3. Materials and methods

3.1 Sample size estimation

With HCV prevalence of 2% in India, sample size is calculated as 53 predicting the population size of maximum and confidence level of 95% with 5% margin of error. According to this statistical analysis a total of 5000 patients of chronic liver disease when screened over a period of three years and at least of 53 patients who fulfill inclusion criteria of chronic hepatitis C will be selected for the study of polymorphism and Expression studies of complement components.

The control group comprising 53 healthy individuals age and sex matched who have no evidence of hepatobiliary disease will also be enrolled. These patients shall be evaluated for previous history of arthritis or Hypocomplementemia and excluded if found positive.

Study subjects: The patients recruited for the study composed of the following groups were as follows:

**Group A:** Chronic hepatitis C n= 84

**Group B:** Healthy Controls n= 75

All these patients were recruited from the medicine OPD and also from those admitted in the medical wards of Lok Nayak hospital.

Diagnostic criteria for Inclusion

**HCV Infection:** Patients whose serum samples showed positivity to Anti HCV or HCV RNA were considered as HCV positive case.
**Materials and methods**

**Chronic hepatitis C**: Patients who showed HCV RNA positive, who have been exposed to HCV within the past six months or with normal/abnormal liver function test for > 6 months, were subjected to liver biopsy for confirmation and diagnosis of chronic hepatitis and staging of liver disease (Charlton MR, Gane E, Manns M, et al, 2013).

**Exclusion criteria**

1. Any liver disease of non-HCV etiology: This includes acute hepatitis A/B/E or chronic Hepatitis B or other viral etiology, drug- or alcohol related liver disease, autoimmune hepatitis, hemochromatosis, Wilson’s disease, alpha-1 antitrypsin deficiency, non-alcoholic steatohepatitis and primary biliary cirrhosis.

2. Co-infected cases

3. Hepatocellular carcinoma cases

4. Pregnant cases

**Treatment consideration**

All the 84 patients were instructed to take peginterferon alfa-2b of 1.5 μg/kg/week dosed according to body weight subcutaneously and Ribavirin was given at 800 mg for genotype 3 and 4 and for genotype 1 was 1000 mg for ≤75 kg and 1200 mg for ≥75 kg.

The duration of the treatment was decided on the basis of the HCV genotype. For patients with genotype 1, the duration was 48 weeks and for genotype 3 and 4 patients the duration was 24 weeks.
Clinical SVR and Liver Histology

Sustained Virological Response was defined when serum HCV–RNA was undetectable at the end of therapy and at 24-week follow-up after the treatment. Non-responders were defined as patients who failed to clear HCV RNA from serum after 24 weeks of therapy for genotype 3 and 48 weeks for genotype 1 patients.

Liver biopsy samples were collected from patients after getting their consent. Histological Activity Index (HAI) was assessed by pathologists who had no knowledge of the clinical or viral load state. Histological Activity Index was quantified ranging from 0-18 and fibrosis from 0-6 (0: No fibrosis, 1-2: Mild fibrosis, 3-4: Moderate fibrosis, 5+: Cirrhosis).

Ethical considerations

An authorization to carry out the study was obtained from the institutional ethics committee. The objective of the study was explained to all participants and their consent was taken.

3.2 Sample collection

Patients who fulfilled the above criteria were included in the study after taking consent. A detailed demographic, clinical history and other information of the patients were recorded in pre-designed and pre-tested proforma. Further the evaluation was done on the basis of LFT profile, serological tests and PCR.

A total of 10 ml of blood sample was collected from all subjects using all aseptic precautions at the initiation of the treatment. Three ml. of the sample was used for biochemical investigations. Four ml. of sample was taken in standard E.D.T.A. vials.
and stored at -70°C eppendorf tubes for DNA extraction and detection of gene polymorphisms using PCR-RFLP and mRNA expression study of C3, C4 and CFH. Three ml. of sample was preserved in plain vials for quantification of C3, C4 and CFH using ELISA. All aseptic precautions were taken during handling and processing of the samples.

3.3 Specialised Investigations

Specialised Investigations included complete haemogram (Hemoglobin, total leucocyte count, differential leucocyte count) and complete liver function tests (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, total serum bilirubin, total serum protein) and prothrombin time were performed at treatment initiations. Patients were followed up at 24 weeks after the initiation of treatment for genotype 3 and 48 weeks for genotype 1 and 4 and after 24 weeks of the completion of treatment to assess their treatment response.

3.4 Serological tests

Serological tests were performed using commercially available Elisa kits according to manufacturers’ instructions. The various serological tests included IgM anti-HAV (Radim diagnostic, Rome, Italy), HBsAg (General Biologicals Corp.Taiwan), IgM anti-HBc (Radim diagnostic, Rome, Italy), Anti-HCV (General Biologicals Corp.Taiwan) and IgM anti-HEV (Immunovision, USA). The various serological tests performed were as follows:-
3.4.1 IgM anti-HAV: (Radim diagnostic, Rome, Italy)

Principle of the test

The assay is based on the principle of "IgM capture" where IgM class antibodies in the sample are first captured by the solid phase coated with anti IgM antibody. After washing out all the other components of the sample and in particular IgG antibodies, the specific IgM captured on the solid phase are detected by the addition of a purified preparation of inactivated HAV, labeled with an antibody conjugated with peroxidase (HRP). After incubation, microwells are washed to remove unbound conjugate and then the chromogen/substrate is added which in the presence of peroxidase the colorless substrate is hydrolysed to a colored end-product, whose optical density may be detected and is proportional to the amount of antibodies to HAV present in the sample.

Assay procedure

The assay was carried out according to that reported below and care was taken to maintain the same incubation time for all the samples in testing.

1. Samples were diluted 1:101 by dispensing first 10 μl of sample and then 1 ml of Sample diluent into a dilution tube and mixed gently on vortex.

2. The required number of Microwells in the microwell holder was placed, leaving the 1st well empty for the operation of blanking.

3. 100 μl of Negative Control in triplicate, 100 μl of Positive Control in single and 100 μl of Calibrator in duplicate in proper wells were dispensed.
4. 100 µl of diluted samples in the proper sample wells was dispensed and then checked that all the samples wells are blue coloured and that controls and calibrator have been dispensed.

5. Incubated the microplate for 60 min at 37°C.

Important note: Strips have to be sealed with the adhesive sealing foil only when the test is carried out manually.

6. About 5-10 minutes before use, the HAV Antigen/Antibody immunocomplex was prepared as described previously.

7. The microplate was washed 4-5 times with 350 µl/well of diluted Washing Solution.

8. 100 µl of HAV Antigen/Antibody complex was pipetted into each well, except the 1st blanking well, and covered with the sealer. Checked that all wells are red coloured, except A1.

9. Incubated the microplate for 60 min at 37°C.

10. Microwells was washed as in step 7.

11. 100 µl of Chromogen/Substrate mixture was pipetted into each well. Then the microplate was incubated at room temperature (18-24°C) for 20 minutes.

12. 100 µl of Sulphuric Acid was Pipetted into all the wells to stop the enzymatic reaction using the same pipetting sequence as in step 10. Addition of acid turns the positive control and positive samples from blue to yellow.
Materials and methods

13. The colour intensity of the solution in each well was measured at 450nm filter (reading) and possibly at 620-630nm (background subtraction), blanking the instrument on A1.

Calculation of the cut-off

The test results are calculated by means of the mean OD 450nm value of the Negative Control (NC) and a mathematical calculation, in order to define the following cut-off formulation:

\[
\text{Cut-Off} = \text{NC} + 0.250
\]

The value found for the test is used for the interpretation of results

Test results are interpreted as a ratio of the sample OD 450nm and the Cut-Off value (or S/Co) according to the following table:

<table>
<thead>
<tr>
<th>S/Co</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 0.8</td>
<td>Negative</td>
</tr>
<tr>
<td>0.8 – 1.2</td>
<td>Equivocal</td>
</tr>
<tr>
<td>&gt;1.2</td>
<td>Positive</td>
</tr>
</tbody>
</table>

A negative result indicates that the patient is not undergoing an acute infection by HAV. Any patient showing an equivocal result was re-tested by examining a second sample after 1-2 weeks from first testing. A positive result is indicative of an HAV infection event and therefore the patient was treated accordingly.
3.4.2 HBsAg: (General Biologicals Corp. Taiwan)

Principles of the test

SURASE B-96(TMB) is based on “sandwich principle” (antibody-antigen-antibody) assay. When anti-HBs coated wells and anti-HBs • HRPO conjugate are incubated with specimens containing HBsAg, (antibody)-(antigen)-(antibody • HRPO) complexes are formed on the wells. After washing, the activity of peroxidase on the wells reflects the presence of HBsAg in the specimen.

Test procedures

1. Before beginning the assay all reagents and specimens were brought to room temperature (20-30°C). The reagents were gently mixed before use.

2. Two wells were reserved for blanks. 50 μl of each control or specimen was added to appropriate wells of reaction plate (3 Negative Controls and 2 Positive Controls).

3. 50μl of Anti-HBs • Peroxidase Solution was added to each well except 2 blanks.

4. The plate was tapped gently.

5. The reaction plate was covered with adhesive slip and incubated in 37±1°C water bath or incubator for 80 minutes.

6. At the end of the incubation period, the adhesive slip was removed and discarded and washed plate by following “PLATE WASHING PROCEDURE”.

7. One of the following two methods was used for color development:

   A- Equal volumes of TMB substrate solution A and B were mixed in a clean container immediately prior to use. 100μl of the mixture solution was added to each well including 2 blank wells.
Materials and methods

B- Adding 50µl of TMB Substrate Solution A first, followed by addition of 50µl of TMB Substrate Solution B into each well including 2 blanks.

8. The plate was covered with black cover and incubated at room temperature for 30 minutes.

9. 100µl 2N H₂SO₄ was added to each well including 2 blanks to stop the reaction.

10. Absorbance of controls and test specimens was determined within 30 minutes with a precision spectrophotometer at 450/650nm. Using the lighter one of two blank wells to blank spectrophotometer.

Calculations and determination:

1. Calculation of NCx (Negative Control Mean Absorbance) if NC >0.015, the list of Negative Control values were checked and deleted the aberrant value as follows:- The three values were aligned in order of magnitude. If one of two neighboring values is equal to or greater than twice the value of the other, the value on the side position is considered as the aberrant value and was deleted, if one is deleted, the other two were used to calculate the NCx. If two are deleted, the test is considered as invalid and was repeated.

Example: Sample No. Absorbance

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.054</td>
</tr>
<tr>
<td>2</td>
<td>0.026</td>
</tr>
<tr>
<td>3</td>
<td>0.024</td>
</tr>
</tbody>
</table>
Of the above three value, 0.054, 0.024 and 0.026, the first value 0.054 is greater than twice of its neighbor, 0.026. The value on the side position, 0.054, is therefore deleted, and the NCx should be:

\[ NCx = \frac{(0.026 + 0.024)}{2} = 0.025 \]

NCx should be ---- 0.1, otherwise, the test is invalid.

2. Calculation of PCx (Positive Control Mean Absorbance):

<table>
<thead>
<tr>
<th>Example: Sample No.</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.432</td>
</tr>
<tr>
<td>2</td>
<td>1.508</td>
</tr>
</tbody>
</table>

\[ PCx = \frac{(1.432 + 1.508)}{2} = 1.470 \]

PCx should be------ 0.6, otherwise, the test is invalid.

1. Calculation of P-N Value

\[ P-N = PCx - NCx \]

Example: P-N =\(1.470 - 0.02 \) = 1.446

P-N Value must be------- 0.5, otherwise, the test is invalid.

4. Calculation of the Cutoff Value

\[ \text{Cutoff Value} = 0.024 + 0.025 \]

\[ = 0.049 \]
Materials and methods

Result interpretation:

Negative results:

Patient's specimens whose absorbance values were less than the Cutoff Value are non-reactive and were considered NEGATIVE for HBsAg.

Positive results:

Patient's specimen with absorbance value GREATER than or equal to the Cutoff Value is considered INITIALLY REACTIVE.

3.4.3 IgM anti-HBc: (Radim diagnostic, Rome, Italy)

Principle of the assay

This immunoenzymometric (IEMA) method is based upon a capture of anti-HBc IgM antibodies in the sample by means of a specific monoclonal anti-human IgM antibody in solid phase. During the second incubation a DNA recombinant antigen (HBcAg) and an anti-HBcAg monoclonal antibody conjugated with horseradish peroxidase (HRPO) are allowed to react with the captured IgM, if any, thereby forming a so-called "sandwich" complex. The residual enzyme activity found in the wells, which is directly proportional to the sample anti-HBc IgM concentration, will be evidenced by adding a chromogen solution (Tetramethylbenzidine, TMB) in substrate buffer to the wells. Colorimetric reading was performed by using a spectrophotometer at 450nm and 405nm wavelength.
Materials and methods

Assay procedure

All reagents and samples are warm to room temperature, and samples are mix by inversion before use.

1. The wells required for the assay were prepared and four replicates of the Negative Control and duplicate Blanks as well as the Positive Control were included.

2. 10 µl of the Negative Control, Positive Control and diluted Samples into the corresponding wells were added and another 200 µl of Sample Diluent to all wells, except for the Blank wells.

3. The microplate was covered with adhesive plate sealer and mixed gently.

4. The wells were incubated for 60±5 minutes at 37±2°C.

5. The adhesive plate sealer was carefully removed and carefully aspirated the incubation mixture from all wells.

6. The wells were washed 4 times with 350 µl of diluted washing solution. Aspirated out all liquid from the wells.

7. 100 µl of HBcAg was pipetted into each well, except for the Blank wells.

8. 100 µl of Enzyme Conjugate was added to each well other than the Blank wells.

9. The microplate was covered with adhesive plate sealer and mixed gently.

10. The wells were incubated for 60±5 minutes at 37±2°C.

11. The adhesive plate sealer was removed and the incubation mixture was carefully aspirated from all wells.

12. Wells were washed as described in point 6.

13. 200 µl of Chromogen-Substrate solution was pipetted into all wells and incubated for 20±2 minutes at 37±2°C.
14. 100 µl of Blocking Reagent was pipetted into all wells.

15. The absorbance of the wells was read with a preferably bichromatic spectrophotometer at 450 nm, with reference wavelength at 620 nm. In case of overflow absorbance values, read at 405 nm.

**Calculation of results**

**Mean absorbance of Negative Control:** calculated the mean absorbance value of the Negative Controls (Mean Negative Control). The test is valid if the mean absorbance of the Negative Controls was lower than 0.070, otherwise the assay was considered invalid.

**Mean absorbance of the Positive Control:** calculated the mean absorbance value of the Positive Controls (Mean Positive Control). The difference between the Mean Positive Control and the Mean Negative Control was greater than 0.500, otherwise it was considered invalid.

**Cut-off Value:** Mean Negative Control + 0.300

**Interpretation of results**

Samples with an absorbance higher than the cut-off value were considered reactive for anti-HBc IgM. Samples with an absorbance lower than the cut-off value was considered non-reactive for anti-HBc IgM. Samples non-reactive in the first assay (absorbance lower than the cut-off value) were considered negative for anti-HBc IgM. Samples reactive upon initial testing (absorbance higher than the cut-off value) were retested for confirmation. If the sample is not repeatedly reactive, then it was considered negative for anti-HBc IgM. Repeatedly reactive samples was considered
positive for anti-HBc IgM. Samples with an absorbance within the range of the cutoff \( \pm \) 10\% (grey zone) should be considered questionable and must be retested for confirmation.

- Absorbance less than the cut-off value: anti-HBc IgM negative samples.
- Absorbance greater than the cut-off value: anti-HBc IgM positive samples.
- Absorbance within the range of the cut-off \( \pm \) 10\% (grey zone): anti-HBc IgM questionable samples.

3.4.4 Anti-HCV (General Biologicals Corp. Taiwan)

**Principle of the test:**

The reagent kit, SP-NANBASE C-96 3.0, developed by the General Biologicals Corporation, ROC. Adopts the second antibody “sandwich principle” as the basis for the assay to detect antibodies to Hepatitis C Virus. The SP-NANBASE C-96 3.0 is an enzyme immunoassay kit, which employs synthetic HCV peptides (core and NS4 antigens) and recombinant HCV antigens (NS3 and NS5 antigens) for the detection of antibodies to HCV in human serum or plasma. These antigens, which are reactive with the predominant antibodies of HCV, constitute the solid phase antigenic absorbent. When human serum or plasma is added to the well, the HCV antigens and Anti-HCV will form complexes on the wells if anti-HCV is present in the specimen. The wells are washed to remove the unbound materials. The Conc. Anti-Human-IgG·HRPO Conjugate is added to the well results in the formation of \((\text{HCV})\cdot(\text{Anti-HCV})\cdot(\text{Anti-human-IgG·HRPO})\) complex. After washing out the unbound conjugate, TMB substrate
solution is added for color development. The intensity of color development is proportional to the amount of antibodies present in the specimen.

**Test procedure:**

1. Before beginning the assay all reagents and specimens were brought to room temperature (20-30°C) and mixed gently.

2. 2 wells were reserved for Blanks. Any specimen or specimen diluent was not added.

3. The needed numbers of wells were prepared, including 2 wells for Blanks, 2 wells for Negative Control, 3 wells for positive control, and 1 well for each specimen.

4. Sampling
   
   A 1:21 dilution of each control and specimen with specimen diluent was made.

**A. Manual Procedure**

a. 10μl of each control or specimen into the well of predilution plate was dispensed.

b. 200μl of Specimen Diluent was added to each well and mixed well.

c. 100μl of each diluted control or specimen was transferred to the corresponding wells in HCV Antigens plate.

**B. Automatic EIA microplate immunoanalyzer.**

a. 200μl specimen diluent was added to each appropriate well in HCV Antigen Plate.
b. 10µl of each control and specimen was added to each appropriate well.

c. Mixed well by tapping the plate gently.

5. The plate was sealed with an adhesive slip and incubated the plate in a 37±1°C water bath or circuited incubator for 60 minutes.

6. At the end of the incubation period, the plate was washed by following the plate washing procedure.

7. Added 100µl of the Diluted Conjugate in each well except the two Blanks.
Sealed the plate with an adhesive Slip, and incubated the plate in a 37±1°C water bath or circuited incubator for 30 minutes.

8. At the end of the incubation period, the plate was washed by following the plate washing procedure.

9. One of the following two methods for color development was used.

   A. Mixing equal volume of TMB Substrate Solution A and B in a clean container immediately prior to use and adding 100ul of the mixture to each well including 2 blank wells.

   B. Adding 50 ul of TMB Substrate Solution A first, then adding 50ul of TMB Substrate Solution B into each well including 2 blanks. Mixing well gently.

10. The plate was covered with Blank Cover and incubated the plate at RT for 30 minutes.

11. The reaction was stopped by adding 100ul of 2N Sulfuric Acid to each well including 2 blanks.
Materials and methods

12. The absorbance of Controls and Test Specimens were determined within 30 minutes at 450nm or 450/650nm if a dual filter instrument by using the lighter one of 2 blanks.

Calculation and determination:

1. Calculation of NCx:

Example:

<table>
<thead>
<tr>
<th>NC</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.045</td>
</tr>
<tr>
<td>2</td>
<td>0.060</td>
</tr>
</tbody>
</table>

NCx = (0.045+0.060)/2 = 0.053

2. Calculation of PCx:

Example:

<table>
<thead>
<tr>
<th>PC</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.510</td>
</tr>
<tr>
<td>2</td>
<td>1.826</td>
</tr>
<tr>
<td>3</td>
<td>1.305</td>
</tr>
</tbody>
</table>

PCx =(1.510+1.826+1.305)/3 = 1.547

Calculation of P-N Value:

P-N=PCx-NCx

Example:
Materials and methods

\[ P-N = 1.547 - 0.053 = 1.494 \]

P-N Value must be \( \geq 0.40 \); otherwise the test is invalid.

4. Determination of Cutoff Value and Cutoff Index:

Cutoff Value = NCx + 0.025 \times PCx

Example:

Cutoff Value = 0.053 = 0.25 \times 1.547

\[ = 0.053 + 0.387 \]

\[ = 0.440 \]

Cutoff Index = Sample OD Value / Cutoff VALUE

Example:

Sample Value is 0.596

\[ \text{Cutoff Index} = 0.596 / 0.440 = 1.355 \]

Interpretation of result:

1. Specimens with absorbance values LESS than the CUTOFF VALUE were considered NON-REACTIVE by the criteria of GBC’S SP-NANBASE C-96 3.0.

2. Specimens with absorbance values GREATER than or EQUAL to the CUTOFF VALUE were considered initially REACTIVE. If both CUTOFF INDEXES of the duplicate are GREATER than 1.5, the specimen was considered to be repeatedly REACTIVE for antibodies to HCV by the criteria of GBC’S SP-NANBASE C-96 3.0.
3. Initially reactive specimens, of which both CUTOFF INDEXES of the duplicate retest are LESS than 1.0, were considered NON-REACTIVE for antibodies to HCV.

4. If one of the two CUTOFF INDEXES of the duplicate is GREATER than 1.0 but LESS than 1.5, the specimen was interpreted as, QUESTIONABLE and this patient should be continuously monitored in the future.

5. If one of the two CUTOFF INDEXES of the duplicate is GREATER than 1.5 and the other one is LESS than 1.0, this indicated unusual experimental error. The test was repeated again.

3.4.5 IgM anti-HEV: (Immunovision, USA)

Introduction:

Principle of the assay

Wells of micro titer plate are coated with HEV-specific immuno-dominant recombinant antigens. The solid phase is then first treated with the diluted sample and HEV IgM is captured, if present, by the antigens. After washing out all the other components of the sample, in the 2nd incubation bound HEV IgM is detected by the addition of anti IgM antibody, labeled with peroxidase (HRP). The enzyme captured on the solid phase, acting on the substrate/chromogen mixture, generates an optical signal that is proportional to the amount of anti HEV IgM antibodies present in the sample.

The immunoVision HEV IgM ELISA kit uses ORF2 as well as ORF3 recombinant antigens instead of synthetic peptides making it more sensitive and specific than other kits which are commercially available.
Materials and methods

Assay procedure:

1) All the components of the kit was brought to room temperature, at least one hour before use, and the liquid reagents were mixed carefully on vortex.

2) The controls were used in duplicate.

3) The excess washing solutions from wells were washed by blotting them gently on a paper adsorbent pad.

4) The A1 well was left empty for blanking operations. The samples were diluted 1:101 with the sample dilute (5 μl sample + 500 μl diluent).

5) Controls and samples were dispensed into wells according to the following table:

<table>
<thead>
<tr>
<th>Position</th>
<th>Calibrator/Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>blanking well</td>
</tr>
<tr>
<td>B1+C1</td>
<td>100 μl of negative control</td>
</tr>
<tr>
<td>D1+E1</td>
<td>100 μl of positive control</td>
</tr>
<tr>
<td>F1.....H12</td>
<td>100 μl of diluted samples</td>
</tr>
</tbody>
</table>

The microplate was covered with the plate sealer and incubated the strips for 30 min at room temperature.

6) The plate sealer was peeled and the microplate was washed according to instructions.

7) Added 100 μl enzyme conjugate to all the wells, but A1. Incubated the microplate sealed for 30 min at room temperature.

8) Peel out the plate sealer and wash the microplate according to instructions.
9) Add 100μl of substrate to all the wells. Incubate the microplate for 15 min at room temperature, protected from light.

10) The enzymatic reaction was stopped by adding 50μl stop solutions to all the wells.

11) The microplate was read at 450nm and 620-630nm blanking the instrument on A1 well.

Validity of the assay

The assay was considered valid if:

1. The OD 450nm of the A1 blanking well was < 0.100.

2. After blanking on A1, the OD 450nm mean value of the negative control (NC) was <0.200.

3. The OD 450 nm mean value of the positive control (PC) was >0.500.

Calculation of results:

The cut-off value was calculated through the following formula:

\[
\text{Cut-off} = \text{NC} + 0.200
\]

Samples with an OD450nm value lower than the cut-off were classified as negative for HEV IgM.

Samples with an OD450nm value higher than the cut-off were classified as positive for HEV IgM.

Examples of calculations:
3.5 Principle of PCR

PCR was originally developed by Kary Mullis and other scientists at Cetus Corporation, USA. It involves the in vitro enzymatic synthesis of millions of copies of a specific DNA segment. The reaction utilises annealing and extension of two oligonucleotide primers than flank the target region in duplex DNA after denaturation of the DNA. PCR utilizes the principle of a pair of primers for repetitive, bi-directional synthesis of a segment of double stranded DNA. The primers, which are short 20 to 25 nucleotide long sequences, are chosen such that the extension product of each cycle will form the template for the subsequent DNA synthesis cycles. For this to occur, the primers must be complimentary to the flanking regions, on opposing DNA strands, of the particular segment of double stranded DNA molecule. The primer DNA complex provides a substrate for DNA polymerase called Taq DNA polymerase, which is a heat stable enzyme. In the presence of dNTP the DNA polymerase will extend the primers in a DNA synthesis reaction. Each newly synthesized strand will be complimentary to the template DNA and will acquire at its 3' end, the sequence complimentary to the other primer used in PCR. On reheating to 90-95°C, the newly formed hybrids will denature, thereby providing, two additional template molecules during the next primer
Materials and methods

annealing step. Each successive cycle of denaturing, primer annealing and primer extensions give an exponential (2 fold) increase in the targeted segment of DNA. This reaction becomes rate limiting due to competition between primer binding, and reannealing of the greatly amplified single DNA molecules synthesized during the PCR. A typical reaction achieves amplification in the order of 10^6 fold. The product will be of uniform size, corresponding to the distance separating the 5'ends of the two primer binding sites on the opposing strands of the target segment of DNA.

In this study the variant of PCR used to amplify is Allele Specific Amplification (ASA). In this technique oligo nucleotides complementary to a given DNA sequence except for a mismatch at their 3'hydroxyl residue will not function as primers in the PCR under appropriate conditions. A typical Allele Specific Amplification consists of two complementary reactions, each containing a common primer, an allele specific primer and Taq DNA polymerase lacking 3'-→5'proofreading activity. The first reaction consists of a primer specific for the normal (or wild type) DNA sequence and refractory to amplification from mutant DNA at given locus. Similarly, the second reaction contains a mutant specific primer unable to amplify wild-type DNA. Molecular conformation is achieved by analysis of the resulting PCR amplicon profiles. A normal individual with wild type homozygous will generate product in the first reaction only. The mutant individual will generate product in the mutant specific reaction only and heterozygous individual will generate product in both the reaction and (Newton, et al., 1989).

Detection of C3, C4 and CFH gene polymorphisms

Whole blood samples from Cases and Controls were collected in E.D.T.A. vials for the study.
Isolation of genomic DNA from freshly collected blood samples

DNA was isolated from peripheral blood mononuclear cells employing the standard methods using Proteinase K and Phenol-chloroform extraction.

1. 300µl of freshly collected blood was taken and added 900µl of blood lysis buffer (155mM NH₄Cl, 10mM KHCO₃, 0.1mM EDTA). (Blood: lysis buffer = 1:3) It was kept for 15-20 min. at room temperature and centrifuged at 5000 rpm at 4°C for 15 mins.

2. Supernatant was discarded and to the white pellet of WBCs equal volume (300µl) of SE buffer (75mM NaCl, 20mM EDTA) (blood sample: SE = 1:1) was added. Mixed by pipetting so no clumps remain.

3. Proteinase K was added to a final concentration of 100µg/ml and 1/5 volume of 10% SDS. Incubated the solution at 37 °C overnight.

4. Added equal volume of 0.1M TE equilibrated phenol (pH 8.0).

5. Mixed on an overhead shaker for 20 min. and centrifuged at 6,500 rpm at 4°C for 15 min.

6. Supernatant was added with equal volume of phenol and CIA (chloroform: iso-amyl alcohol 24:1) and repeated the step 5.

7. Supernatant was taken and added equal vol. of CIA and repeated the step 5.

8. The supernatant was taken and to it 1/10 volume of chilled CH₃COONa (3M, pH 5.2) and 2.5 volume of absolute alcohol or equal volume of isopropanol was added.

9. Mixed gently by rotating the tube upside down and the DNA was spooled out or centrifuged it at 12,000 rpm at 4°C for 10 min.
10. The pellet of DNA was washed twice with 70% ethanol and centrifuged at 12,000 rpm at 4°C for 10 min.

11. The pellet was air-dried at room temperature or vacuum for 15 min.

12. The pellet was dissolved in 1X TE (10X Tris EDTA-100mM Tris Cl (pH 8.0), 10mM EDTA (pH8.0)).

**Quantitation of DNA**

The genomic DNA isolated was quantitated by gel electrophoresis on 1% Agarose gel prepared in 1X Tris acetate EDTA buffer (40mM Tris acetate, 1mM EDTA) and stained with Ethidium Bromide (10mg/ml) till final concentration of 0.5µg/ml. 1 µg of Hind III digested λ DNA was used as a marker to compare the UV-induced fluorescence emitted at 590nm.

**3.5.1 Allele Specific PCR amplification of CFH gene SNP**

Determination of the CFH gene mutations was accomplished with Allele Specific Amplification (Newton, et al., 1989). The total volume of the PCR was 20 µl containing 100 ng of genomic DNA, 10xPCRbuffer (XT-5 PCR system, Merck, India), 0.25 mM of each dNTP (Merck, India), 0.6 units of XT-5 Taq™DNA polymerase (Merck, India) and 0.3 µM of common primer and 0.3 µM of allele specific primer (Table 1). The PCR was performed in a Thermocycler Bioemtra with an initial denaturation at (95°C for 5 min), 14 cycles (94°C for 30 sec, 59°C for 30 sec, 72°C for 30 sec), 19 cycles (94°C for 30 sec, 54.5°C for 30 sec, 72°C for 30 sec) and a final extension step (72°C for 5 min) for rs10922103. The conditions for amplification of rs4658046 were (95°C for 5 min), 34 cycles (94°C for 30 sec, 59.3°C for 30 sec, 72°C for 30) sec and a final extension step (72°C for 5 min). The products were than
Materials and methods

electrophoresed on a 3 % agarose gel. The products were than electrophoresed on a 3 % agarose gel. Representative images of rs 10922103 and rs4658046 shown in Figure 66 and Figure 67 respectively.

3.5.2 Allele Specific PCR amplification of C4 gene SNP

Determination of the C4 gene mutations was accomplished with Allele Specific Amplification (Newton, et al., 1989). The total volume of the PCR was 20 µl containing 100 ng of genomic DNA, 10xPCR buffer (XT-5 PCR system, Merck, India), 0·25 mM of each dNTP (Merck, India), 0·6 units of XT-5 Taq™DNA polymerase (Merck, India) and 0·3 μM of common primer and 0·3 μM of allele specific primer (Table 2). The PCR was performed in a Thermocycler Bioemtra with an initial denaturation at (95°C for 5 min), 14 cycles (94°C for 30 sec, 61°C for 30 sec, 72°C for 30 sec), 19 cycles (94°C for 30 sec, 56.5°C for 30 sec, 72°C for 30 sec) and a final extension step (72°C for 5 min) for rs2857009. Representative images of rs2857009.

Table 1: Primers for CFH SNP screening

<table>
<thead>
<tr>
<th>SNP</th>
<th>Primer Sequence</th>
<th>Tm</th>
<th>size</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs10922103</td>
<td>Wild 5'-TATACATAGGGTTTTCATCTCATCC-3'</td>
<td>59</td>
<td>119 bp</td>
</tr>
<tr>
<td></td>
<td>Mutant 5'-TATACATAGGGTTTTCATCTCATCC-3'</td>
<td>54.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Common 5'-TACCCACTGGATTTCCGAGA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs4658046</td>
<td>Wild 5'-CAGCAGGCTACAAATCTGACAATCTC-3'</td>
<td>59.3</td>
<td>178 bp</td>
</tr>
<tr>
<td></td>
<td>Mutant 5'-CAGCAGGCTACAAATCTGACAATCTC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Common 5'-GGGAGAAGTAAGGGACAGCC-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.5.3 Allele Specific PCR amplification of C3 gene SNP

Determination of the C3 gene mutations was accomplished with Allele Specific Amplification (Newton, et al., 1989). The total volume of the PCR was 20 μl containing 100 ng of genomic DNA, 10xPCRbuffer (XT-5 PCR system, Merck, India), 0.25 mM of each dNTP (Merck, India), 0.6 units of XT-5 Taq™DNA polymerase (Merck, India) and 0.3 μM of common primer and 0.3 μM of allele specific primer (Table 3). The PCR amplification was performed in a Thermocycler Bioemtra with an initial denaturation at (95°C for 5 min), 14 cycles (94°C for 30 sec, 67.5°C for 30 sec, 72°C for 30 sec), 19 cycles (94°C for 30 sec, 64.5°C for 30 sec, 72°C for 30 sec) and a final extension step (72°C for 5 min) for rs2230201. The conditions for amplification of rs7951 were (95°C for 5 min), 35 cycles (94°C for 30 sec, 57.5°C for 30 sec, 72°C for 30 sec) and a final extension step (72°C for 5 min). The products were then electrophoresed on a 3 % agarose gel. Representative images of rs 2230201 and rs7951 shown in Figure 69 and Figure 68 respectively.
Table 3: Primers for C3 SNP screening

<table>
<thead>
<tr>
<th>SNP</th>
<th>Primer Sequence</th>
<th>Tm</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild</td>
<td>5'- CCCGTCCAGCAGTACCTTC -3'</td>
<td>67.5</td>
<td>186 bp</td>
</tr>
<tr>
<td>Mutant</td>
<td>5'- CCCGTCCAGCAGTACCTTT -3'</td>
<td>64.5</td>
<td></td>
</tr>
<tr>
<td>Common</td>
<td>5'- AACAGAGGATTTCCCTGCCT -3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wild</td>
<td>57.5</td>
<td>207 bp</td>
</tr>
<tr>
<td></td>
<td>5'- AAGTATGAGCTGGACAAAGCC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mutant</td>
<td>5'- AAGTATGAGCTGGACAAAGCT-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Common</td>
<td>5'- CCTACAACCTCAGCAGCACA -3'</td>
<td></td>
</tr>
</tbody>
</table>

Precautions while performing PCR

During carrying out PCR, two very important factors have to be taken care of, namely,

1. Fragility of RNA/DNA fragments.
2. Exquisite amplification power of the procedure which can result in a lot of non-specific amplification products.

For this a number of precautions were taken to exclude or minimize the possibility of contamination during sampling and subsequent handling. This includes:

1. Use of strict aseptic precautions during venepuncture and during subsequent separation of serum and further handling.
2. Sera for purposes of PCR were aliquoted at the beginning and kept at -70°C within 4 hrs of collection.
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3 Repeated freezing - thawing was avoided.

4 Clean pipettes meant only for PCR purposes were used.

5 Positive and negative controls were included in every PCR assay.

3.6 Expression study of C3, C4 and CFH.

Expression of C3, C4 and CFH was been done using commercially available ELISA Kits (Abnova) following the manufacturer’s protocol described below:-

3.6.1 Expression study of C3

Principle of the assay

The C3 (Human) ELISA Kit is designed for detection of human complement C3 in plasma, serum samples. This assay employs a quantitative competitive enzyme immunoassay technique that measures human complement C3 in less than 3 hours. A polyclonal antibody specific for human complement C3 has been pre-coated onto a 96-well microplate with removable strips. Complement C3 in standards and samples is competed by a biotinylated complement C3 sandwiched by the immobilized antibody and streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Assay procedure

Standard

Standard: Reconstitute the 60 µg of Complement C3 Standard with 2mL of EIA Diluent to generate a solution of 30 µg/mL. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by
Materials and methods

serially diluting the standard solution (30 µg/mL) 1:2 with EIA Diluent to produce 15, 7.5, 3.75, 1.875, 0.938 and 0.469 µg/mL solutions. EIA Diluent serves as the zero standard (0 µg/mL). Any remaining solution should be frozen at -20°C and used within 30 days.

<table>
<thead>
<tr>
<th>Tube</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
<th>S7</th>
<th>S8</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/ml</td>
<td>30</td>
<td>15</td>
<td>7.5</td>
<td>3.75</td>
<td>1.875</td>
<td>0.938</td>
<td>0.469</td>
<td>0.00</td>
</tr>
</tbody>
</table>

All the reagents and samples were brought to room temperature before use.

1. Serum samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 3000 x g for 10 minutes. Remove serum and assay. Dilute samples 1:800 into EIA Diluent. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

2. Add 25 µL of Human Complement C3 Standard or sample per well, and immediately add 25 µL of Biotinylated Complement C3 to each well (on top of the Standard or sample) and mix gently. Cover wells with a sealing tape and incubate for 2 hours. Start the timer after the last sample addition.

3. Wash five times with 200 µL of Wash Buffer manually. Invert the plate each time and decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid. If using a machine wash six times with 300 µL of Wash Buffer and then invert the plate, decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.

4. Add 50 µL of Streptavidin-Peroxidase Conjugate to each well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
5. Wash a microplate as described above

6. Add 50 µL of Chromogen Substrate per well and incubate for about 15 minutes or until the optimal blue color density develops. Gently tap plate to ensure thorough mixing and break the bubbles in the well with pipette tip.

7. Add 50 µL of Stop Solution to each well. The color will change from blue to yellow.

8. Read the absorbance on a microplate reader at a wavelength of 450 nm immediately. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

Calculation of Results

Duplicate readings for each standard, control, and sample were averaged and subtracted the average zero standard optical density. A standard curve was created by reducing the data using a freely available online software (ELISA Analysis, www.elisaanalysis.com) capable of generating a four parameter logistic (4-PL) 10 curve-fit.

3.6.2 Expression study of C4

Principle of the assay

The Human complement C4 ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for detection of human complement C4 in plasma and serum. This assay employs a quantitative competitive enzyme immunoassay technique that measures
Materials and methods

human complement C4 in less than 3 hours. A polyclonal antibody specific for human complement C4 has been pre-coated onto a 96-well microplate with removable strips. Complement C4 in standards and samples is competed by a biotinylated complement C4 sandwiched by the immobilized antibody and streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Assay procedure

Standard

Reconstitute the 50 µg of Human complement C4 Standard with 5 ml of MIX Diluent to generate a solution of 10 µg/ml. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting the stock solution (10 µg/ml) 1:2 with MIX Diluent to produce 5, 2.5, 1.25, 0.625, and 0.313 µg/ml solutions. MIX Diluent serves as the zero standard (0 µg/ml). Any remaining solution should be frozen at -20°C and use within 30 days.

<table>
<thead>
<tr>
<th>Tube</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/ml</td>
<td>5</td>
<td>2.5</td>
<td>1.25</td>
<td>0.625</td>
<td>0.313</td>
<td>0.00</td>
</tr>
</tbody>
</table>

All the reagents and samples were brought to room temperature before use.

1. Serum samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 3000 x g for 10 minutes. Remove serum and assay. Dilute samples 1:200 into MIX Diluent or within the range of 100x-1000x. The undiluted
samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

2. Add 25 μl of Human Complement C4 standard or sample per well, and immediately add 25 μl of Biotinylated Complement C4 to each well (on top of the Standard or sample) and mix gently. Cover wells with a sealing tape and incubate for two hours. Start the timer after the last sample addition.

3. Wash five times with 200 μl of Wash Buffer manually. Invert the plate each time and decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid. If using a machine wash six times with 300 μl of Wash Buffer and then invert the plate, decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.

4. Add 50 μl of Streptavidin-Peroxidase Conjugate to each well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.

5. Wash the microplate as described above.

6. Add 50 μl of Chromogen Substrate per well and incubate for about 10 minutes or till the optimal blue color density develops. Gently tap plate to ensure thorough mixing and break the bubbles in the well with pipette tip.

7. Add 50 μl of Stop Solution to each well. The color will change from blue to yellow.

8. Read the absorbance on a microplate reader at a wavelength of 450 nm immediately. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.
Calculation of Results:
Duplicate readings for each standard, control, and sample were averaged and subtracted the average zero standard optical density. A standard curve was created by reducing the data using a freely available online software (ELISA Analysis, www.elisaanalysis.com) capable of generating a four parameter logistic (4-PL) 10 curve-fit.

3.6.3 Expression study of CFH

Principle of the assay

The CFH (Human) ELISA Kit is designed for detection of human FH in urine, saliva, milk, plasma, serum and cell culture supernatant samples. This assay employs a quantitative sandwich enzyme immunoassay technique that measures FH in less than 4 hours. An polyclonal antibody specific for FH has been pre-coated onto a 96-well microplate with removable strips. Human FH in standards and samples is sandwiched by the immobilized antibody and the biotinylated polyclonal antibody specific for human FH, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Assay procedure

Standard Standard Curve: Reconstitute the 144 ng of Complement Factor H Standard with 4 ml of EIA Diluent to generate a stock solution of 36 ng/ml. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting the stock solution (36 ng/ml) 1:2 with
Materials and methods

equal volume of EIA Diluent to produce 18, 9, 4.5, 2.25, 1.125, 0.563, and 0.281 ng/ml. EIA Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20°C and used within 30 days.

<table>
<thead>
<tr>
<th>Tube</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
<th>S7</th>
<th>S8</th>
</tr>
</thead>
<tbody>
<tr>
<td>ng/ml</td>
<td>18</td>
<td>9</td>
<td>4.5</td>
<td>2.25</td>
<td>1.125</td>
<td>0.563</td>
<td>0.281</td>
<td>0.00</td>
</tr>
</tbody>
</table>

All the reagents and samples were brought to room temperature before use.

1. Serum samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 3000 x g for 10 minutes. Remove serum. Dilute samples 1:200000 into EIA Diluent and assay. The undiluted samples can be stored at -20°C or below for up to 90 days. Avoid repeated freeze-thaw cycles.

2. Add 50 µl of Complement Factor H Standard or sample per well. Cover wells with a sealing tape and incubate for 2 hours. Start the timer after the last sample addition.

3. Wash five times with 200 µl of Wash Buffer manually. Invert the plate each time and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If using a machine, wash six times with 300 µl of Wash Buffer and then invert the plate, decanting the contents; hit 4-5 times on absorbent material to completely remove the liquid.

4. Add 50 µl of Biotinylated Complement Factor H Antibody to each well and incubate for 1 hour.

5. Wash the microplate as described above.
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6. Add 50 µl of Streptavidin-Peroxidase Conjugate per well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.

7. Wash the microplate as described above.

8. Add 50 µl of Chromogen Substrate per well and incubate for approximately 20 minutes or till the optimal blue color density develop. Gently tap the plate to ensure thorough mixing and break the bubbles in the well with pipette tip.

9. Add 50 µl of Stop Solution to each well. The color will change from blue to yellow. Read the absorbance on a microplate reader at a wavelength of 450 nm immediately.

Calculation of Results:
Duplicate readings for each standard, control, and sample were averaged and subtracted the average zero standard optical density. A standard curve was created by reducing the data using a freely available online software (ELISA Analysis, www.elisaanalysis.com) capable of generating a four parameter logistic (4-PL) curve-fit.

3.7 RNA Extraction
RNA isolation from whole blood was done by the standard TRIZOL method. Briefly 250 µl of whole blood was added to 750 µl of Trizol reagent and 100 µl of 1-bromo-3-chloropropane and centrifuged at 12000g for 15 minutes. The aqueous phase was separated and transferred to a new eppendorf to which 500 µl isopropanol was added and centrifuged at 12000g for 8 minutes. The supernatant is then discarded and 1 ml 75% ethanol is added and vortexed. This is then again centrifuged at 7500g for 5 minutes. The supernatant is discarded and allowed to air dry for 10-15 minutes. The RNA is suspended in nuclease free water and incubated for 10 minutes at 55 °C.
Materials and methods

RNA was quantified by using Nanophotometer P300. Samples with $A_{260}/A_{280} > 1.8$ and $A_{260}/A_{230} > 1.8$ were selected for further down processing.

3.7.1 RNA purification

To remove any carry over genomic DNA, the isolated RNA is digested using DNase I treatment. DNase I is an endonuclease that digests single and double stranded DNA. It hydrolyses phosphodiester bonds producing mono- and oligodeoxyribonucleotides with 5' - phosphate and 3' -OH groups. The steps for DNasel treatment is as follows:

1μg of RNA was mixed with 1μl 10x Reaction buffer and 1 unit DNaseI and the volume was made upto 10μl and incubated at 37°C for 30 min. Add 1μl 50mM EDTA and incubate at 65°C for 10 min.

3.7.2 cDNA preparation

From the above DNasel treated samples, 2μg total RNA was used for conversion into cDNA using Thermo scientific first strand cDNA synthesis kit. The process has been described as follows:

- 2μg total RNA is mixed with 1μl of Oligo(dT)$_{18}$ and made to a final volume of 11μl and incubated at 80°C for 5 min.
- After incubation, the above tubes are kept on ice quickly.
- cDNA reaction mixture is prepared by adding 4μl of 5xRT buffer, 1 μl Rinolock Inhibitor, 2 μl of 10mM dNTP mix, 2 μl M-MuLV Reverse transcription and is added to eppendorf containing mixture of RNA and Oligo(dT).
- The above reaction mixture is incubated at 42°C for 1 hr and then at 70°C for 5 mins.
Materials and methods

The cDNA prepared is then checked in nanophotometer P300 and samples with $A_{260}/A_{280} \geq 1.8$ and $A_{260}/A_{230} \geq 1.8$ were selected for further processing.

3.8. RT-PCR of C3, C4 and CFH gene

C3, C4 and CFH mRNA gene was amplified by PCR in appropriate reaction conditions using suitable set of primers from the Primer Bank and are shown in table below. The instruments used for real-time PCR have a thermal cycler and a fluorescence detection system controlled by software and can monitor real-time product accumulation by measuring the increase in fluorescence during each cycle of the PCR to generate quantitative results. SYBR1 Green I dye is thought to bind in the minor groove of DNA; in its unbound state it has relatively low fluorescence but when bound to DNA it fluoresces brightly. As the amount of DNA in the PCR increases, the amount of fluorescence from the dye increases proportionally. The incorporation of SYBR1 Green I into real-time RT-PCR allows the detection of any double-strand DNA generated during PCR. This provides great flexibility because no target specific probes are required; however, both desired and undesired products will generate a signal. This problem can be overcome by analysing the PCR product melting curve. The melting temperature ($T_m$) of an amplicon depends markedly on its size and nucleotide composition; hence, it is feasible to distinguish the fluorescence signal of the desired product from the signal(s) of undesirable products that melt at lower temperatures. This is the only post-PCR analysis that is necessary, making the technique both rapid and reliable.

The $2^{-\Delta\Delta Ct}$ method was used to carry out the relative expression of C3, C4 and CFH mRNA. The reaction was carried out in Rotor Gene 3000. The entire method has been mentioned below briefly.
Materials and methods

To obtain dilution curve and calculate PCR efficiency for each pair of primer

- 5 fold serial dilution of cDNA was prepared (50ng, 10ng, 2ng, 0.4ng, 0.08ng)
- 2 duplicates of each diluted template and NTC were set up in a PCR reaction.
- The slope generated by rotor gene 3000 software was evaluated. The primer pair and Ta which presented slope of <0.1 was selected for relative quantification.

Cycling condition and data acquisition

The total volume of the PCR was 20 µl containing 50 ng of genomic DNA, 10xPCR buffer (166mM (NH₄)₂SO₄, 67mM Tris-HCL, 20mM MgCl₂, 10% DMSO, 0.8 mg/ml BSA, 50mM β-ME, 1.5% Triton X-100) 0.25 mM of each dNTP (Merck, India), 0.9 units of XT-5 Taq™DNA polymerase (Merck, India) and 0.25 µM of both primers and 0.7x Sybr Green I (Molecular Probes). The PCR amplification for was performed in a Rotor Gene 3000 with an initial denaturation at (95°C for 5 min), 40 cycles (95°C for 5 sec, Ta for 5 sec, 72°C for 5 sec, acquisition at 82°C for 5 sec), and a final extension step (72°C for 3 min). Melt curve analysis was done by ramping from 75°C to 98°C with a resolution of 1°C. The primers used for amplification are mentioned below with their Ta and Tm (Table 4). The machine generated Ct of C3, CFH and C4 have been shown in Figure 71, Figure 74 and respectively. The melt peak image of C3, GAPDH, CFH and C4 has been shown in Figure 72, Figure 73, Figure 76, Fig

Table 4: Primers for qPCR amplification

<table>
<thead>
<tr>
<th>Gene (Primer Bank ID)</th>
<th>Forward</th>
<th>Reverse</th>
<th>Ta</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3(115298677c1)</td>
<td>GGGGAGTCCCATGTA CTCTATC</td>
<td>GGAAGTCGTGGACAG TAACAG</td>
<td>61°C</td>
<td>125</td>
</tr>
</tbody>
</table>
Materials and methods

<table>
<thead>
<tr>
<th></th>
<th>TCCAGAGAGGGTA C59°C 177</th>
<th>CTCAGGAACACTGAT C59°C 177</th>
<th>CFH(184172391c2) CACACAAGATGGAT C58°C 132</th>
<th>GAPDH (378404907c1) GGAGCGAGATCCCTC C58°C 197</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4 (443671a1)</td>
<td>GATCCGTC</td>
<td>CCTTTCAC</td>
<td>GGATGCAGGCAACG TCTAT</td>
<td>GGCTGTTGTACTCTT CTCATGG</td>
</tr>
<tr>
<td>CFH(184172391c2)</td>
<td>CACACAAGATGGAT GGTGC</td>
<td>GGATGCAGGCAACG TCTAT</td>
<td>GGATGCAGGCAACG TCTAT</td>
<td>GGCTGTTGTACTCTT CTCATGG</td>
</tr>
<tr>
<td>GAPDH (378404907c1)</td>
<td>GGAGCGAGATCCCTC CAAAAT</td>
<td>GGCTGTTGTACTCTT CTCATGG</td>
<td>GGCTGTTGTACTCTT CTCATGG</td>
<td>GGCTGTTGTACTCTT CTCATGG</td>
</tr>
</tbody>
</table>

3.9 Viral RNA extraction

RNA was extracted using acid guanidium-phenol-chloroform method as described by Chomczynski and Sacchi with slight modification.

**Procedure:**

1. To 100μl of serum sample, 600μl of lysis buffer with BME (for 4ml of lysis solution 30μl of 14 M BME (final concentration 0.1M) at the time of experiment was added, 50μl of 2M sodium acetate (pH 4.0), 600μl of phenol mix (DEPC water saturated phenol), and 131 μl of CIA (49:1) were added, mixed well and kept in ice for 15 minutes.

2. It was then centrifuged at 12000 rpm for 20 minutes at 4°C.

3. CIA step was repeated and aqueous phase was transferred to fresh eppendorf tube.

4. Equal volume of ice cold isopropanol was added and kept at 70°C for 90 minutes to precipitate the RNA.

5. Then again centrifuge was performed at 12000 rpm for 20 minutes at 4°C.

6. The pellets was resuspended in 150μl lysis buffer without BME, equal volume of ice cold isopropanol was added and kept 70°C for 90 minutes.
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7. It was again centrifuged at 12000 rpm for 20 minutes at 4°C.

8. The pellet was washed in 70% ethanol.

9. Air dried and dissolved the pellets in 30μl of DEPC water.

3.9.1 Detection of HCV RNA

HCV RNA was detected in serum by reverse transcriptase PCR. Complementary DNA (c-DNA) was synthesized from 5μl viral RNA using 5X reaction buffer, 0.2 μg random hexamer, 1 mM dNTP, 20U ribonuclease inhibitor and 40U M-MuLV Reverse transcriptase in a 20 μl reaction mixture. The reaction was carried out at 42°C for 1 hr followed by 10 min incubation at 70°C.

For the amplification of the 404 bp HCV RNA from the viral genome, 2 consecutive PCR was done. First PCR was done using 2 μl c-DNA as template, 10 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 400μM dNTPs, 0.4 μM of each primer and 1.2 U of Taq in a total of 25 μl reaction mixture. The temperature profile used was as follows- initial denaturation at 94°C/2 min, 25 repeating cycles of DNA denaturation at 94°C/1 min, primer annealing at 55°C/1 min and nucleotide extension at 72°C/2 min followed by final extension at 72°C/5 min (Table 5).

<table>
<thead>
<tr>
<th>Table 5: Primers used for amplification of HCV cDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer sequence</td>
</tr>
<tr>
<td>HCV outer 5’ACTGCTCTGATAGGTGCTTG3’</td>
</tr>
<tr>
<td>HCV 5’AGGTCTCGTACGCTGCA3’</td>
</tr>
</tbody>
</table>
### 3.9.2 Detection of PCR products

10μl of the final PCR product was visualized in ethidium bromide-stained 3% Nusieve agarose gel under a UV transilluminator. Amplicon size was of 404bp. Both positive and negative controls were included at the RNA extraction and amplification step.

### 3.10 Quantification of HCV viral load by real time PCR

**Principle of Real-Time PCR**

During PCR, forward and reverse primers hybridize to a specific sequence product. A TaqMan probe, which is contained in the same reaction mixture and consists of an oligo-nucleotide labeled with a 5'-reporter dye and a downstream, 3'-quencher dye, hybridizes to a target sequence within the PCR product. A Taq polymerase which possesses 5'-3' exonuclease activity cleaves the probe. The reporter dye and quencher dye are separated upon cleavage, resulting in an increase in fluorescence for the reporter. Thus, the increase in fluorescence is directly proportional to the target amplification during PCR. The quantification of HCV was done by commercially available HCV Real Time PCR kit.

The master mix contains reagents and enzymes for the specific amplification of HCV and for direct detection of the specific amplicon in the fluorescence channels Cycling A.Fam of the Rotor Gene 3000 & the reference gene on Cycling A.Joe. External positive Standards (HCV S 1-5) have been supplied which allow the determination of the gene load. Inhibition Control Gene was used which allowed determining and
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controlling possible PCR inhibition. The reagents of this gene were present in the premix and only the internal control gene was added in the reaction mix.

The quantification standards were treated in the same way as extracted samples and the same volume was used i.e. 30μL instead of the sample. To generate a standard curve in the Rotor Gene 3000, all the 5 Standards were used as defined in the menu window Edit Samples of the RotorGene™ software and the standards concentration was also specified.

The Cooling block was pre-cooled to +4°C in a Refrigerator. For each sample the HCV Super mix 14μL, MgCl₂ 5μL and internal control 1μL was added to 30μL of the RNA or Standard, thus a total 50μL was subjected for the amplification.

Programming the RotorGene™3000

The RotorGene™3000 PCR program for the detection of HCV was divided into following steps:

A. Setting of general assay parameters & reaction volume: First it was confirmed that the PCR tubes used were No Domed PCR tubes and the volume of the reaction was set to 50μL.

B. Thermal Profile & Calibration: Programming of the temperature profile was done by activating the Edit Temperature Profile. The cycling profile was First hold 50°C for 15 minutes (cDNA synthesis step), Second hold 95°C for 10 minutes (initial activation of the Hot Start enzyme) then the cycling profile was set denaturation step 95°C for 15 seconds, annealing step 55°C for 20 seconds and extension step 72°C for 15 seconds and the
number of cycles were set to 45 cycles. Final confirmation was done by pressing OK button. Next the calibration was set by clicking the Calibrate button. The calibration temperature was set to 55°C and Calibration was set before 1st acquisition. Then the RotorGene™ was started by pressing Start Run Button.

Specifications

Sensitivity and Reproducibility: A dilution series had been set up from $10^6$ down to $10^0$ copies/μL of HCV RNA and analyzed. The assays were carried out on three different days in the form of 8-fold determinations. The results were determined by a probit analysis. The detection limit determined was consistently 25 copies/mL. This means that there is 95% probability that 25 copies/mL will be detected.

Specificity: HCV Real time PCR kit did not led to a positive signal to HBV, HAV, HEV, HIV-1, HIV-2, Human Herpes Virus 1&2. Gene sequence analysis of the amplified region of HCV showed a pronounced homology among the various HEV strains, and no homology with other RNA.

3.11 Genotyping of HCV

Viral RNA was extracted from the serum of the HCV RNA positive patients using the QIAamo viral RNA kit (Qiagen) following manufacturer’s protocol. The extracted viral RNA was subjected to c-DNA synthesis using random hexamer as primer. The cDNA prepared from the isolated viral RNAs were subjected to RT-PCR analysis using primers mentioned in the above table (). The amplified PCR products of the HCV RNA after the second PCR were subjected to RFLP by enzymes AccI, MboI and MvaI using the manufacturer’s protocol. Genotypes
were detected according to the fragments obtained and have been shown in the table below (Table 6).

Table 6: specific fragment pattern relative to genotype after RFLP

<table>
<thead>
<tr>
<th>Type 405 bp</th>
<th>1a</th>
<th>1b</th>
<th>1c</th>
<th>2a</th>
<th>2b</th>
<th>2c</th>
<th>3a</th>
<th>3b</th>
<th>4a</th>
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<td>175</td>
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<td>181</td>
<td>142</td>
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</tbody>
</table>

3.12 Statistical analysis

Statistical evaluation was carried out using the Statistical package for the Social Sciences (SPSS) version 20 for windows. Quantitative data was expressed as mean ± standard deviation. It was analyzed using Mann Whitney U test and Student's t-test as applicable. The categorical data was expressed as proportions and was analyzed using Chi square test or Fisher's test as applicable. 2-tailed p value was used to test significance. The data was considered significant when p < 0.05. Odds ratio was also calculated with 95% confidence limit. Regression analysis was done by using linear
regression analysis model and ROC curve was done by using linear regression analysis model and ROC curve was drawn using MedCalc 12.7.