was used as per manufacturer protocol.

3.3.1.3 Intracellular TLR staining:

For intracellular staining, 100µl of the whole blood was lysed with 2ml of BD FACS lysing solution. Cells were fixed and permeabilized with 1 ml of BD cytofix/ cytoperm solution (BD Biosciences, USA) and processed for staining with the anti-human TLR3, TLR7 and TLR8 antibodies. The concentration of antibodies was used as per manufacturer protocol. Intracellular stained cells were resuspended in 500µl PBS with 1% Paraformaldehyde and used for flow cytometry analysis.

3.3.1.4 Signaling molecule staining

Intracellular levels of IRAK4, IκBα, NFκβ, p38, TBK1, IRF7 were detected by using the BD PhosFlow kit (BD Pharmingen, USA) using protocol provided by manufacturer. Briefly, 100µl of whole blood was stimulated with TLR ligands (polyI:C, LPS and R848) and incubated at 37°C and incubation period was optimized for different signaling molecules. The optimum time of incubation for activation was 15 min for IRKA4, 30 min for TBK1 and IRF7, 45 min for IκBα and 60 min for NFκβ. Cells were fixed with BD PhosFlow lysed/Fix Buffer (BD Pharmingen, USA) at 37°C for 15 min, pelleted, washed with PBS and permeabilized the cells by adding BD Phosflow Perm Buffer II or III and incubated for 30 minutes on ice. Permeabilized with BD PhosFlow Perm/Wash Buffer (BD Pharmingen, USA) at RT for 10 min, pelleted and washed with Perm/Wash Buffer, pelleted and resuspend in same buffer. Cells were incubated with fluorochrome–conjugated antibodies [IRAK4, Iκbα, NFκβ (pS529), p38 (pT180/pY182), TBK1, IRF7 (BD Pharmingen, USA)] in the dark at room temperature for 30 min, washed twice with Perm/Wash Buffer, and resuspended in the Stain Buffer (BD Pharmingen, USA) for FACS analysis.

3.3.1.5 FACS analysis:

(a) Gating strategy for TLRs and signaling molecules

On the basis of dot plots lymphocytes and monocytes were gated and analyzed using FACS Diva software (BD, Bioscience, USA). Levels of TLR and
signaling molecules were represented as median fluorescence intensity (MFI).

(b) Gating strategy for monocytes and T cells

On the basis of dot plots monocytes and T helper cells were gated with the help of their respective fluorochrome, like for monocytes CD14-PeCy7 and for T helper cells CD4-APC and analyzed using FACS Diva software (BD). Levels of monocytes and T helper cells were represented in percentage cells.

3.4 Peripheral blood mononuclear cells (PBMCs) isolation

Ficoll-hypaque method used for PBMCs isolation, employs a liquid density gradient medium of Ficoll (poly sucrose) and sodium metrizoate or sodium diatrizoate solution [density=1.077±0.001 g/ml (Histopaque-1077)]. In this procedure anti coagulated blood collected by routine phlebotomy is carefully layered onto the Histopaque column. This preparation is then centrifuged (400 x g for exactly 30-40 minutes at room temperature) and mononuclear cells forms ring at the interface between the Ficoll and plasma wherein erythrocytes and granulocytes forms pellet at the bottom of the centrifuge tube. Centrifugation at lower temperatures, such as 4°C, may result in cell clumping and poor recovery. After centrifugation, plasma and cells are harvested by pipetting. Plasma were aliquoted and stored at -80°C. PBMCs were washed with RPMI-1640 medium, three times (250 x g for 10 minutes at room temperature) and cell were resuspended in to RPMI-1640 medium with 10% FBS. 10ul of cell suspension was used for viable cell count with a hemacytometer using tryphan blue.

3.4.1 Protocol:

1. whole blood was carefully layered onto a sterile 15 ml tube (Nunclon, Nunc International, Naperville, USA) containing half the blood volume of Ficoll-hypaque.

2. Centrifugation was carried out at 2500 rpm for 30-40 minutes at room temperature.

3. After centrifugation, the upper layer of plasma was carefully aspirated, the opaque interface containing mononuclear cells was carefully removed with a Pasteur pipette and transferred into a clean conical 15 ml centrifuge tube.

4. 10 ml of RPMI-1640 medium (Invitrogen, Carlsbad, CA) was added and mixed by gentle aspiration.
5. Centrifugation was carried out at 1000 rpm for 10 minutes at room temperature. The supernatant was aspirated and discarded.

6. Cell pellet was resuspended with 8-10 ml of RPMI-1640 medium and mixed by gentle aspiration with a Pasteur pipette and centrifugation was carried out at 1000 rpm for 10 minutes.

7. Step 6 was repeated and cell pellet was resuspended in RPMI-1640 with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA).

8. Viable cell count was performed by tryphan blue dye exclusion test.

3.4.3 Viable cell count by dye exclusion

This method is based on the principle that live (viable) cells will not take up certain dyes, whereas dead (non-viable) cells will. The plasma membrane of a viable cell does not permit the entry of non-electrolyte dye substances. This phenomenon is used to distinguish dead cells from living cells. Many dyes are suitable for this purpose, for example, trypan blue, eosin, nigrosin, and alcian blue. Trypan blue is one of several stains recommended for use in dye exclusion procedures for viable cell counting. It is not always an accurate test because it indicates only the structural integrity of the cell membrane.

10 ul of cell suspension of PBMCs was mixed with 10 ul of trypan blue (0.2% w/v in PBS/3mM NaN3) and immediately the mixture was loaded beneath the cover slip on a hemacytometer. This volume ensured that the hemocytometer was not overfilled. The unstained (viable) and stained (non-viable) cells from the upper left primary square of the hemacytometer grid were counted using microscope (Figure 3). Cells had been counted and total number of viable cells was calculated as follows:

Total viable cells = Viable cells counted \times 10^4 \times \text{dilution factor (per ml)}
Figure 3  Grid patterns of improved Neubauer ruled haemocytometer. Inset shows cells (enlarged for clarity) distributed over a primary square. Cells that are within, or on the left or top boundary are counted, while those that are outside the lower or right hand boundary are not counted.

3.5 Cytokine assay

The TLR-specific inducible cytokine production was measured in the supernatants of TLR ligands-stimulated peripheral blood mononuclear (PBMC) cultures while systemic cytokines were estimated from the plasma samples. 9 cytokines i.e. IL1α, IL1β, IL6, IL8, IL12p40, IL12p70, TNFα, IFNα were analyzed in the supernatants of cultured PBMCs while 4 cytokines i.e., IL6, IL8, IL12p70 and TNFα were assayed in the plasma. The cytokines assay was done using commercial premixed Milliplex Map Kit (Millipore, USA) and measured on Bio-Plex Protein Array System (Bio-Rad, Hercules, CA, USA). For IFNβ assay, IFNβ ELISA kit (PBL interferon source, NZ, USA) was used. PBMCs were stimulated with specific TLR ligands, i.e., LPS (TLR4), Poly I:C (TLR3) and R848 (TLR7/8). Unstimulated or mock stimulated cells were used as controls for background release. The cells were incubated at 37°C with 5% CO₂. Supernatants were collected from stimulated and unstimulated cultures. The supernatants were stored at -80°C until use.

In accordance to the above protocol, PBMCs from 25 AVH non-ANC
patients, 13 ANC patients, 20 non-ANC healthy controls and 12 healthy ANC controls were stimulated in vitro with TLR3 and TLR4 ligands (poly I:C and LPS) for 6 hours, at 37°C with 5% CO₂.

3.6 Gene expression analysis

Frozen PBMCs were thawed, centrifuged to pellet down the cells and pellets were used to isolate RNA. Total RNA was extracted from PBMCs by using Ribopure Kit (Ambion) as per the manufacturer’s instructions. RNA eluted in 100µl elution buffer, was quantitated using Nanodrop (ND-1000) and checked for quality using Agilent bioanalyzer (Agilent, U.S.A). Equal quantities of RNA (500ng) with ≥ 7 RIN value were processed further for cDNA synthesis using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, U.K.). To ensure efficient cDNA synthesis, all cDNAs were tested for 18S rRNA in real time PCR assay using TaqMan primers and probe for (Applied Biosystems, U.K.). cDNAs were then loaded per port of the TaqMan Low Density Array card (TLDA) of the Human Immune panel (Applied Biosystems, U.K.) and run on 7900HT Fast Real-Time PCR system (Applied Biosystems, U.K.). Relative gene expression values were obtained employing comparative ∆∆Ct method using Applied Biosystems’ Relative Quantification (RQ) Manager Software v1.2. cDNAs from healthy Non-ANC (n=16) and healthy ANC (n=10 for each trimester) individuals processed similarly were considered as calibrators. 18s RNA was used as an endogenous control. Relative quantitation values of each study group were used to calculate mean RQ values.

3.6.1: RNA isolation:
- PBMCs from different study subjects were isolated and stored in RNAlater (Ambion, USA) in volume sufficient to submerge the sample.
- The PBMCs were homogenized in 1.0 ml of TRI-reagent completely.
- The samples were incubated for 5 min at RT.
- 200µl of Chloroform was added, vortexed for 15 sec and incubated at RT for 5 min.
- The samples were centrifuged at 12000 rpm for 10 min at 4°C.
- 400 µl of aqueous phase was transferred in fresh 1.5 ml tube.
• 200µl of 100% ethanol was added and mixed immediately to avoid RNA precipitation.
• For each sample, a filter cartridge was placed in one of the collection tubes provided with the kit.
• The samples were transferred to a filter cartridge-collection tube assembly, the lid was closed and the assembly was centrifuged at 12,000 rpm, at RT for 1 min.
• The flow-through was discarded and the filter cartridge was returned to the same collection tube (The RNA was now bound to the filter in the filter cartridge).
• 500µL of wash solution was applied to the filter cartridge-collection tube assembly, and the lid was closed.
• The tubes were centrifuged for 30 sec at RT or until all of the liquid is through the filter.
• The flow-through was discarded and the filter cartridge was returned to the same collection tube.
• The washing step was repeated.
• The centrifugation step was repeated.
• The filter cartridge was transferred to a new collection tube.
• 100µL of elution buffer was added to the filter column.
• The column was incubated at RT for 2 min and then centrifuged for 30 sec to elute RNA from the filter.
• The recovered RNA was stored at -70°C.

3.6.2: RNA quantitation and quality analysis
• The concentration of RNA was determined using nano drop 1000A spectrophotometer.
• A260/A280 was found to be in the range of 1.8–2.1,
• Bioanalyzer, was then used to determine quality of RNA.
  o RIN (RNA Integrity Number) was calculated to further evaluate RNA integrity as follows
  • RIN≥7 (Integerated RNA)
• RIN=7-5 (Partially degraded RNA)
• RIN≤5 (Degraded RNA)
• The 28S to 18S rRNA ratio often used as an indicator of RNA integrity and was found to be >7.0.

3.6.3: cDNA synthesis:
• High capacity cDNA reverse transcription kit was used (Applied Biosystems International, Foster City, CA International, Foster City, CA). the composition for the cDNA synthesis reaction was as follows:
  10X RT buffer- 2.0 µl
  25X dNTPs mix- 2.0 µl
  10X RT random primers- 2.0 µl
  MultiScribe RT- 1.0 µl
  D/w- 3.0 µl
  RNA- 10.0 µl (500 ng)
• The following conditions for thermal cycling were used for cDNA synthesis:
  25°C-10 min
  37°C-120 min

3.6.4: cDNA dilution:
• 50 µl of D/W was added to the cDNA tube to make the final volume to 70 µl.
• Quality of cDNA was checked by running individual assay (18 S rRNA, pre designed assay, Applied Biosystems International, Foster City, CA International, Foster City, CA International, Foster City, CA) using 10µl of cDNA.
• To the cDNA equal amount of 2X master mix (60 µl cDNA+ 60 µl 2X Master mix) was added.
3.6.5 Taqman Low Density Array (TLDA)

3.9.5.1 Loading and running the TLDA reaction:

- The TLDA card was incubated at RT for 30 min before use.
- The solution containing cDNA + 2X master mix was loaded carefully in each port (110 µl).
- TLDA card was centrifuged at 1200 rpm in bucket centrifuge MULTIFUGE 3SR) for 2 min (twice).
- The microfluidic card was then sealed and run on 7900 fast real time PCR system.

3.6.5.2 Data analysis:

- Relative quantitation (RQ) software provided with the system was used for the analysis of data.

3.7 Statistical Analysis:

A value of 0.2 pg/ml was used in case of undetectable concentration of cytokines in the tested samples. The Mann-Whitney U and student T test were used for group comparisons. For all analyses, a P value of less than 0.05 derived from a two tailed test was considered significant. All statistical analyses were performed with ‘SPSS11.0 for Windows’ software (SPSS Inc.).