MATERIALS AND METHODS

5.1 Patient enrolment, Demographical, Biochemical and serological profile
To investigate the issues related to the proposed objectives, random cases of patients suffering from liver disease with clinical presented jaundice (n=224) receiving care at Central Hospital, N.F Railway, Maligaon, Guwahati, Assam were enrolled in the study with informed consent. Initial diagnosis of the patients were made on the basis of biochemical test such as liver function test, serological marker for liver diseases such as anti-HCV, HAV-IgM, HEV-IgM, HBsAg etc and the patients were stratified accordingly. The patients were further clinically co-related with severity by virtue of clinical diagnosis such as CT scan, USG, endoscopy and histopathological examinations to stratify into subgroups under different etiological factors. A detail datasheet of each enrolled individual patient with demographical, clinical, food and drinking habit details was collected and maintained. About 5ml of blood was collected from each patient with their informed consent with the help of a registered practitioner. The plasma or serum was separated and stored at -20°C. To avoid repeated freezing and thawing of the serum or plasma, aliquot was prepared and stored at -70°C. The whole blood was stored in 4°C. Further, almost an equal number of non-related voluntary healthy age and sex matched individuals (n=230) were enrolled in the present study with all clinical history and dietary habits details and was used as comparative control group. Blood samples (5ml) was collected from these control individuals, and was screened for any viral infections by serology and liver profiling blood by biochemical analysis. The procedure of sample storage of healthy controls was same as in case of the patients. The Performa and informed consent for every patient and control was maintained with all the data according to the ICMR norms and regulations.

Inclusion and exclusion criteria for patient enrollment
The patients who were clinically diagnosed as acute viral hepatitis, fulminant hepatitis, chronic hepatitis, Cirrhosis, alcoholic liver disease and cryptogenic on the basis of diagnostic criteria were included in the present study. The liver disease patients, who were associated with high-risk group like intravenous drug abusers, chronic renal failure, thalassemia, haemophilia, diabetes mellitus, psychiatric illness, and confection with other viruses, were excluded from the study.
Diagnosis criteria for the Liver disease patients recruited in the study

A. Clinical Examination
All the liver disease patients were examined by qualified Gastroenterologists to determine the different symptoms of liver disease which includes nausea, vomiting, loss of appetite, jaundice, dark urine and pale stool.

B. Biochemical Analysis
The following biochemical tests were performed using patient’s and controls serum samples on presentation using a blood biochemistry semi-auto analyzer:

B1. Alanine transaminase (ALT): Formally known as Serum glutamic pyruvic transaminase (SGPT). It is an enzyme necessary for energy production. Though present in a number of tissues, including heart, liver and skeletal muscles, but it is expressed in highest concentration in liver. It is released in the blood stream as a result of damage of liver cells. Normal range of values for ALT is from 5 to 60 units per liter of serum. Significantly elevated levels of ALT are generally result of primary liver diseases including viral and toxic hepatitis, cirrhosis without jaundice and carcinoma.

B2. Aspartate transaminase (AST): Also known as Serum glutamic oxaloacetic transaminase (SGOT). AST is an enzyme found in cells throughout the body but mostly in heart and liver and to a lesser extent in the kidneys and muscles. When liver cells are injured they release AST in the bloodstream making it an useful test for detecting liver damage. Normal range of values for AST in female is from 6-34 units/L and in males from 8-40 units/L of serum respectively. Increased levels of SGOT are associated with liver diseases, myocardial infarction and muscular dystrophy. In most types of liver disease, the ratio of AST: ALT are low except in alcoholic hepatitis, cirrhosis and during bile duct obstruction.

B3. Serum Bilirubin: Biliruin is a yellow colour pigment formed as a breakdown product of haemoglobin. It is transported to the liver bound to albumin and is known as indirect or unconjugated bilirubin. In the liver it is conjugated to glucoronic acid
to form direct or conjugated bilirubin. Conjugated bilirubin is secreted into the bile by the liver. Serum bilirubin is considered a true test of liver function as it reflects’s the liver’s ability to take up, process, and secrete bilirubin into the bile. Bilirubin concentrations are elevated in the blood either by increased production, decreased conjugation, decreased secretion by the liver, or blockage of the bile ducts. Total bilirubin measures both direct and indirect bilirubin. Normal bilirubin levels are less than 1mg/dl. In acute liver disease, the bilirubin is usually increased in relation to the severity of the acute process. In chronic acquired liver diseases, the serum bilirubin concentration is usually normal until a significant amount of liver damage has occurred and cirrhosis is present. Elevation of total bilirubin may be an indication of obstructive conditions of the bile duct, hepatitis, cirrhosis and several inherited enzyme deficiencies.

B4. Alkaline phosphatase: Alkaline phosphatase (Alk.P) represents a group of hydrolase enzyme that are responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids generating an organic radical and inorganic phosphate. Alk.P is present in all tissues throughout the body, but particularly concentrated in liver, bile duct, kidney, bone and the placenta. Normal range of serum ALK.P is 20 to 140 international units/L of serum. Elevated level of serum Alk.P is associated with a variety of disease such as extrahepatic obstruction of bile duct, intrahepatic cholestasis, infiltrative liver disease and hepatitis.

C. Serological analysis
The following serological tests were performed using patient’s and controls serum samples on presentation using specialized commercial diagnostic serological kits and microplate ELISA reader (Biorad) following manufacturers protocol:

C1. HAV IgM

*Principle of the test:*

Serological assay for determination of IgM antibodies to Hepatitis A virus in the plasma/serum is based on the principle of Sandwich ELISA. The assay is performed with the help of an Enzyme Immunoassay kit developed by Immuno vision.
which involves micro plate coated with HAV-specific immuno-dominant recombinant antigens. The solid phase is treated with the sample and HAV IgM (if present) gets captured by the antigens. Bound HAV IgM is detected by the addition of anti human IgM antibody, labeled with peroxidase (HRP). Action of the enzyme captured on the solid phase with the substrate mixture generates an optical signal that is proportional to the amount of anti IgM antibodies present in the sample.

**Test Procedure:**
Briefly, all the reagents and specimens were brought to room temperature. Serum samples (100µl) were pipetted into the 96 wells micro plate, each coated with HAV specific immune-dominant recombinant antigens. Two negative controls and two positive controls were used in four wells. The plate was covered and incubated at room temperature (37°C) for 30 minutes on a shaker. Each well of the plate was washed four times with washing solution which comprised of phosphate buffer and Tween-20. 100µl of enzyme conjugate was added to all the wells and the plate was incubated at 37°C for 30 minutes on a shaker in dark after properly sealing the plate. After multiple washes, 100µl of substrate was added to all the wells and the microplate was incubated at room temperature for 15 minutes, protected from light. The enzymatic reaction was stopped by adding 50 µl of stop solution consisting of 0.2M sulfuric acid to each well. The ELISA reader was blanked on air (without strip holder and strips) and the micro plate was read for absorbance in each well at 450nm.

**Calculation of Results:**
The cut off value was calculated using the formula: 
\[ \text{Cut off} = \text{NC} + 0.250 \]
where NC is the mean optical Density (OD) of the negative controls.

**Result interpretation**
Positive Result: Specimen with absorbance value greater than the cut off value is considered positive for HAV IgM. However the samples were retested before final interpretation and classifying the patient to be HAV IgM positive.

**C2. ANTI-HCV**

**Principle of the test:**
The principle of the test is based on sandwich principle. The SP-NANBASE C-96 3.0 is an enzyme immunoassay kit (General Biologicals Corp. Taiwan), 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-HRP Conjugate is added to the well results in the formation of (HCV)-(Anti-HCV)-(Anti-human-IgG-HRP) complex. After washing out the unbound conjugate, TMB substrate solution is added for colour development. The intensity of colour development is proportional to the amount of antibodies present in the specimen.

**Test procedure:**

All the reagents were brought to room temperature and mixed well before the assay. 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. A 1:21 dilution of each control and specimen with specimen diluent was made. 10µl of each control or specimen into the well of pre-dilution plate was dispensed. 200µl of Specimen Diluent was added to each well and mixed well. 100µl of each diluted control or specimen was transferred to the corresponding wells in HCV Antigens plate. 200µl specimen diluent was added to each appropriate well in HCV Antigen Plate. 10µl of each control and specimen was added to each appropriate well and mixed by tapping the plate gently. The plate was sealed with an adhesive slip and incubated at 37°C for 60 minutes. At the end of the incubation period, the plate was washed by following the plate washing procedure. 100µl of the Diluted Conjugate was added in each well except the two blanks. The plate was again sealed and incubated in a 37°C for 30 minutes. At the end of the incubation period, the plate was washed by following the plate washing procedure. For colour development, equal volume of TMB Substrate Solution A and B were mixed in a clean container immediately prior to use and 100µl of the mixture was added to each well including two blank wells gently. The plate was then covered with Blank Cover and incubated at room temperature for 30 minutes. The reaction was stopped by adding 100 µl of 2N Sulphuric Acid to each well including the 2 blanks. The absorbance of Controls and Test specimens were determined within 30 minutes at 450nm.

**Calculation of results**
Calculation of P-N Value: P-N = PCx - NCx

Determination of Cutoff Value and Cutoff Index:
Cutoff Value = NCx + 0.025 x PCx
Cutoff Index = Sample OD Value / Cutoff VALUE

Result Interpretation:
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. 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. 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.

C3. Anti HEV

Principle:
Microplates are coated with HEV-specific synthetic antigens encoding for conservative and immunodominant determinants derived from HEV virus strains. The solid phases are first treated with the diluted sample and anti HEV IgM are captured, if present, by the antigens adsorbed on wells. After washing out all the other components of the sample, in the 2nd incubation bound anti HEV IgM antibodies are detected by the addition of polyclonal specific anti hIgM antibodies, 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 antibodies present in the sample. A cut-off value lets optical densities be interpreted into HEV antibody negative and positive results. Neutralization of IgG anti-HEV and Rheumatoid Factor, carried out directly in the well, is performed in the assay in order to block such kind of interferences.

Test Procedure:
Initially after bringing all the kit component to room temperature, all the samples diluted in a ratio of 1:101 into a properly defined dilution tube except the negative Control and the Positive Control which were supplied in diluted ready to use state. All the liquid components were mixed carefully by vortexing. The required
number of Micro wells were placed in the micro well holder leaving the A1 well of the microplate empty for the operation of blanking. Next, 50µl of the Neutralizing Solution was dispensed in all the wells of the samples except in the wells used for Controls and in blank. Next 100ul of Negative Control in duplicates and 100µl of Positive Control in single were dispensed, followed by dispensing 100µl of diluted samples in each properly identified well. The microplate was then incubated for 60 min at 37°C after sealing with an adhesive sealing foil, followed by stringent washing by delivering and aspirating 300ul/well of diluted washing solution supplied with the kit. Next, 100µl of enzyme Conjugate was put into each well, except the A1 well, and covered with the sealer. The microplate was incubated for 60 min at 37°C, and washed as earlier. 100µl of Chromogen/Substrate mixture was pipetted into each well including the blank well, and was incubated at room temperature for 20 minutes. 100ul of Sulphuric Acid was added into all the wells to stop the enzymatic reaction. The colour intensity of the solution in each well was measured at 450nm filter.

**Calculation of results:**
The tests results are calculated by means of the mean OD450nm value of the Negative Control (NC) and a mathematical calculation, in order to define the following cut-off formulation: Cut-Off = NC mean OD450nm + 0.250

**Result interpretation:**
Test results are interpreted as ratio of the sample OD450nm and the Cut-Off value according to the following: <1.0 Negative, 1.0-1.2: equivocal, >1.2- Positive. Any patient showing an equivocal result was tested again on a second sample taken 10 days later from the patient and was examined again. A positive result is indicative of HEV infection.

**C4. HBsAg**

**Principle:**
HBsAg detection is done by ELISA based on “Direct Sandwich” principle. The microplates are coated with Monoclonal antibodies (anti-HBs) with high reactivity for HBsAg. Addition of serum sample containing HBsAg after incubation with enzyme conjugate (Polyclonal antibodies linked to Horseradish Peroxidase (HRP) leading to the formation of a sandwich complex [(antibody)-(antigen)-
(antibody.HRP). After washing the amount of bound conjugate present in the well is proportional to the concentration of HBsAg present in the sample.

**Test Procedure:**

Before beginning with the assay all the reagents and samples were brought to room temperature. All the reagents were mixed well before use. Two wells of the micro plate were reserved for blanks. Three negative and two positive controls were used in five wells. Next, 50µl of specimens was pipetted into each well, followed by the addition of 50µl Anti-HBs-HRP conjugate to each well except the blanks. The plate was gently shaken for a few seconds to mix the sample and conjugate. The plate was covered and incubated at 37°C for 1 hour. After incubation, the plate cover was removed carefully and each well was washed five times with wash buffer comprising of phosphate buffer and surfactant. The wells were tap dried and 100µl of substrate solution (50µl of TMB concentrate + 50µl of TMB Diluent) was added to each well including the two blanks. The plate was covered and incubated at room temperature for 30 minutes in dark. After incubation, 50µl of 2N sulphuric acid solution was added to each well and mixed gently to stop the reaction. Absorbance of control and test specimens was obtained in an ELISA reader at 450/630nm (450nm as reading wavelength and 630nm as reference wavelength).

**Calculation of Results**

The mean of negative and positive controls was computed. To be accepted the negative control, (NC) < 0.015. If the 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 was equal to or greater than twice the value of the other, the value on the side position was considered as the aberrant value and was deleted, if one was deleted, the other two were used to calculate the NCx. If two were deleted, the test is considered as invalid and was repeated. The test to be valid, the mean absorbance of positive controls, (PC) must be > 0.5, If not so the test was considered invalid was repeated. Calculation of Cutoff Value: Cut off value was determined by using the following formula: Cut off value = NCX + 0.1

where NCX is the mean absorbance (O.D) of negative control.

**Interpretation of results:**
Negative Result: The test specimens with absorbance value less than the cutoff value are nonreactive and were considered as negative for HBsAg.
Positive result: The test specimens with O.D value more than the cut off value are reactive for HBsAg and considered as HBsAg positive.

5.2 Patient stratification based on diagnosis criteria’s, clinical details and duration of disease

**Group: Hepatitis A virus (HAV) infected patients**

**HAV related acute viral hepatitis (AVH-A):** The clinical onset of an acute viral hepatitis (AVH) A is defined as the beginning of early symptoms including fever, general malaise, fatigue, nausea, vomiting, anorexia and right upper quadrant discomfort. It is mainly characterized by the onset of jaundice and positive serological test for IgM anti-HAV.

**HAV related fulminant hepatitis (FHF):** Fulminant hepatitis A is defined as clinical syndrome develops as a result of severe impairment of hepatic functions or massive necrosis of hepatocytes in the absence of preexisting liver disease. Fulminant hepatic failure (FHF) patients were diagnosed by the presence of encephalopathy within 4 weeks of onset of illness with a prothrombin time of less than 40% that of the standardized value.

**Group: Hepatitis C virus (HCV) infected patients**

**HCV related acute viral hepatitis:** An acute illness with a discrete onset of any sign or symptom consistent with acute viral hepatitis (e.g., anorexia, abdominal disco-mfort, nausea, vomiting), and either jaundice, or serum alanine aminotransferase (ALT) levels >400 IU/L, antibodies to hepatitis C virus (anti-HCV) screening-test-positive with a signal to cut-off ratio predictive of a true positive as determined for the particular assay as defined by CDC.

**Chronic HCV infection:** Chronic hepatitis C is defined as infection with the hepatitis C virus persisting for more than six months based on the presence of its RNA. Chronic infections are typically asymptomatic during the first few decades, and thus are most commonly discovered following the investigation of elevated liver enzyme levels or during a routine screening of high risk individuals.
**Group: Hepatitis E virus (HEV) infected patients**

**Acute viral Hepatitis (AVH-E):** The criteria for diagnosis of acute viral hepatitis were defined as those cases that had an acute self-limiting disease and a serum aspartate transaminase elevation of at least five fold or jaundice or both.

**HEV related Fulminant hepatic failure (FHF):** The fulminant hepatic failure was diagnosed when after a typical acute onset, patient become deeply jaundiced and went into hepatic encephalopathy within 8 week of onset of disease without any past history of chronic liver disease.

**Group: Hepatitis B virus (HBV) infected patients**

**HBV related acute viral hepatitis (AVH-B):** The criteria for diagnosis of Acute hepatitis B is defined as those cases that shows positive test for B surface antigen (HBsAg) and IgM antibody to hepatitis B core (anti-HBc) in the serum of a patient with clinical and biochemical evidence of acute hepatitis.

**Chronic Hepatitis B (CHB):**
An individual testing positive for HBsAg on two occasions spaced six months apart and clinical data showing features of chronic hepatitis (Raimondo et al., 2003) i.e., (i) HBsAg positive for at least six months or more, (ii) HBeAg positive ‘or’ HBeAg negative/anti-HBe positive, (iii) having elevated transaminases (>1.5 times upper limit of normal), (iv) HBV DNA positive by a quantitative assay (Dot blot hybridization or b-DNA, Bayer Diagnostics Ltd., NY, USA, or Digene Co., USA). Chronic hepatitis B patients were further categorized as Hepatitis B e antigen positive HBV infection, and Hepatitis B e antibody positive HBV infection based on e antigen and Anti-HBe antibody status (Raimondo et al., 2003).

**Group: Hepatitis virus co-infection**
Cases positive for more than one hepatitis virus based on the serological screening were categorised as having hepatitis virus co-infection cases.

**Group: Alcoholic liver disease patients**
The diagnosis and staging of alcoholic liver disease (ALD) is based on a combination of features, including a history of significant alcohol intake, clinical evidence of liver disease, and supporting laboratory abnormalities.

*Group: Cryptogenic cases*
Cases of liver disease with no hepatitis virus or alcohol aetiology were categorised as cryptogenic cases.

**5.3 Hepatitis B virus infected cases**
Based on HBsAg positivity at presentation and after 6 months follow-up, anti-HBe-total positive, HBV DNA positive and PCR positive status were categorized as chronic carriers and were sub-categorized as chronic hepatitis B or cirrhosis after co-relating with the clinical details; else they were categorized as acute HBV cases. The HBeAg status was further screened by ELISA using commercially available kits.

**Detection of hepatitis B precore antigen (HBeAg):**

*Principle:*
Bioelisa HBeAg is a sandwich type immuno enzymatic method in which the wells of a microplate are coated with anti-HBe. The specimen to be analysed is incubated in one of the microfilter plate wells. If the sample contains HBeAg, it will not bind to the anti-HBe on one plate well. After washing in order to eliminate any unbound material, anti-HBe conjugated with peroxidase is added, which will react with possible antigen-antibody complex formed during the first incubation. After this second incubation and second washing, an enzyme substrate solution containing a chromogen is added which will develop a blue colour, if the sample is positive. The blue colour changes to yellow after blocking the reaction with sulphuric acid. The intensity of the colour is proportional to the concentration of HBeAg in the specimen.

*Test Procedure:*
Initially 100 µl of HBeAg positive controls two in number and three negative controls were transferred to wells, followed by 100µl each of patient specimens were also transferred to the marked wells. Next, 50 µl of conjugate was added to each well except in blanks. The microplate was then covered with an adhesive seal and shaken gently then incubated for 3 hours at 37°C. During the last 5-10 minutes of this incubation period, chromogen solution was diluted with substrate buffer. Adhes-ive seal was
removed; contents of the wells were aspirated and filled with diluted washing solution. This process was repeated four times. After final washing, microtiter plate was blotted on absorbent tissue to remove any excess liquid form the wells, and incubated uncovered at room temperature (20-25°C) for 30 minutes. Thereafter, 100 ul of stopping solution was added to stop the reaction. Spectrophotometer was used at 450nm, first for blank then for reading the absorbance of each well.

**Calculation of results:**
The cut off value was calculated using a formula 0.5 (N+P)

**Result interpretation:**
Samples with an absorbance higher than the cut-off value were considered reactive for HBeAg. Samples with an absorbance lower than the cut-off value were considered non-reactive for HBeAg. Samples with an absorbance within the range of the cut-off ± 10% (grey area) was considered doubtful and retested.

**Detection of HBe Antibody:**

*Principle:*
It is a sandwich type immnoenzymatic method in which wells are coated with anti-HBe using anti-HBe marked with peroxidase as conjugate. This test is based on the principle of the neutralization of anti-HBe by a solution containing HBeAg. The greater the concentration of anti-HBe present in the specimen, the smaller the amount of HBeAg which will bind to the well and which will react with anti-HBe conjugated with peroxidase.

*Test procedure:*
First, 50 µl of anti-HBe positive control to 2 wells and 50 µl of negative control to 3 wells were transferred. Next 50 µl each of the specimens was also transferred to wells. 100 µl of neutralization buffer was added to each well except the blanks. 50 µl of conjugate was also added to each well except blanks. The plate was covered with an adhesive seal and incubated for 3 hours at 37°C. After 3 hours incubation, contents of the wells were aspirated and filled completely with diluted washing solution. This process was repeated 4 times. Then, 100 µl of substrate TMB solutions was added to the wells, including the blanks. Incubated uncovered at room temperature for 30 minutes.
Reaction was stopped by adding 100 µl of stopping solution. Then absorb-ance was read for each well at 450 nm in the spectrophotometer.

**Calculation of results:**
The cut off value was calculated using a formula 0.5 (N+P).

**Result interpretation:**
Samples with an absorbance higher than the cut-off value were considered as Anti-Hbe positive. Samples with an absorbance lower than the cut-off value were considered negative for Anti-HBe. Samples with an absorbance within the range of the cut-off ± 10% (grey area) was considered doubtful and retested.

**Clinical examination:**
Each patient was carefully examined by me and/or a qualified gastroenterologist to determine signs of chronic liver disease. Endoscopic and CT based examination was performed for assessing the severity of liver disease in patients.

**Histopathological studies:**
Many of the enrolled patients scoring positive for HBsAg and HBV DNA with evidence of hepatitis (ALT > 1.5 times that of normal) with clinical or radiological evidence of advance chronic liver disease including cirrhosis were advised to go for liver biopsy (in and outside the hospital), which was performed with prior informed cones-nt. Liver sections were stained with eosin and haematoxylin. All the liver biopsies were examined by registered pathologists to assess the Histological Activity Index (HAI, calculated according to Knodell et al. 1981) and fibrosis score, and confirm for the presence of cirrhosis.

**Quantification of Serum HBV DNA Levels**
Serum HBV DNA levels were quantified commercially using ultrasensitive hybrid capture assay by Digene Co. (Gaithersburg, MD) with a lower limit of detection being 4700 copies/mL (940 IU/ml). The protocol used is briefly discussed.

**Procedure:**
Firstly 30 µl of each calibrator, control and specimen was pipetted after vortexing into the bottom of the designated Microplate well followed by the following steps:
Denaturation:
The denaturation reagent with indicator dye was transferred into reagent reservoir. With multichannel pipette, 30 µl of denaturation reagent was pipetted into the bottom of each well. The microplate was covered tightly with a plate sealer by pressing down firmly and the plate was placed on rotary shaker set at 1100 rpm for 1 minute for mixing thoroughly. The controls, calibrators and specimens should be purple. Then incubated at 65°C for 30 minutes in the microplate heater.

Hybridization:
The plate was removed from the incubator immediately and plate sealer was carefully peeled off to avoid condensation and splashing. Each well was inspected and noted for any location where evaporation has occurred; specimen in that well was re-tested. The probe mix was transferred into reagent reservoir and then with a multi channel pipette, 30 µl of probe mix was pipetted into the bottom of each well. Then, microplates were placed onto a rotary shaker set at 1100 ± 100 rpm for 1 minute to mix thoroughly. The controls, calibrators and specimen turned yellow. Then again incubated at 65°C for 60 minutes in the microplate heater.

Hybrid Capture:
After removing plate from the incubator peeling off by the sealer was done immediately to avoid condensation and splashing. Using multi channel pipette, carefully transferred 75 µl from each microplate well to the corresponding well of the capture microplate. After covering the microplate tightly with a new plate sealer by pressing down firmly, it was placed on the rotary shaker set at 1100±100 rpm for 60 minutes at room temperature. Wash buffer was prepared during this incubation and the liquid was removed from the capture microplate by aspirating it out with a multi channel pipette set at 100 to 150 µl. The plate was tilted to about 45 degrees to capture maximum volume without touching the bottom of the well. Extreme care was taken at this step to avoid cross contamination and scratches at the bottom of the capture microplate. To remove maximum amount of liquid from the capture microplate after aspiration, blot plate was placed on adsorbent blotting pad available from Digene, with enough force to create imprints of individual wells.

Hybrid Detection:
An appropriate amount of Detection Reagent 1 was aliquoted into a reagent reservoir, and then 75 µl of Detection Reagent 1 was carefully pipetted into each well of the capture microplate using the multi channel pipette. Then plates were covered with clean parafilm and incubated at 20-25°C for 30 minutes.

Washing:
The plate was decanted and was shaken 2-3 times with a downward motion above the absorbing blotting pad. The plate was hand washed 6 times with the help of Digene's wash apparatus.

Signal Amplification:
The Detection Reagent 2 was aliquoted into a new reagent reservoir, then carefully with multi channel pipette, 75 µl of detection reagent 2 was pipetted into each well of the capture microplate. The microplate was covered with clean parafilm and incubated for 15 minutes. Finally reading of the microplate was taken on the DML 2000TM Illuminometer immediately after 15 minutes of incubation period.

**Extraction of HBV DNA from sera samples:**
The DNA was extracted form 100 µl of patient serum with the help of standard phenol/chloroform extraction method. Briefly, 100 µl sera samples were incubated with 5 µl proteinase K (15mg/ml), 10 µl of 10X sera lysis buffer and 5 µl of 20% SDS at 37°C for 3 hours. Subsequently, the supernatant obtained after phenol, chloroform and chloroform Iso-amyl alcohol (24:1) steps were left for overnight DNA precipitation in the presence of 3M Sodium Acetate pH 5.2, and absolute alcohol. After centrifugation, the obtained pellet was washed with 70% alcohol, dried, and dissolved in 30µl of TE (10mM Tris and 1mM EDTA) buffer.

**HBV Genotyping by Multiplex PCR**
HBV genotypes were determined by multiplex PCR method designed by Oliver Krischberg et al. Briefly 3µL of extracted HBV DNA was subjected to PCR using primers used by Oliver et al. PCR amplification with the help of set of twelve primers was carried out with initial denaturation at 94°C for 10 min., followed by 35 cycles of 94°C for 1 min., 60°C for 1 min., and 72°C for 2 min. A single cycle for primer
extension was done at 72°C for 7 min. Ten µl of amplified product was analyzed on 2.5% agarose gel in 1X TAE.

The size of the expected genotype specific amplification was: genotype A 370 bp, genotype B 190 bp, genotype C 701 bp, genotype D 147 bp, genotype E 787 bp, genotype F 481 bp (Table 5.1). The samples showing multiple band patterns, i.e. same sample showing different amplicon size corresponding to the sizes of the PCR product for specific genotype by multiplex PCR genotyping method were categorized as mixed genotype. To avoid false positive results, 20% of the cases were re-genotyped and 10% of such representative cases including mixed HBV genotype were subjected to direct sequencing (surface region) and compared with the standard gene bank isolates for respective HBV genotypes, to further validate our results.

Table 5.1. The sequences of the primers used for HBV genotyping were:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Amplicon size</th>
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<tbody>
<tr>
<td>As</td>
<td>5’CGGAAACTACTGTTGTTAGACGACGGGAC-3’</td>
<td>370 bp</td>
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<tr>
<td></td>
<td>5’-AATTCCTTTTGTCTAGCAAAATATTTAGTGTGGG-3’</td>
<td></td>
</tr>
<tr>
<td>Bs</td>
<td>5’CCGCTTGGGCTCTACCGCCCG3’</td>
<td>190 bp</td>
</tr>
<tr>
<td></td>
<td>5’CTCTTATGCAAGACCTTTGGGCAGTTCC3’</td>
<td></td>
</tr>
<tr>
<td>Cs</td>
<td>5’CCTGAACATGCAGTTAATCATTACTCAAATACTAGG3’</td>
<td>701 bp</td>
</tr>
<tr>
<td></td>
<td>5’AGCAGGGGTCTCTAGGAATCCTGTATGTG3’</td>
<td></td>
</tr>
<tr>
<td>Ds</td>
<td>5’ACAGCATGGGGCAGAATCTTTCCACCAG3’,</td>
<td>147 bp</td>
</tr>
<tr>
<td></td>
<td>5’CTACCTTTGTGGCGTCTGCGCAGG3’</td>
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<td>Es</td>
<td>5’CTAATGACTCTAGCTACCTGGGTGGTGTGTA3’</td>
<td>787 bp</td>
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<td>5’CCATTGAAAGGCGCCATCCAAAGGCA3’</td>
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<tr>
<td>Fs</td>
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</tr>
<tr>
<td></td>
<td>5’AGAGGCAATAGTCGGAGCGGTTCTG3’</td>
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</table>

Amplification of the surface and overlapping polymerase regions:

Further to validate the multiplex genotyping results as well as to genotype the isolates which couldn’t be genotyped by multiplex PCR method, a PCR-direct sequencing-phylogenetic analysis based approach was taken. The part of the HBV genome encoding the Major Hydrophilic Region (which also includes the ‘a’ determinant) up to the last amino acid (aa 226) of surface gene and overlapping catalytic domains of Polymerase gene (nucleotide position 425 to 840) was amplified by nested polymerase chain
reaction (PCR). Three µl of extracted DNA from sera samples was amplified in a 25µl reaction mixture containing dNTP, MgCl₂, PCR buffer and Taq DNA polymerase in the appropriate quantity. The amplification step was performed for 30 cycles with the outer primers SA1: 5'- AT-CGCTGGATGTGTCTGCGG-3’ and SA2: 5’-GGCAACGGGTTAAAGGTTCA-3’ (position 369 to 388 and 1136 to 1155 respectively) in the HBV genome. Ten µl of amplified product was analyzed on 1.2% agarose gel in 1X TBE. Samples, which did not show amplification, were directly used for nested amplification. Where 787 bp fragment was generated, nested PCR was done after appropriate dilution. Three µl of the first round PCR product (directly or after dilution depending on the band intensity visualized on the gel) was used as a template in a second amplification step performed for 30 cycles with inner primers SB1:5’-TTAGGGTTTAATGTATACCC-3’ (position 822 to 842) and SB2: 5’-CATCTTCTTTGTTGGTTCTTCTG-3’ (position 427 to 448). PCR was carried out using thermal cycler (Eppendorf) with denaturation at 94°C for 1 min., annealing at 55°C for 1 min., primer extension at 72°C for 2 min. for total of 30 cycles and finally a single cycle for primer extension was performed at 72°C for 7 min. Ten µl of nested PCR product (416bp) was analyzed by agarose gel electrophoresis on 1.2% gel in 1X TAE buffer.

Automated DNA sequencing:

Purified PCR products were sequenced commercially with respective forward and reverse primers in an automated DNA sequencer. Briefly, 200-250 ng of purified template (using gel extraction method, Qiagen) in 5µl vol. was mixed with 4µl of sequencing reaction mixture (supplied) and 1µl each of respective forward and reverse primers of 5 pmole/µl concentration in two different sets of reactions. Sequencing reaction was carried out under the cyclic conditions of 96°C for 30 sec., 55°C for 30 sec. and 60°C for 4 min. After completion of 30 cycles, the sequencing reaction product was precipitated by adding 90µl H₂O, 10 ul 3M NaOAc (pH 4.6) and 250µl absolute alcohol at room temperature for 10 min., and centrifuged at 12,000 rpm for 15 min. Subsequently, 70% alcohol wash was given to the pellet at the same speed for 10 min. and the dried pellet at 37°C was dissolved in 3.5 ul of gel loading dye (Form-amide and Dextran Blue). Samples were denatured for 10 min. at 90°C, and then quenched on ice for 5 min., prior to loading on polyacrylamide gel in the automated DNA sequencer.

Sequence and phylogenetic analysis
Each electrophorogram was carefully read to repair the appearance of 'N' in the sequence and to ensure if the laser reader has correctly read the peaks in the electrophorogram. The corrected electrophorograms thus generated were carefully analyzed using the bioinformatics tools such as multalin and expasy; and the genotype was determined by phylogenetic tree analysis using the MEGA4 software. The standard sequence available from NCBI genbank database for HBV A-H (genotype A, X70185; genotype B, D00331; genotype C, X01587; genotype D, X72702; genotype E, X75664; genotype F, X75663; genotype G (Stuyver et al., 2000) etc were considered for the preparation of the phylogenetic tree.

**Viral RNA extraction:**
Total RNA was isolated from the patient’s serum sample by the Tri-reagent of Sigma-Aldrich, USA. 750µl of trizol was added to 250µl of plasma or serum, vortexed briefly and incubated at room temperature for 10-15 mins. 200µl of chloroform was added to the mixture, vortexed to give a whitish curdy appearance and allowed to stand at room temperature for 5mins. The mixture was centrifuged at 13000rpm for 15 mins at room temperature.

Centrifugation separates the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colourless upper aqueous phase (containing RNA). The upper aqueous phase was collected in a fresh micro-centrifuge tube and 600 µl of ice cooled isopropanol was added to it. The mixture was incubated at -20°C for 20mins pr overnight. Centrifugation was done for 15mins at13000rpm .The supernatant was carefully discarded and the pallet was washed with 70%ethanol by centrifuging at 13000rpm for 10mins. After washing the alcohol was cautiously discarded. The pellet was air dried and resolved in molecular grade water the extracted RNA was stored at -20°C until further use. For few of the samples viral RNA extraction was done by using the Qiagen viral RNA extraction kit following the manufacturer’s protocol.

**Complimentary DNA (cDNA) preparation:**
The cDNA was synthesized by reverse transcription (MMuLV-Reverse Transcriptase, Fermentas) method. To 6µl of template RNA 8µl of mix A comprising of 10mM dNTP, random hexamer and RNase inhibitor (composition table 5.2) was added.

Table 5.2. Reaction mixture for mix A:

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular grade water</td>
<td>5.5 µl</td>
</tr>
<tr>
<td>Random hexamer</td>
<td>1 µl</td>
</tr>
<tr>
<td>10nM dNTP</td>
<td>1 µl</td>
</tr>
<tr>
<td>RNase Inhibitor</td>
<td>0.5µl</td>
</tr>
</tbody>
</table>

The mixture was run in a thermal cycler for 10mins at 70°C and snap freezed on ice. To it 6 µl of mix B comprising on 0.1MdTT and 5X RT buffer (table. 5.3) was added.

Table 5.3. Reaction mixture mix B:

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5XRT buffer</td>
<td>4 µl</td>
</tr>
<tr>
<td>0.1M dTT</td>
<td>2 µl</td>
</tr>
</tbody>
</table>

The mixture was run in a thermal cycler for 15mins at 25°C and snap freezed on ice. After snap freezing 2 µl of RT (20U/µl) was added and run in a thermal cycler under the following conditions, 42°C for 1 hour and 72°C for 10 mins. The cDNA thus prepared was aliquoted and stored at -20°C for further use.

5.4 Hepatitis A virus infected cases
The diagnosis of HAV infection was screened on the basis of IgM Anti-HAV positivity and were categorized into AVH and fulminant cases based on clinical details and status as discussed earlier.

Detection of HAV RNA
Detection of HAV RNA in the HAV IgM positive samples was done by Real time-PCR using primers specific for the VP3-VP1 region of HAV genome (Table 4.4), which are enlisted in table 5.4, and the cDNA prepared from the isolated viral RNA of IgM-Anti-HAV positive cases as templates. The amplification conditions comprised of initial
denaturation at 94\(^0\)C for 5mins, followed by 35 cycles of 94\(^0\)C for 15secs, 55\(^0\)C for 15secs and 72\(^0\)C for 15secs followed by a single final extension cycle of 72\(^0\)C for 7mins.

**Table 5.4: Primer sequences for HAV PCR amplification-for VP3-VP1 region**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>PCR amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5’CCACACAAGTTGGGGATGAT3’</td>
<td>154 bp</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’TGCTTGCACTCCTGAAACAT3’</td>
<td></td>
</tr>
</tbody>
</table>

**HAV genotyping**

Cases showing positive detection for HAV RNA by real time PCR were targeted for genotyping by nested PCR method. HAV genotyping was initially done by nested PCR using two sets of primers specific for the VP3-VP1 region of HAV genome, which are enlisted in table 5.5, and the cDNA prepared from the isolated viral RNA of IgM-Anti-HAV positive cases as templates. First round of PCR amplification was carried out using primers A1 and A2 and 1µl of cDNA as template, with amplification conditions comprising of initial denaturation at 94\(^0\)C for 5mins, followed by 40 cycles of 94\(^0\)C for 45secs, 51\(^0\)C for 45secs and 72\(^0\)C for 45secs. Final extension at 72\(^0\)C for 7mins was given and the block was hold at 4\(^0\)C. 10µl of PCR product was analysed on 1.2% agarose gel prepared in1X TAE buffer. Three µl of first round product was used as template for second round of PCR using A3 and A4 as the forward and reverse primer respectively, under the same amplification conditions. Both positive and negative controls were used for both for every PCR amplification carried out. Cases with positive amplification of 154bp were purified and were subjected to direct sequencing commercially. The sequencing electrophorograms thus generated were evaluated carefully and was analyzed phylogenetically with isolates reported from different global pockets.

**Table 5.5: Primer sequences for HAV PCR amplification-for VP3-VP1 region**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>PCR amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5’CCACACAAGTTGGGGATGAT3’</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>5’TGCTTGCACTCCTGAAACAT3’</td>
<td></td>
</tr>
</tbody>
</table>
Secondly, as most of the available literature suggests that the VP1/2A region is more conserved than the VP3-VP1 region of HAV and thus better for genotyping, we validated our initial genotype results as well as performed the HAV genotyping for the cases enrolled in the later phase of our study using PCR-direct sequencing-phylogenetic analysis approach using the VP1/2A region of the HAV isolates (table 5.6). The phylogenetic analysis was performed by using the MEGA4.0 software, and the statistical evaluation for distribution of HAV genotype in AVH vis-à-vis FHF cases was performed by using the SPSS13.0 software.

<table>
<thead>
<tr>
<th>Primers</th>
<th>sequence</th>
<th>PCR amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP1/2A external</td>
<td>5’TGTCTGTCAAGAACAATCAG3’</td>
<td>361 bp</td>
</tr>
<tr>
<td></td>
<td>5’AGTCACACCTCAGGAAAACTT3’</td>
<td></td>
</tr>
<tr>
<td>VP1/2A internal</td>
<td>5’TCCCAGACTTCCTGAA3’</td>
<td>234 bp</td>
</tr>
<tr>
<td></td>
<td>5’AGGAGGTCAGACTTCATTGG3’</td>
<td></td>
</tr>
</tbody>
</table>

* Primers adapted from Ahmad Nejati et al, Journal of Medical Virology, 2012

5.5 Hepatitis C virus infected cases

Detection of HCV RNA

Detection of HCV RNA in the serum samples of anti-HCV positive patients was done by amplification of the 5’UTR region by Reverse transcription polymerase chain reaction using two different set of outer and inner primers (Nested-PCR). The oligonucleotide used for diagnosis are listed in table 5.7

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer Forward (HC1)</td>
<td>CTGTGAGGAACTACTGTCTT</td>
</tr>
</tbody>
</table>
Briefly, 1µl of cDNA was used for first round of PCR amplification, using master mix with composition as enlisted in table 5.8 and reaction conditions consisting of Initial denaturation of the cDNA at 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute. Final extension at 72°C for 8 minute and block was finally allowed to cool down to 4°C.

<table>
<thead>
<tr>
<th>Components</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA</td>
<td>1µl</td>
</tr>
<tr>
<td>10X Buffer</td>
<td>1X</td>
</tr>
<tr>
<td>dNTP’s</td>
<td>200µM</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>40 picomoles</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>40 picomoles</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2 mM</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>5 Units</td>
</tr>
<tr>
<td>Milli Q water</td>
<td>To make up vol.</td>
</tr>
<tr>
<td>Total Volume (C)</td>
<td>25µl</td>
</tr>
</tbody>
</table>

The first round PCR amplification was performed with outer forward and reverse primers (HC1 and HC2) and the generated PCR product which was seeded as DNA template (1µl) for second round nested PCR with inner forward and reverse primers (HC3 and HC4) with same buffer system and cycling conditioning as for the first round. Both positive and negative controls were run parallel along with the test samples. An expected final PCR product of 251bp was detected in an EtBr stained 2.5% agarose gel in UV transilluminator. All possible measures were taken to avoid contamination and DEPC treated plastic ware was used to prevent RNAase activity.

**HCV genotyping based on amplification of 5’UTR-Core**

PCR amplification of HCV 5’ UTR core3 region was carried out using two sets of primers as listed in table 5.9. The reaction setup for first round of PCR amplification consisted of 2µl of 10X PCR buffer, 1.2 µlof 25mM MgCl₂.0.5 µl of 10mM dNTP 0.4 µl of forward (HC5)and reverse primers (HC6) and 0.1 µl of Taq polymerase (5U/ µl).1
µl of cDNA was used as template. Amplification condition consisted of initial denaturation at 94°C for 4 mins followed by 35 cycles at 94°C for 1 min, 58°C for 1 min, 72°C for 1 min. Final extension was done at 72°C for 8 mins and allowed to cool to 4°C. The generated PCR product was seeded as DNA template (1µl) for second round nested PCR with Inner forward (HC7) and reverse (HC8) primers with same buffer system and cycling conditions as for the first round of PCR.

Table 5.9: Primers for HCV-5'UTR Core region

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer Forward Primer (HC5)</td>
<td>ACTGCCTGATAGGGTGCTTGCGAG</td>
</tr>
<tr>
<td>Outer Reverse primer (HC6)</td>
<td>ATG TAC CCC ATG AG/T A/G TCG GC</td>
</tr>
<tr>
<td>Inner Forward Primer(HC7)</td>
<td>AGGTCTCGTAGACCGTGCATCATG</td>
</tr>
<tr>
<td>Inner Reverse primer(HC8)</td>
<td>CACT GT AG AGG GTA TCG ATG A</td>
</tr>
</tbody>
</table>

An expected final PCR product of 405 bp was detected in an ethidium bromide-stained 2.0% agarose gel in UV transilluminator. The samples for HCV genotyping were sequenced (Macrogen Inc, Korea) on commercial basis. Sequence identity matrices and multi-sequence alignment from DNA databases were generated using Basic Local Alignment Search Tool (BLAST) available at National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST). The evolutionary history was inferred using the neighbor-joining method (Zhang and Sun, 2008). The reliability of different phylogenetic groupings was evaluated by the bootstrap consensus tree inferred from 500 replicates (Felsenstein J, 1992). The phylogenetic analysis of HCV isolates was performed with Phylip (Expasy) and Molecular Evolutionary genetic Analysis (MEGA 4.0) software.

5.6 Hepatitis E virus infected cases

HEV RNA detection and genotyping

Screening for HEV infected cases was done on the basis of IgM-Anti-HEV positivity for cases having jaundice at presentation. The cDNA prepared from the isolated viral RNAs were subjected to RT-PCR analysis using primers based on ORF1 region of HEV (Table 5.10). The PCR amplified products were checked on agarose gel, and an amplicon of 343 bp designated HEV RNA positivity. Since viral genotypes are known to influence the disease severity, further the PCR positive cases were evaluated for HEV genotyping analysis. The amplified products were purified by gel extraction kit...
(Qiagen) and subjected to direct sequencing commercially. HEV genotyping was performed by sequencing analysis of the amplicon and comparing with the standard HEV genotype sequences from the GenBank using suitable softwares (MEGA4.0).

Table 5.10. Primers for HEV detection by PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Tm</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEV outer</td>
<td>5’AAGGCATCCATGGTGTTTGAGAATGAC3’</td>
<td>50°C</td>
<td>574bp</td>
</tr>
<tr>
<td></td>
<td>5’CACACATCTGAGCTACATTGTGAGC3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEV inner</td>
<td>5’GACTCCACCCAGAATAACTT3’</td>
<td>50°C</td>
<td>343 bp</td>
</tr>
<tr>
<td></td>
<td>5’CACAGCCGGCGATCAGGACAG3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5.7 HOST GENETIC FACTOR ANALYSIS

5.7.1 Genomic DNA Isolation

The host genomic DNA was isolated from 200µl of whole blood from the patients as well as the controls for genetic analysis by standard Proteinase K digestion followed by phenol chloroform extraction procedure. Briefly, 200µl of whole blood was taken in a microcentrifuge tube and 800µl of lysis buffer was added to it and incubated in ice for 30-45mins. The mixture was centrifuged at 1200rpm for 15mins at 4°C. The supernatant was discarded, and the previous step was repeated to lyse any RBCs left. To the pellet 750µl of SE buffer, 10µl of 10%proteinase K and 50µl of 20%SDS was added. The mixture was thoroughly mixed and incubated overnight at 37°C or 1-2 hrs at 56°C for digestion. 750µl of tris saturated phenol was added to the mixture, was gently shaken for 10mins and centrifuged at room temperature for 10 mins at 10000 rpm. Upper phase of supernatant was transferred in a fresh microcentrifuge tube. 750µl of mixture of phenol: chloroform: isooamyl alcohol (25:24:1) was added to the upper phase. The mixture was gently shaken for 10mins and centrifuge at 10000rpm for 10mins at room temperature. The upper phase was transferred into a new microcentrifuge tube and 750µl of chloroform: isoamyl alcohol (24:1) was added. After shaking the mixture for 10 mins, the mixture was centrifuge at room temperature for 10mins at 10000rpm. The upper phase was collected in a fresh microcentrifuge tube addition of 1/10th volume of 3M sodium acetate was added to it followed by addition of 1ml isopropanol. The mixture was shaken gently and centrifuged at 10000rpm for 10mins. Supernatant was
discarded, and 500µl of 70% ethanol was added to the pellet and the pallet was gently
tapped. Centrifugation was done at 1000rpm for 10mins. The extracted pallet of DNA
was suspended in adequate amount of TE buffer. The quality and quantity of extracted
DNA was checked in UV spectrophotometer by obtaining absorbance at 260nm.

5.7.2 hoGG1 ser326Cys polymorphism analysis
Polymorphism of hoGG1 codon 326 gene was studied by PCR-RFLP method. PCR
amplification of hoGG1 codon 326 gene was performed using primers with sequences
sense Primer-5’ACTGTCACTAGTCTCACCAG3’and anti sense primer- 5’GGAAGG
TGCTTGGGAAT3’. The PCR master mix was prepared for 25µl reaction with a
composition comprising of 2.5 µl of 10X PCR Buffer, 1.8 µl of 25mM MgCl2, 0.5µl
each of 10mM dNTP, sense and anti sense primers (10pmols/µl) , 0.1 µl of Taq
polymerase (5U/µl) and 1µl of DNA template, finally making the volume to 25µl with
nuclease free water. The amplification conditions were set as, initial denaturation at
95°C for 5mins followed by 35 cycles of 94°C for 45secs, 58.2°C for 45secs and 72°C
for 45 secs with a final extension for 10mins at 72°C. The interpretation of
hoGG1 codon326 gene PCR amplification was done on the basis of the size of band at
200bp with respect to the molecular marker of 100bp as observed in 2% agarose gel
stained with ethidium bromide. Genotype analysis of hoGG1 codon326 was done by
digestion of the PCR amplicon with Fnu4H1 restriction enzyme. Composition for RFLP
reaction mixture is listed in table 5.11. Restriction digestion was carried out by
incubating overnight at 37°C. The digested DNA was electrophoresed on 3% agarose gel
stained with ethidium bromide along with a 100bp molecular marker for interpretation
of genotypes as per their electrophoresis pattern.

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fnu4H1 restriction enzyme</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>NEB Buffer4</td>
<td>2 µl</td>
</tr>
<tr>
<td>PCR amplified product</td>
<td>17.8 µl</td>
</tr>
</tbody>
</table>
Presence of two characteristic band at 100bp was considered to represent the mutant Cys allele or homozygous variant hoGG1 codon326 genotype as compared to an uncut band at 200bp representing wild type ser allele or wild genotype for hoGG1 codon 326.

5.7.3 XRCC1 codon 399 polymorphism analysis
XRCC1 SNP at codon 399 in exon10 was studied by PCR-RFLP method. XRCC1 gene fragment was PCR amplified by using the primers designed by Arizono et.al (2008). The sequences of the primer used for XRCC1 codon 399 PCR amplification were 5’GGACTGTCACCGCATGCGTCGG3’ (sense primer) and 5’GGCTGGGACCACCTGTGTT3’ (anti sense primer). The PCR master mix was prepared for 25µl reaction with the constituents as listed in table 5.12. Amplification conditions were initial denaturation at 95°C for 5min followed by 35 cycles of 94°C for 45 s, 58°C for 45s, and 72°C for 45 s, with final extension for 7 min at 72°C. The PCR amplified products were run on 2.5% agarose gel stained with ethidium bromide. A PCR amplicon of 149bp compared with standard 100bp marker (Fermentas) was considered as a positive amplification.

Table 5.12: Composition of PCR Reaction mixture for XRCC1 codon399 amplification

<table>
<thead>
<tr>
<th>Components</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease free water</td>
<td>18.1 µl</td>
</tr>
<tr>
<td>10X PCR Buffer (MgCl2 free)</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>25mM MgCl2</td>
<td>1.8 µl</td>
</tr>
<tr>
<td>10mM dNTP</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>10pmols/ µl Sense primer</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>10pmols/ µl anti sense primer</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Taq polymerase (5U/ µl)</td>
<td>0.1 µl</td>
</tr>
<tr>
<td>DNA template</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

The genotyping of XCC1 codon 399 was done by digesting the PCR amplicon using MspI restriction enzyme. The interpretation of the genotype was done on the basis of the size of the band with respect to the molecular marker as observed in 3% agarose gel stained with ethidium bromide. Restriction digestion was carried out as per the supplier’s instructions by incubating at 37°C (table 5.13).
Table 5.13: Restriction digestion reaction set up

<table>
<thead>
<tr>
<th>Components</th>
<th>concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Msp1 (restriction enzyme)</td>
<td>0.5µl</td>
</tr>
<tr>
<td>NEB 2 buffer</td>
<td>2µl</td>
</tr>
<tr>
<td>PCR Product</td>
<td>17.5µl</td>
</tr>
</tbody>
</table>

Two bands at 115bp +34bp characterizes the wild-type (Arg allele) XRCC1 codon 399 genotype, a single band at 149bp characterizes the homozygous variant (Gln allele) XRCC1 codon399 genotype, and presence of three bands, 149bp +115bp +34bp represented a heterozygote XRCC1 codon 399 genotype.

5.7.4 XRCC1 expression analysis

XRCC1 protein expression was checked by immunohistochemistry in five available formalin fixed paraffin embedded tissue sections of 5µm section each of cirrhosis cases of different grades of cirrhosis (as evident earlier from CT and endoscopy based examination and histopathology details) and three autopsy based control liver sections. Initially, the sections were dewaxed by keeping on a heat plate followed by three washes in xylene, followed by acetone wash and keeping in water. Next, the slides were dipped completely in citrate buffer (pH=6.0), and antigen retrieval was done by subjecting the slides dipped in buffer to microwave oven based boiling at full power for 5 minutes and half power for 2mins. The slides were kept as such till the citrate buffer cooled down, and was then washed once with TBS buffer (tris buffer saline). The XRCC1 antibody (Santacruz Biotechnologies) was diluted to a ratio of 1:200 and was spread over the slides, except one which was spread with TBS buffer and was used as negative staining control. The slides were incubated as such overnight in a moist chamber in 4°C freeze. On the second day the slides were thoroughly washed with 3 washes of TBS buffer for 5 mins each, and was incubated with secondary antibody supplied with the LSAB kit (DAKO), and incubated for 1 hour at room temperature, followed by thorough washing with TBS buffer. Next, the slides were incubated with tertiary antibodies supplied with the kit for 1 hour. The slides were then washed thrice with TBS buffer and incubated with DAB chromogen in dark for 10 mins, followed by stringent washing with water and counterstained with haematoxylin. The slides were kept under indirect running water for 5 mins, dried on the hot plate and mounted in
DPX. The differential expression profile was analyzed by a registered senior pathologist, and correlated with disease stage.

5.7.5 CYP2E1 rsal (-1019) polymorphism analysis

Cyp2E1 rsal polymorphism consists in a transition from cytosine to thymine upstream of the gene in the regulatory region: 1,019. The functional relevance of the RsaI polymorphism is due to its location in the binding site of the hepatic nuclear factor 1 (HNF-1), a liver-specific transcription factor that enhances the transcriptional activity of the human CYP2E1 promoter (Hayashi et al., 1991; Watanabe et al., 1994). Variation in CYP2E1 rsal allele was studied by PCR-RFLP method. PCR amplify-cation for CYP2E1 Rsa1 allele was done using primers for the 5’ flanking region with sequences 5’CCAGTCGAGTCTACATTGTCA3’ (Sense primer) and 5’TTCATTCTGTCTTCTA ACTGC3’ (anti-Sense). The reaction mixture consists of 2µl of 10X PCR buffer, 1.2 µl of 25mM MgCl2, 0.4µl of 10mM dNTP, 1.2 µl of each of forward and reverse primers (10pmol/µl), 0.1µl of Taq DNA polymerase (5U/µl) and 1 µl of template DNA, adjusted to 20µl with addition of nuclease free water. The amplify-cation protocol comprised of initial denaturation at 95°C for 5 mins followed by 35 cycles of 94°C for 45 secs, 60°C for 45 secs, 72°C for 45 sec with a final extension for 10 mins at 72°C. The PCR ampli-cons were checked on 2% agarose gel stained with ethidium bromide against a molecular marker of 100bp. An amplified product at 410bp was considered as positive PCR amplification.

RFLP analysis:

Variation in CYP2E1 rsal genotype was determined by digesting the PCR amplicon with RsaI restriction enzyme. Restriction digestion was carried out as per the instruction mentioned by the supplier by incubating at 37°C for overnight. Composition of RFLP reaction mixture is enlisted in table 5.14. The digested fragm ents were separated on 2.5% agarose gel by electrophoresis along with 50bp/100bp molecular weight marker for interpretation of genotypes on the basis of their electrophoresis pattern.

<table>
<thead>
<tr>
<th>Table 5.14. Restriction digestion reaction mixture set up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Components</td>
</tr>
<tr>
<td>Rsal restriction enzyme</td>
</tr>
<tr>
<td>Tango buffer</td>
</tr>
</tbody>
</table>
Presence of two characteristic bands at 360bp and 50bp was considered to represent the wild C1 allele or homozygous wild CYP2E1 genotype as compared to an uncut band at 410 bp representing the variant C2 allele or homozygous variant genotype for CYP2E1. Mutated heterozygous CYP2E1 genotype (C1/C2) was determined by the presence of characteristic three bands at 360bp, 50bp and 410bp respectively.

5.8 ENVIRONMENTAL FACTOR ANALYSIS

5.8.1 Determination of nitrite concentration

Nitrite is carcinogenic. Kirkali et al had previously demonstrated increased serum levels of nitrite and nitrate, which are metabolites of nitric oxide, in patients with liver cirrhosis (Turk J, 2000). Serum nitrite and nitrate concentrations may also be elevated in patients with chronic hepatitis which may be due to enhanced cytokine expression, as mentioned by Simpson et al. No data on Indian acute and chronic hepatitis patients, which was evaluated in the present study. Secondly, several indigenously prepared fermented food products and the raw material used to prepare them were short listed and collected based on a questionnaire on the food habits of the jaundice patients and the community controls enrolled in the study. Concentration of nitrite was estimated in different fresh and fermented food products of Northeast India and in the plasma of liver disease patients and healthy controls using the standard Griess’s reagent method followed by spectrophotometric detection at 540nm.

Principle:

Estimation of nitrite using Griess reagent is based on the principle as represented in the chemical reaction (Fig 5.1). Sulfanalic acid is quantitatively converted to a diazonium salt by reacting with nitrite under acidic condition. The diazonium salt then couples with N-(1-naphthyl) ethylenediamine forming an azo dye that can be spectrophotometrically quantitated based on its absorbance at 550 nm.
**Test Procedure**

Ten grams of food product was taken, ground using distilled water and then diluted four-fold. In case of plasma samples 100µl of plasma was taken and diluted four-fold with sterile distilled water. The samples were deproteinized by adding 1/20th volume of zinc sulphate (300g/l) to give a final concentration of 15g/L. The samples were then centrifuged at 9500 rpm for 5 minutes at room temperature. 1.5 ml of supernatant was applied to a clean tube and 4.5ml of Griess’s Reagent (.5% sulphanilic acid, 0.002%N-(1-napthyl) ethylenediamine dichloride and 14%glacial acetic acid) was added. The tubes were mixed well and left in dark for 30 minutes at room temperature. At the end of the reaction time the absorbance was measured at a wavelength of 550nm on a spectrophotometer. A blank was prepared in a similar way by using 150 ml of potassium phosphate buffer (50mM) instead of the sample.

**Calculation and interpretation of the result:**

Standard curve was prepared using sodium nitrite and potassium nitrate in distilled water (linear range 0-100µmol/L). The absorbance of each sample was plotted against the standard curve and the concentration was calculated for each sample.

**5.8.2 Detection of volatile Nitrosoamines**

Nitrite alone can cause cancer. An even more serious concern is the well documented potential for nitrates/nitrites to cause cancer through the formation of nitrosamines, formed when nitrites react with secondary amines. Nitrates and nitrites are agents in endogenous nitrosamine formation in the gastrointestinal tract (Spiegelhalder et al., 1976; Tannenbaum et al., 1976). Nitrosamine formation in the stomach depends on consumption of foods containing amines (Ohshima and Bartsch, 1981; Shephard and
Lutz, 1989). Possible role of nitrosamines in Thai food (especially fermented foods) in the etiology of liver cancer has been already reported (Mitacek EJ et al, 1999), but no data is available from NE India where fermented food products are a regular part of dietary habits.

Based on the food habit list, fermented bamboo shoot and fish, and areca catechu (tamul or supari) were found to be the most products consumed by the local and indigenously people from NE India. The fermented bamboo shoot and fish and their raw materials were processed for detection of presence of volatile nitrosoamines by GC-MS analysis commercially.

**Preparation of sample for GC-MS and analysis:**

About 10g of food sample was accurately weighed, minced into small pieces and homogenized in a mixing blender. To the mixture 50ml of phosphate-citric acid buffer at PH 4.5 was added followed by the addition of 175mg ascorbic acid and 1ml of 5ppm solution of N-nitrosodipropylamine as an internal standard. The blended mixture was then transferred to a 250ml flask to which 150 ml of buffer and 700mg ascorbic acid were added. The mixture was stirred for 2 hours and then filtered through celite 545. The aqueous filtrate was extracted four times with 300 ml dichloromethane (DCM). The pooled DCM extracts were dried over anhydrous sodium sulfate, concentrated to 3-5ml and then chromatographed on basic alumina with 200ml DCM and carefully concentrated to 0.5 to1ml prior to gas chromatography. Detection for nitrosamines was finally done by GC-MS analysis commercially.

**5.8.3 Measurement of oxidative stress (8-hydroxy-2-deoxy Guanosine)**

Cellular oxidative stress was measured in the plasma of liver disease patients and healthy controls by ELIZA using 8-hydroxy-2-deoxy Guanosine EIA kit (Cayman).8-OH-dG serves as an established marker of oxidative stress in urine, plasma or serum and tissue samples. Normal concentration of 8-OH-dG in plasma was reported to be 4-21 pg/ml. 8-OH-dG levels and 8-OH-dG based mutations are repaired by hoGG1 enzyme.

**Principle:**
The EIA assay uses an anti-mouse IgG-coated plate and a tracer consisting of an 8-OH-dG –enzyme conjugate and is based on the principal of competitive ELISA between 8-OH-dG and 8-OH-dG-acetylcholinesterase (AChE) conjugate (8-OH-dG-tracer) for a limited amount of 8-OH-dG monoclonal Antibody. The concentration of the 8-OH-dG tracer is kept constant while the concentration of 8-OH-dG varies. The amount of 8-OH-dG Tracer that binds to the 8-OH-dG Monoclonal Antibody is inversely proportional to the concentration of 8-OH-dG present in the well. The antibody-8-OH-dG complex binds to goat polyclonal anti-mouse IgG which is previously attached to the well of the EIA plate. Addition of particular substrate (Ellman’s Reagent) to the plate followed by an enzymatic reaction produces a colored end product of specific intensity. Spectrophotometric determination of the intensity of the color at 412nm is proportional to the amount of 8-OH-dG Tracer bond to the well, which is inversely proportional to the amount of free 8-OH-dG present in the well during incubation.

\[
\text{Absorbance} \propto \frac{[\text{Bound 8-OH-dG Tracer}]}{[8-OH-dG]} \propto \frac{1}{[8-OH-dG]}
\]

**Test Procedure:**

The plate was set up in a specific format consisting of two blanks, two non-specific binding wells, two maximum binding wells, one Total activity well and an eight point standard curve. Eight standards S1–S8 of known concentration were prepared in separate tubes from a bulk standard consisting of 30ng/ml of 8-OH-dG sample. 100µl of EIA buffer was added to the Non-specific Binding wells and 50µl of EIA buffer was added to Maximum Binding wells. 50µl standard from S8 tube was added to the assigned lowest standard well. 50 µl from S7 tube was added to the next standard well. The procedure was repeated until all the standards were aliquoted. 50µl of plasma sample was added per well. 50µl of 8-OH-dG AChE Tracer was added to each well except the Total Activity (TA) and the Blank wells. 50µl of 8-OH-dG Monoclonal Antibody was added to each well except the total activity, the Non-Specific Binding and the Blank wells.

The plate was covered with plastic film and incubated for 18 hours at 4°C. The wells were emptied and rinsed five times with Wash Buffer. 200µl of freshly reconstituted
Ellman’s Reagent was added to each well. Next, 5µl of tracer was added to the total activity well. The plate was again covered with plastic film and put inside an orbital shaker in dark for 120 minutes to obtain optimum development. The bottom of the plate was whipped with clean tissue paper to remove any dirt. The plastic cover was removed and the plate was read at a wavelength between 405 and 450nm.

**Calculation and Interpretation of Result**

**Preparation of standard curve:**
Average of the absorbance readings from the NSB wells and the Bo wells was obtained. Corrected Bo or Maximum binding was calculated by subtracting the NBS average from the Bo average. The percentage B/Bo was calculated for the remaining wells. %B/Bo for standards S1-S8 was plotted versus 8-OH-dG concentration using linear(y) and (x) axes and the data was fit to a 4-parameter logistic equation.

\[
\text{Logit} \left( \frac{B}{Bo} \right) = \ln \left[ \frac{B/Bo}{1 - B/Bo} \right]
\]

**Result interpretation:**
The %B/Bo value was calculated for each sample. Concentration of 8-OH-dG in pg/ml in each sample was obtained using the equation obtained from the standard curve plot.

**5.9 Statistical Evaluation**
Data were expressed as means ± standard deviations (SD). Statistical analyses were performed using a Chi square test and Fisher’s exact test for categorical variables and Mann–Whitney’s U-test or one-way analysis of variance for continuous variables, as appropriate. Differences were considered significant for P values less than 0.05. The statistical analysis software that was used was SPSS, version 13.0. Logistic regression analysis was used to assess the influence of each viral factor on the risk of cirrhosis development.