Materials & Methods
MATERIALS AND METHODS

Patients and Materials

Patient details like age, sex, history of jaundice/surgery/transfusion/liver function tests and virological markers were recorded in the prescribed proforma formatted and approved by the Institute Review Committee for this study. The patients attending the following hospitals and research center of Chennai were enrolled for this study:

- Department of Medical and Surgical Gastroenterology: Govt. General Hospital, Chennai.
- Department of Digestive and Health services: Govt. Peripheral Hospital, Chennai.
- Department of Surgical Gastroenterology: Stanley Medical College & Hospital, Chennai.
- Department of Microbiology, Dr. ALM PGIBMS, Taramani, Chennai.

Specimen

5 ml of blood was collected aseptically from each study subject and the sera were separated and stored at -70°C till tested.

Study Subjects

Chronic Liver Diseases

421 serum samples from patients with clinically proven CLD and increased ALT & AST (21- CPH, 29- CAH, 294–Cirrhosis and 77–HCC) were collected and tested for HBV & HCV serological and molecular markers.
Hepatitis Virus Serological Markers

HBV Markers

Hepatitis B surface antigen (HBsAg) (*BIO-RAD, Monolisa*, HBsAg, France)

The presence of HBsAg was detected using the commercial kit procured from Bio-Rad, Monolisa, HBsAg, France.

**Principle of the test**

In the Monolisa HBsAg assay, micro wells coated with a specific goat antibody to HBsAg are incubated with the sample and a mixture of mouse monoclonal antibodies, specific for different epitopes on HBsAg conjugated with horse-radish peroxidase. If the HBsAg is present in the sample, it will bind to both capture antibody and conjugated antibody. Thus the bound conjugate will develop a purple color which can be determined photometrically and is directly proportional to the amount of conjugate bound and hence the concentration of HBsAg in the sample.

**Test Procedure**

**Step 1**: The conjugate was reconstituted with conjugate diluent.

**Step 2**: Wells were set up.

**Step 3**: 25 μl of sample diluent was added to each well.

**Step 4**: 75 μl of samples or controls were added to the wells. For each series of tests the first column of wells were used for the assay controls.

**Step 5**: 50 μl of the conjugate was added to all the wells.
**Step 6:** The plate was shaken for 10 seconds and incubated for 90 mins at 37°C under humid conditions.

**Step 7:** The plate was washed 5 times with the diluted wash buffer.

**Step 8:** 100 µl of the substrate solution was added to each well

**Step 9:** Plate was covered and incubated for 30 mins at 37 °C under humid conditions.

**Step 10:** 50 µl of the stop solution was added to all wells.

**Step 11:** Within 15 minutes the absorbance of each well was read at 450 nm using 690 nm or 620 nm as the reference wavelength.

**Calculation of the cut-off value**

The cut-off value was calculated by adding 0.05 to the mean of the Negative control replicates.

**Interpretation of Test Results**

1. **Negative Results:** Samples giving an absorbance less than the cut-off values were considered non reactive in the assay.

2. **Positive Results:** Samples giving an absorbance equal to or greater than the cut-off value were considered reactive in the assay.
HBeAg/Anti-HBe (Wellcozyme, Murex Diagnostics Ltd, UK)

The presence of hepatitis B e-antigen (HBeAg) and antibody to hepatitis B 'e'-antigen (anti-HBe) was detected using the commercially available Wellcozyme, HBeAg / anti-HBe Murex Diagnostics Ltd, UK.

Principle of the test

Murex HBeAg/anti-HBe is an enzyme labeled 'sandwich' immunoassay, which detects HBeAg and anti-HBe in serum or plasma. For each test, two wells coated with two specific mouse monoclonal antibodies are used, to test simultaneously for both HBeAg and anti-HBe. To measure the HBeAg level, the test specimen was incubated with a third monoclonal antibody conjugated to horseradish peroxidase. HBeAg, if present, simultaneously couples to both antibodies on the solid phase and the conjugate creating an antibody-antigen-antibody 'sandwich'. After washing, a sensitive peroxidase substrate was added which produces color development if HBeAg is present.

The test for anti-HBe was carried out using essentially the same protocol except that a small quantity of HBeAg prepared from genetically engineered HBeAg was added. This test depends on competitive binding between antibody in the sample and the conjugated antibody for the added HBeAg, which is bound to the well surface. If anti-HBe is present in the sample, the addition of the pre-determined quantity of HBeAg (Neutralising antigen) will give a decrease in colour due to competition for the added HBeAg between antibody in the sample and the conjugated antibody resulting in less conjugate becoming bound to the wells.
Test Procedure

Two wells were allotted for each sample or control. Column 1 was allotted for the anti-HBe test and column 2 for the corresponding HBeAg test.

Step 1: 100 µl of each control/samples was added as follows:

<table>
<thead>
<tr>
<th>ROW</th>
<th>COLUMN 1</th>
<th>COLUMN 2</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(Anti-HBe test)</td>
<td>(HBeAg test)</td>
</tr>
<tr>
<td>A</td>
<td>anti-HBe Positive</td>
<td>HBeAg Positive</td>
</tr>
<tr>
<td>B</td>
<td>anti-HBe Positive</td>
<td>HBeAg Positive</td>
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<td>C</td>
<td>Negative</td>
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<tr>
<td>F</td>
<td>Sample 2</td>
<td>Sample 2</td>
</tr>
</tbody>
</table>

Step 2: 50 µl of Neutralizing antigen was added into the wells of column 1 (i.e., the anti-HBe Tests).

Step 3: 50 µl of conjugate was added to all wells.

Step 4: The plate was incubated in a water bath at 45 to 50ºC for 1 hour.

Step 5: At the end of the incubation period the plate was washed 5 times with the diluted buffer.

Step 6: 200 µl of substrate solution was added to each well.

Step 7: The plate was incubated at RT for 30 minutes in dark.

Step 8: 50 µl of the stop solution was added to all wells.

Step 9: The absorbance was read at 450nm.
Interpretation of Test Results

1. Anti-HBe assay (Column 1)
   a) The mean absorbance of the two negative control values and the two anti-HBe positive controls were calculated.
   b) The cut-off was calculated by multiplying the total mean of positive and negative control by 0.5.
      \[ \text{Cut-off} = 0.5 \times (\text{mean of Negative control} + \text{Mean of Positive control}) \]
   c) Samples giving an absorbance equal or less than the cut-off were considered positive for anti-HBe.

2. HBeAg Assay (Column 2)
   The mean absorbance of the two negative control values were calculated.
   a) The cut-off value was calculated by adding 0.1 to the mean of the negative control values.
      \[ \text{Cut-off} = \text{Mean of Negative control} + 0.1 \]
   b) Samples giving an absorbance equal or greater than the cut-off were considered positive for HBeAg.
Anti HBc ELISA (Wellcozyme) *(Murex Diagnostics Ltd, UK.)*

Wellcozyme anti HBc is an enzyme immuno assay kit for the detection of antibody to hepatitis B core antigen (anti HBc).

**Principle of the test**

In the Wellcozyme anti HBc assay the sample is incubated in a micro-well coated with HBcAg, when any anti HBc present in the specimen binds to all or part of the HBcAg. The sample is then removed and after washing the well, human anti HBc conjugated to horse radish peroxidase is added. The second incubation is carried out during which the conjugate binds to any HBcAg on the well surface not blocked by anti HBc from the test sample. After washing to remove unbound conjugate the sensitive peroxidase substrate is added and colour develops in proportion to the amount of conjugate bound. The level of colour is greatest in the absence of anti HBc and falls from this level with increasing concentrations of anti HBc in the sample.

**Test Procedure**

1. Use only the number of wells required for the test.
2. Add 200µl of samples under test or controls to each well. For each series of tests use three negative control and two positive control wells.
3. Incubate in a water bath at 37°C for two hours.
4. Wash the wells for three or four washes with a forty five second soak time.
5. Add 200μl of conjugate to each well, cover the wells with lid and incubate for 60 min at 37°C.

6. At the end of incubation, wash four times.

7. Add 200μl of substrate solution to each well.

8. Incubate for 30 min at room temperature.

9. Add 50μl stop solution (2M sulphuric acid to each well)

10. Read the well at 450 nm.

Calculation of Results

Cut-off = Mean of negative control x 0.33 + Mean of Positive control

A sample is considered positive if the OD is less than the cut-off.
HDV Serology

HDAg (Wellcozyme, Murex Diagnostics Ltd., UK)

The presence of hepatitis D antigen (HDAg) was detected using the commercially available Wellcozyme HDAg kit.

Principle of the test

To detect the HDAg, the test specimen was incubated in the wells coated with specific human antibody to HDAg (anti-HDAg) and the delta extraction buffer. If the delta antigen is present in the sample, it binds to anti-delta on the solid phase. After washing, anti-delta peroxidase conjugate was added to each well and incubated. Again after washing, the enzyme remaining bound to the wells is visualized using 3,3′,5,5′ – tetramethyl benzidine and hydrogen peroxide to give a yellow colour after termination of the enzymatic reaction with sulphuric acid.

Test Procedure

1. The number of wells was set up.
2. 50 µl of negative control, positive control and the samples were added into the respective wells.
3. 50 µl of Delta antigen extraction buffer was added to all wells.
4. The strips were sealed with a individual disposable strip sealer, and incubated at room temperature overnight.
5. At the end of incubation period the plate was washed 4 times with the diluted wash buffer.
6. Immediately after washing the plate, 100μl of conjugate was added to all wells.

7. The strips were sealed with new individual strip sealers. The plate was incubated for 60 min at 37°C in a water bath.

8. At the end of incubation period the strips were washed as in step 5.

9. 100 μl of substrate solution was added to all wells.

10. The plate was incubated for 30 min at 18-25°C in the dark.

11. At the end of the incubation time 100μl of stop solution (2M Sulphuric Acid) was added to all the wells.

12. The absorbance was read at 450 nm (ELx 800 ELISA reader. Biotech Instruments Inc., USA)

**Calculation of Results**

1. The mean absorbance of the replicates of the negative control was calculated.

2. Cut-off Value: The cut-off value was calculated by adding 0.5 to the mean of the negative control replicatives.

3. Positive results: Samples giving an absorbance equal to or greater than the cut-off value were considered positive for HDAg.
Anti-HD (Total) *(Wellcozyme, Murex Diagnostics Ltd., UK)*

Wellcozyme anti-HD is an enzyme immunoassay, which detects antibody to hepatitis delta antigen (anti-delta) in human serum or plasma. In order to do this sample or control is incubated in a well coated with delta antigen captured onto purified anti-delta. After washing to remove unreacted serum, anti-delta/peroxidase conjugate is added to each well and incubated. Excess conjugate is washed and the bound conjugate is visualized using TMB and hydrogen peroxide to give a yellow colour after termination of the enzymatic reaction with sulphuric acid.

Anti-delta in the sample will block the binding of the conjugate, resulting in the decrease in absorbance.

**Test Procedure**

1. The required number of wells was set up.
2. 100 μl of negative control, positive control and the samples were added into the respective wells.
3. The wells were covered with lid and incubated for 2 hours at 37°C under humid conditions.
4. At the end of incubation period the plate was washed 4 times with the diluted wash buffer.
5. Immediately after washing the plate, 100μl of conjugate was added to all wells.
6. The strips were sealed with new individual strip sealers. The plate was incubated for 60 min at 37°C in a humid condition.
7. At the end of incubation period the strips were washed with the wash buffer.
8. 100 μl of substrate solution was added to all wells.
9. The plate was incubated for 30 min at 18-25°C in the dark.
10. At the end of the incubation time 100μl of stop solution (2M Sulphuric Acid) was added to all the wells.
11. The absorbance was read at 450 nm (ELx 800 ELISA reader, Biotech Instruments Inc., USA)

Calculation of Results

The mean absorbance of the three negative controls (NC) values and the two positive control (PC) values were calculated. The cut-off value was calculated using the following formula: Cut-off value = 0.5 (Mean OD NC + Mean OD PC). Samples with absorbance less than or equal to the cut-off value are presumed positive for antibody to delta antigen.
HCV Serology

Anti-HCV ELISA: III Gen. (Murex Diagnostics, UK)

Principle of the test

In the Murex anti-HCV test, diluted sample is incubated in microwells coated with highly purified antigens, which contain sequences from the putative C, NS3, NS4 and NS5 regions of HCV. During the course of the first incubation any anti-HCV antibodies in the sample will bind to the immobilized antigens. Following washing to remove unbound material, the captured anti-HCV antibodies are incubated with peroxidase conjugated antibody-Anti-human antibody/enzyme complex will be formed in those microwells which contained samples with antibodies to HCV. After removal of excess conjugate, bound enzyme is detected by the addition of a solution containing 3,3',5,,- tetramethylbenzidine (TMB) and hydrogen peroxide. A purple color will develop in the wells, which contain anti-HCV positive samples. The enzyme reaction is terminated with sulphuric acid to give an orange color, in the wells, is directly related to the concentration of antibody in the sample.

Test Procedure

Step 1 : The conjugate was reconstituted with conjugate diluent and the substrate solution was prepared according to the manufacturer’s instruction.

Step 2 : 180 µl of sample diluent was added into each well.

Step 3 : 20 µl of samples and controls was added into each well.

Step 4 : The wells were covered with the lid and incubated for 1 hour at 37°C under humid conditions.
Step 5 : At the end of the incubation period, the wells were washed 5 times as described under wash procedures.

Step 6 : Immediately after washing the plate, 100 µl of conjugate was added to each well.

Step 7 : The wells were covered with the lid and incubated for 30 minutes at 37°C under humid conditions.

Step 8 : At the end of the incubation period the plate was washed as described under wash procedures.

Step 9 : Immediately after washing the plate, 100 µl of substrate solution was added to each well.

Step 10 : The wells were covered with the lid and incubated for exactly 30 mins at 18-25°C while color developed. A purple color developed in wells with positive samples.

Step 11 : 50 µl of stop solution (2M sulphuric acid) was added to each well.

Step 12 : Within 15 minutes, the absorbance was read for each well at 450 nm using a microwell plate reader. The instrument was blanked on air.

Step 13 : The mean of the three negative control wells and of the two positive control wells was calculated.
Calculation of the cut-off value

The cut-off value was calculated by adding 0.6 to the mean of the negative control replicates.

Interpretation of Test Results

1. **Negative Results:** Samples giving an absorbance less than the cut-off values were considered non reactive in the assay.

2. **Positive Results:** Samples giving an absorbance equal to or greater than the cut-off value were considered reactive in the assay.

Quality Controls

WHO panel serum samples obtained from Institute for Standardisation and Documentation in the Medical Laboratory (INSTAND), Dusseldorf, Germany. (WHO collaborating center for Quality Assurance and Standardization in Laboratory Medicine), were used as quality controls in our all PCR assays.
Hepatitis Viral Molecular Markers

HBV-DNA Extraction from serum

Extraction of DNA was carried as per the method of Higuchi. (1989). The advantage of this method over the basic method for the extraction of DNA is that the phenol extraction and ethanol precipitation steps are avoided in this protocol.

1. 100 μl of serum was taken in an eppendorf tube and 450 μl of proteinase K solution (2X proteinase K solution: 20 mM Tris HCl, pH 8.0; 10 mM EDTA; 1% SDS; 200 μg/ml proteinase K) was added to it.

2. This was incubated at 56°C for 2 hours.

3. After 2 hours the tube was incubated at 95°C for 10 minutes to inactivate the protease.

4. The solution was stored frozen and 10μl was used for PCR reaction.

Amplification of HBV-DNA

HBV surface antigen primers were used for amplification as per method of Cheung et al. (1991)

F1679: 5’ – GGG TGG AGC CCT CAG GCT CAG GGC A – 3’

R2254: 5’ – GAA GAT GAG GCA TAG CAG CAG GAT – 3’
PCR Reaction Mix

The reaction mix was constituted in the following manner:

10X Taq buffer – 10 µl; 10 mM dNTPs – 2 µl; Sense primer – 10 µl; Antisense primer – 10 µl (20pmoles of each primer); Taq Polymerase – 2.5 U; Sterile nuclease free water- 57.5 µl and Template – 10 µl.

The template was denatured at 94°C for 5 minutes, quick-chilled on ice and then added to the reaction mix tube.

Thermocycling Profile

The reaction mix was subjected to the following thermocycling profile: 94°C for 1 minute (denaturation); 55°C for 2 minutes (annealing); 72°C for 3 minutes (extension) in a PCR machine (PTC-100, MJ Research, MA, USA). The cycle was repeated 35 times and the final extension was carried out at 72°C for 7 minutes. The amplification product was separated on 2% agarose gel and stained with ethidium bromide (0.5µg/ml concentration). The gel was visualized under UV transilluminator and the 575 bp product was compared with molecular weight marker (100 bp ladder, MBI fermentas, NY, USA).
Subtype determination of HBV based on PCR & Restriction Digestion as per method of Cheung et al. (1991)

The Subtyping was done based on the restriction digestion of the amplified DNA of the partial sequences of ‘S’ region containing the restriction nuclease susceptible sites for Bam HI, Hpa II and Xho I. The subtype was confirmed based on the pattern of digestion and fragment size.

Primers

The primer set was selected to amplify the pre S-S region of the S gene. It encodes the 3’ portion of pre S1, the entire pre S2 and 5’ portion of the major S gene. Three restriction nuclease susceptible sites Bam HI, Hpa II and Xho I were observed within this region and they gave unique fragmentation patterns for each subtype.

HBV F : 5’ – GGG TGG AGC CCT CAG GCT CAG GGC A – 3’
HBV R: 5’ – GAA GAT GAG GCA TAG CAG CAG GAT – 3’

Thermocycling Profile

The reaction mix was subjected to the following thermocycling profile: 95°C for 35 seconds (denaturation); 55°C for 1 minute (annealing); 72°C for 1 minute (extension) in a PCR machine (PTC-100, MJ Research, MA, USA). The cycle was repeated 35 times and the final extension was carried out at 72°C for 5 minutes. The amplification product was separated on 2% agarose gel and stained with ethidium bromide (0.5µg/ml concentration). The gel was visualized under UV transilluminator and the 575 bp product was compared with molecular weight marker (100 bp ladder, MBI fermentas, NY, USA).
Restriction Digestion analysis of Amplified DNA- 575 bp PCR product

The amplified DNA from each HBV positive sample was digested with BamHI, Hpa II and Xho I restriction nuclease, restriction susceptible sites were observed in this region. The subtype was confirmed based on the pattern of digestion and fragment size. The cleavage reaction was performed in volume of 30µl reaction constituting the following:

- 10 X reaction buffer – 5 µl; 50 mM MgCl₂ – 1 µl; 5 U/µl of restriction enzymes - 2µl; Sterile double distilled water- 5 µl; Template – 17 µl.

The reaction mix was incubated at 37°C for 2 hours; the digested product was separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide.

Restriction Nuclease/Fragment size of different HBV subtypes Pattern of Digestion

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Bam HI</th>
<th>Hpa II</th>
<th>Xhoh</th>
</tr>
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<tbody>
<tr>
<td>adr</td>
<td>575</td>
<td>357, 218</td>
<td>306. 269</td>
</tr>
<tr>
<td>adw</td>
<td>405, 170</td>
<td>575</td>
<td>575</td>
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<tr>
<td>ayw</td>
<td>575</td>
<td>575</td>
<td>306. 269</td>
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</table>
HBV Genotyping by type specific primers

{Genotyping was carried as per the method of Naito et al. (2001) }

The nucleic acid was extracted from 100 µl serum samples using a nucleic acid extraction Qiagen kit, (QIA amp, USA). The resulting pellet was resuspended in nuclease –free water and then subjected to nested PCR. The HBV genome was amplified by nested PCR. The first round PCR primers (Outer primer pairs) and second round PCR primers (Inner Primer pairs) were designed on the basis of the conserved nature of nucleotide sequences in regions of the pre-S1 through S genes, irrespective of the six HBV genotypes. P1 (sense) and S1-2 (antisense) were universal outer primers.

**Outer Primer**

P1 5’TCA CCA TAT TCT TGG GAA CAA GA – 3’(nt 2823-2845, universal, sense)
S1-2 5’ CGA ACC ACT GAA CAA ATG GC- 3’ (nt 685-704, universal, antisense)

The first round PCR was carried out in a tube containing 40 µl of a reaction buffer made up of the following components; 50 ng of each outer primer; a 200 µM concentration of each of the four deoxynucleotides, 1U of Ampli Taq Gold DNA polymerase, and 1 X PCR buffer containing 1.5 mM MgCl₂.

**Thermocycling**

The reaction mix was kept at 95° C for 10 min. Then it was subjected to the following thermocycling profile:
94°C for 20 sec (denaturation)
55°C for 20 sec (annealing) and
72°C for 1 min (extension)
For 40 cycles and a final extension of 5 min at 72°C.

NESTED PCR

Two second-round PCRs was performed for each sample, with the common universal sense primer (B2) and Mix A, and common universal antisense primer (B2R) and Mix B.

The second round nested PCR were performed for each sample, with the inner set of primers. B2 was used as the inner primer (sense) with a combination called mix A for genotypes A, B and C. Mix A consisted of antisense primers BA1R (type A specific), BB1R (type B specific), and BC1R (type C specific). B2R was used as the inner primer (antisense) with a combination called mix B for genotypes D, E and F. Mix B consisted of sense primers BD1 (type D specific), BE1 (type E specific), and BF1 (type F specific). These primer combinations for second-round PCR were designed on the basis of the differences in the sizes of the genotype-specific bands.

Inner Primers

Mix-A

B2 5’ GGC TCM AGT TCM GGA ACA GT-3’ (nt 67-86, types A to E specific, sense)
BA1R 5’ CTC GCG GAG ATT GAC GAG ATG T-3’ (nt 113-134, type A specific, antisense)
BB1R 5’ CAG GTT GGT GAG TGA CTG GAG A-3’ (nt 324-345, type B specific, antisense)
BC1R 5’ GGT CCT AGG AAT CCT GAT GTT G-3’ (nt 165-186, type C specific, antisense)
Mix-B

BD1  5' GCC AAC AAG GTA GGA GCT-3' (nt 2979-2996, type D specific, sense)
BE1  5' CAC CAG AAA TCC AGA TTG GGA CCA-3' (nt 2955-2978, type E specific, sense)
BF1  5' GYT ACG GTC CAG GGT TAC CA-3' (nt 3032-3051, type F specific, sense)
B2R  5' GGA GGC GGA TYT GCT GGC AA-3' (nt 3078-3097, types D to F specific, antisense)

A 1 µl aliquot of the first PCR product was added to two tubes containing the second sets of each of the inner primer pairs, each of the deoxynucleotides, Ampli Taq Gold DNA polymerase, and PCR buffer, as in the first reaction.

Thermocycling

The reaction mix was kept at 95°C for 10 min. Then it was subjected to the following thermocycling profile:

94°C for 20 sec (denaturation):
58°C for 20 sec (annealing) and
72°C for 30 sec (extension)

For 20 cycles and a final extension of 5 min at 72°C.

Genotypes of HBV for each sample were determined by identifying the genotype specific DNA bands. The two different second-round PCR products from one sample were separately electrophoresed on a 3% agarose gel, stained with ethidium bromide and evaluated according to the migration pattern of a 50 bp DNA ladder.

Mix A allows for the specific detection of PCR products for types A, B, and C, and Mix B allows for detection of types D, E and F.
Detection of Genetic Alterations in HBV Precore & core region

Amplification of HBV Precore and core region

The nucleic acid was extracted from 100 µl serum samples using a nucleic acid extraction Qiagen kit. (QIA amp, USA). The extracted DNA was subjected for amplification using the nucleotide sequences encoding the entire core ORF of HBV in a standard 50 µl PCR reaction using outer and inner set primers as mentioned below, as per the method of Fagan and Harrison, (1994). The reaction mix was constituted in the following manner:

PCR buffer (10X), Mgcl₂ (2.5 mM), dNTPs (200 µM each), primers (0.2 µM each primer), BSA (100 µg/ml), Taq polymerase (1 U).

Outer primers

PM1:5' – GCG AAG CTT GAG GAA TAA AGC CCC GTA AA -3'
PM2:5' – GCG CTG CAG GAG TTG GGG AGG AGA ATT A – 3'

Inner primers

PM3:5' – GCG AAG CTT AGA TCT CTG GAT GCT GGA – 3'
PM4:5' – GCG CTG CAG GAG GCT GTA GGC ATA AAT – 3'

Themocycling Profile

The reaction was subjected to the following thermocycling profile: 94°C for 1.5 min (denaturation); 50°C for 1.5 min (annealing); 72°C for 3 min (extension) in a PCR machine (PTC-100, MJ Research, MA, USA). The cycle was repeated 35 times and the final extension was carried out at 72°C for
10 minutes. At the end of 35 cycles the n-PCR product was separated on 2% agarose gel and stained with ethidium bromide. The gel was visualized under UV transilluminator and the 387bp product was compared with molecular weight marker (100 bp ladder, MBI fermentas, Amherst, NY, USA).

**Gel Extraction and Purification (QIA Quick extraction)**

The HBV positive amplicons were purified using QIA Quick gel extraction kit (Qiagen, USA). The QIA quick system combines the convenience of spin-column technology with selective binding properties of uniquely designed silica-gel membrane. Special buffers provided with each kit are optimized for efficient recovery of DNA and removal of contaminants in each specific application. DNA absorbs to the silica membrane in the presence of high salt while contaminants pass through the column. Impurities are efficiently washed away, and the pure DNA is eluted with Tris buffer or nuclease free water.

**Procedure**

1. The DNA fragment was excised from the agarose gel with a sterile, sharp scalpel.

2. The sliced gel was weighed in a colorless tube. 3 volumes of Buffer QG to 1 volume of gel (100 mg - 100 μl) were added.

3. The mixture was incubated at 50°C for 10 min (or until the gel slice has completely dissolved). The tube contents were mixed by vortexing the tube every 2-3 min during the incubation.

4. After the sliced gel had dissolved completely, it was checked that the colour of the mixture was yellow (Similar to buffer QG without dissolved agarose).
5. A gel volume of isopropanol to the sample was added and mixed thoroughly.

6. A QIA quick spin column was placed in a provided 2 ml collection tube.

7. The sample was applied to the QIA quick column, to bind DNA, and was centrifuged for 1 min.

8. Flow-through the QIA quick column back and was placed in the same collection tube and the flow-through was discarded.

9. 0.5 ml of buffer QG was added to QIA Quick column and centrifuged for 1 min.

10. To wash, 0.75 ml of buffer PE was added to QIA quick column and centrifuged for 1 minute.

11. The QIA quick column was centrifuged for an additional 1 min at 13,000 rpm and the flow-through was discarded.

12. The QIA quick column was placed into a clean 1.5ml microcentrifuge tube.

13. 50 µl of buffer EB (10mM Tris-HCl, pH 8.5) was added to the center of the QIA quick membrane and centrifuged the column for 1 min at 12000 rpm to elute the DNA. Alternatively, for increased DNA concentration, 30µl of elution buffer was added to the center of the QIA quick membrane, and the column was allowed to stand for 1 min and then the column was centrifuged for 1 min.
Direct DNA Sequencing of PCR products

Reagents for DNA sequencing and processing

Products of positive PCR reactions were purified using QIA quick gel extraction kit (Qia quick, QIAGEN, USA). Purified templates were then subjected to direct cycle sequencing, the amplicons were sequenced bidirectionally using (Automated A3700 DNA Analyzer, PE Applied Biosystems). Big Dye Terminator cycle sequencing ready reaction kit (Big Dye™, 50% HiDye Formamide, 70% ethanol, 3M Sodium Acetate) with AmpliTaq DNA polymerase (PE Applied Biosystems) containing Fluorescence labelled-dideoxynucleotide chain terminators.

ABI PRISM® 3700 DNA Sequencer

The ABI PRISM® 3700 DNA Sequencer automatically analyses DNA molecules labeled with multiple fluorescent dyes. It contains of a change couple device (CCD) camera and a power Macintosh computer that includes software for data collection and data analysis. After samples are loaded on to the system’s vertical gel, they undergo electrophoresis, Laser detection and computer analysis electrophoretic separation can be viewed on-screen in real time.

PCR Conditions

The reaction was subjected to the following thermocycling profile:

96°C for 10 seconds; 52°C for 5 seconds; 60°C for 4 minutes – 30 cycles and 4°C.
1. The reaction mixture was prepared in a 1.5 ml tube and then equally dispersed into Micro amp 96 well plate.

2. The PCR of the Micro amp plates containing the DNA mixture was carried out in the Gene Amp 9600 thermal cycler (Perkin-Elmer). The PCR was carried out for 3hrs, after that plate was processed.

3. Processing: 3ml absolute alcohol was added to 120µl of 3N sodium acetate (Freshly prepared) in a tube. The tube was mixed thoroughly. 25 µl of the above mixture was added in each well of the plate.

4. The plate was centrifuged at 4000 rpm for 25 minutes I eppendorf 5810 R centrifuge.

5. The plate was inverted and the supernatant removed, 100µl of fresh 70% ethanol was added to each well and centrifuged at 4000 rpm for 15 minutes.

6. Again the plate was inverted and the alcohol was removed by placing filter paper and centrifuged for few seconds at 500 rpm. The plate was covered properly with foil.

7. At the time of sequencing, 10µl of 50% High Dye™ formamide was added to all the wells. The sample plates were kept and run in ABI 3700 DNA analyzer (Automated A 3700 DNA analyzer, PE Applied Biosystems).

8. Each amplicons were analyzed bidirectionally using fluorescent-labeled dideoxy nucleotide chain terminators (ABI prism Big Dye Terminator cycle sequencing ready reaction with Ampli Taq DNA polymerase, PE Applied Bio systems).
Nucleotide sequences obtained were initially analyzed using "Gene auto assembler software" (version 2.0). It allows on screen alignment of chromatograms. Sequences were later aligned with the consensus sequence for HBV precore & core region obtained from EMBL database (European Molecular Biology Laboratories). The aligned sequence data were later analyzed using the following multiple sequence alignment softwares namely, Clustal X (version 1.8), it is a network analysis software it provides an integrated environment for performing multiple sequence alignments and analyzing the results and GENEDOC, which is a full featured multiple sequence alignment editor, analyzer and is windows based.
RNA extraction from serum samples was performed as per the single step extraction method (Chomczynski and Sacchi, 1987). To 250 µl of serum sample an equal amount of lysis buffer; Solution D containing (4M Guanidium Isothiocyanate, 25mM Sodium citrate, 0.5% Sacrosyl, 0.1 M β-mercaptoethanol) and 1µg of tRNA (Sigma Aldrich Chemicals Pvt Ltd., Bangalore) was added. To this suspension 25 µl of 2 M Sodium Acetate (pH 4.8), 250 µl of water saturated phenol and 50 µl of chloroform-isooamyl alcohol mixture were added. After shaking for 10-20 sec, the suspension was incubated on ice for 10 min. The suspension was separated into aqueous and organic phases by centrifugation at 10,000 g for 20 min at 4°C. Following centrifugation, total RNA remains exclusively in the top aqueous phase, whereas DNA and proteins remain in the interphase and organic phase. The aqueous phase was transferred to a fresh tube and RNA precipitated at -20°C for 30 min, by mixing with an equal volume of 100% isopropanol. The resulting RNA precipitate was sedimented by centrifugation at 10,000g for 20 min at 4°C. RNA pellet was dissolved in 300-500 µl. RNA was re-precipitated by mixing with 1 vol. of isopropanol, storing the mixture at-20°C for 30 min, and centrifugation at 10,000g for 10 min at 4°C. The supernatant was removed and the RNA pellet was suspended in 75% ethanol by vortexing. The suspension was centrifuged at 10,000g for 10 min at 4°C for removal of salts. The supernatant was removed by aspiration with a capillary and pellet allowed to air dry for 5-10 min. The pellet was dissolved in 20 µl of DEPC treated water and stored at -70°C until further use.
cDNA Synthesis

The RNA was denatured at 65°C for 10 minutes using random primers 300 ng random hexamers (Life Technologies Gaithersburg, MD) and then added onto cDNA master mix. First strand cDNA was synthesized using a reaction mixture containing 8 units of Moloney Murine Leukaemia Virus (MMuLV) reverse transcriptase (Superscript II; Life Technologies, Gaithersburg, MD), 500 μM dNTPs and 4 units of RNAsin (Promega, Madison, WI, USA). Reverse transcription reaction was carried out by incubation at 38°C for 1 hr.

Polymerase Chain Reaction

Primers for RT-PCR for HCV-RNA detection were derived from 5’NTR region. HCV RT-PCR was standardized ‘in-house’ as per method Cha et al., (1991).

First Round Amplification

Outer primers

HCV # 1: 5’-CCC AAC ACT ACT CGG CTA G-3’
HCV # 3: 5’-CCA TGA ATC ACT CCC CTG TGA GGA ACT A-3’

PCR mix: The different components of PCR reaction mix were as follows: 10X Taq buffer, dNTPs at concentration of 200 μM, MgCl₂ at 1.5 μM and primer HCV #1 and HCV #3 at conc. of 10 pmoles each and Taq polymerase at 2.5U.
Thermocycling Profile

95°C for 1 min (denaturation); 50°C for 1 min 30 sec (annealing) and 73°C for 1 min 30 sec (extension) for 35 cycles and final extension of 5 min at 73°C (PTC-100, MJ Research, MA, USA)

Inner Primers

Nested polymerase chain reaction was carried out with inner set of primers HCV#2 and HCV#4. Additional amplification was achieved by the nested PCR under the same reaction conditions as mentioned above for 1st round amplification and 1μl of diluted first round amplicon was used as template.

HCV # 2: 5'-TTG CGG GGG CAC GCC CAA-3'
HCV # 4: 5'-GCC ATG GCG TTA GTA TGA GTG TC-3'

At the end of 35 cycles the n-PCR product was separated on 2% agarose gel stained with ethidium bromide. The gel was visualized under UV transilluminator and 162 bp products were compared with molecular weight marker (100bp ladder, MBI fermentas, Amherst, NY, USA).
HCV Genotyping by INNO-LiPA HCV II Line Probe Assay

INNO-LiPA PCR amplification

The RNA positive samples were genotyped by hybridization of amplicons to HCV–genotype specific oligonucleotides using the commercial HCV genotyping kit (InnoLiPA, Innogenetics, Belgium) according to manufacturer’s instructions. PCR products with less banding intensity were subjected to a second round amplification using the inner primers. The banding patterns obtained were interpreted using the reading reference card provided in the kit.

The reagents are used for reverse transcriptase and nucleic acid amplification of the 5’ untranslated region (UTR) of the hepatitis C virus (HCV). The existence of variations in the 5’UTR of the different HCV genotypes enables to use these sequences to determine 6 HCV genotypes and their subtypes. This kit makes use of boitinylated oligonucleotide primers to generate biotinylated amplified target material, to be used in the INNO-LiPA HCV II test for genotyping the HCV virus.

Principle

The purified RNA, together with random primers is denatured and mixed with human placental ribonuclease inhibitor, deoxy nucleotide 5’-triphosphates (dNTP) and avian myeloblastosis virus-reverse transcriptase (AMV-RT). When this mixture is placed at 42°C, the reverse transcriptase will make complementary DNA (cDNA) from the RNA. The amplification is polymerase chain reaction (PCR). The cDNA is introduced in a reagent mixture containing an excess of deoxynucleotide outer primers, and thermostable DNA polymerase. The outer primers (first round of PCR) used in this test will amplify the 5’UTR of the HCV genome. By heating, the two
strands of the DNA helix are separated (denaturation) to expose the target sequences to the biotinylated outer primers. These oligonucleotide primers are complementary to very conserved regions flanking the target sequence. Therefore, upon cooling to a well-defined temperature, the primers will bind to their specific sequence (annealing). At another temperature, using the dNTPs, the thermostable DNA polymerase will extend the annealed primers along the target template (extension). This way, a biotinylated exact copy of the template sequence is produced after one cycle of denaturation, annealing and extension. This process is repeated for 40 cycles, thus yielding a multi-fold amplified biotinylated target sequence.

If the amount of amplified product is not sufficient, a second round (nested) PCR is needed. This amplification is identical to the first with exception that the cDNA is replaced by the amplified product of the first round PCR, and the outer primers are replaced by nested (inner) primers. The nested primers have a sequence complementary to a sequence of the amplified target of the first PCR. The assay is based on variations found in the 5′ untranslated regions (5′UTR) of the different HCV genotypes. Type specific probes are tailed with a poly (T) – tail by terminal deoxynucleotidyl transferase and attached to nitrocellulose membranes.

Biotin-labeled amplified products are reversely hybridized to the probes on the strip. The biotin group is incorporated by employing a 5′-biotinylated primer during amplification. The labeled product obtained from the 5′UTR will only hybridize to a probe that gives a perfect sequence match. After hybridization, streptavidin labeled with alkaline phosphatase is added and bound to any biotinylated hybrid previously formed. Incubation with BCIP/NBT chromogen results in purple / brown precipitate. Consequently, a purple/brown or positive line will occur only when there is a perfect match between the probe and the biotinylated PCR products.
The genotype can be detected using the INNo LiPA HCV II interpretation chart after collecting all the numbers from positive lines. Using the INNO-LiPA HCV II strips, the 6 major HCV types and their most common subtypes can be detected simultaneously. The INNo-LiPA HCV II strip contains a test control line and 20 parallel DNA probe lines. The conjugate control line is a control for the colour development reaction, and the amplification control line contains universal probes hybridizing to amplified products to any HCV type.

The reactivity of an amplified fragment with one or more lines on the strip allows the easy recognition of the HCV genotype. Mutations occurring within the quasispecies pool of sequences from one isolate, do not affect the outcome of this genotyping procedure.

Sample Preparation

1. 50 μl of serum was added to 150μl of TRIzol reagent. Cells were lysed in the sample suspension by pipetting up and down.

2. Incubated at room temperature for 30 minutes.

3. 40 μl of chloroform was added, mixed vigorously by hand for 15 seconds.

4. Incubated for 2 to 25 minutes at room temperature.

5. Centrifuged for 15 minutes at 12000 g in an eppendorf centrifuge at 4°C.

6. The colourless supernatant was transferred into a fresh tube and 20 μl of 1 μg/μl dextran T 500 was added then briefly vortexed.

7. 100 μl of isopropanol was added, briefly vortexed and incubated for 10 minutes at room temperature.
8. Centrifuged for 10 minutes at 12000 g in an eppendorf centrifuge at 4°C.

9. The supernatant was removed and to that 200 μl of ice cold 75% ethanol was added then centrifuged at 7500 g for 5 minutes.

10. The supernatant was removed and the RNA pellet was briefly dried, stored at -70°C until use.

**cDNA Synthesis**

1. The reaction mixture was prepared as follows:
   
   DEPC treated water - 10 μl
   150 ng / μl random primers - 2 μl
   The RNA pellet was dissolved in 12 μl mix.

2. The RNA was denatured for 10 minutes at 70°C.

3. Immediately after the denaturation the whole tube was kept at 42°C.

4. The cDNA master mix was prepared as per the kit brochure:
   
   HPRI (Human placental ribonuclease inhibitor) - 25 U
   5x AMV-RT buffer - 4 μl
   10 mM dNTPs - 1 μl
   AMV-RT - 8 U
   
   Finally 8 μl reaction mixture was added with 12 μl of RNA/random primers.

5. The cDNA reaction mixture was incubated at 42°C for 90 minutes.

6. The converted cDNA was freezed at -20°C until tested for the first round DNA PCR.
Test Procedure

First Round Amplification

1. Master mix was prepared as per the instruction manual
   10X Taq buffer without Mgcl₂ - 5 µl
   Outer HCV primers - 10 µl
   10 mM dNTPs - 1 µl
   Taq polymerase - 1 U
   25 mM Mgcl₂ - 3 µl

2. 5 µl of cDNA was added to the 45 µl of reaction mixture.

3. HCV-RNA was amplified as per the kit procedure
   a. 94°C for 1 minute
   b. 55°C for 1 minute
   c. 72°C for 1 minute
   d. Step a) to c) 40 times
   e. 72°C for 5 minutes
   f. Cool to 4°C until further use.

4. 10 µl of the amplified product was analyzed in a 2% agarose gel.

Second (nested) Amplification round

1. Master mix was prepared as per the instruction manual
   10X Taq buffer without Mgcl₂ - 5 µl
   Inner HCV primers - 10 µl
   10 mM dNTPs - 1 µl
   Taq polymerase - 1 U
   Mgcl₂ - 1.5 mM

2. 1 µl of the First round product was added to the 49 µl of reaction mixture.
3. HCV-RNA was amplified as per the kit procedure
   a. 94°C for 1 minute
   b. 55°C for 1 minute
   c. 72°C for 1 minute
   d. Step a) to c) 40 times
   e. 72°C for 5 minutes
   f. Cool to 4°C until further use.

4. 10 µl of the amplified product was analyzed in a 2% agarose gel.

5. After verification the products were subjected for the INN-LiPA HCV II test.

Quality Control

Both positive and negative control specimens were also amplified along with test samples (first round product 300 bp and second round product 240 bp).

Genotype Detection - Intended Use

INNO-LiPA HCV II is a line probe assay, for in vitro use, for the genotyping of hepatitis C virus (HCV) in human serum and plasma. This test allows the genotyping of 6 major HCV types and their most common subtypes.

Test Procedure

1. Using tweezers, the required number of INNO-LiPA HCV strips were removed from the tube and by pencil an identification number was marked just above the black marker line on the strip.
Always one strip was used as a blank amplified control (no DNA added).

2. The required numbers of test troughs (1 trough per test sample) were taken and placed them in the tray.

3. 10 µl of denaturation solution was added into the upper corner of each trough.

4. 10 µl of the amplified product was added to the denaturation solution and carefully mixed by pipetting up and down. The solution was allowed to denature for 5 minutes at room temperature.

5. The ready-for-use hybridization solution was prewarmed and gently 2ml of the denaturated-amplified product were added into each trough. Troughs along with reagents were shaken gently. The troughs were carefully handled not to contaminate neighboring troughs during pipetting.

6. All the strips were placed immediately with the marked side (black marker line) of the membrane up into the trough.

7. The strips should be completely submerged in the solution.

8. All the trays were kept into the shaking water bath (approximately 80 rpm) at 50°C for 60 minutes.

9. After hybridization the tray was removed from the water bath.

10. Vacuum attached aspirator was used to aspirate the liquid from the trough. 2ml of prewarmed ready for use stringent wash solution (SW) was added into each trough and rinsed by rocking the tray at room temperature (20-25°C) for 10-20 seconds.

11. The stringent wash solution step was repeated for one or more time. Finally 2 ml of prewarmed stringent wash solution (SW)
was added in all the troughs and incubated in the shaking water bath at 50°C for 30 minutes.

12. All the strips were washed twice for 1 minute by using the 2ml of diluted Rinse Solution (RS).

13. 2 ml of the diluted conjugate was added to each trough and was incubated for 30 minutes while agitating the tray on the shaker.

14. Once again all the strips were washed for 1 minute using 2 ml of the diluted Rinse solution (RS) and also washed with 2 ml of Substrate Buffer (SB)

15. 2 ml of the substrate solution was added to each trough and was incubated for 30 minutes while agitating the tray on the shaker.

16. All the strips were washed twice with 2 ml of distilled water for at least 3 minutes.

17. Finally by using tweezers, all the strips were removed and were kept on the absorbent paper. The colour-developed strips were stored in the dark.

**Reading**

After hybridization, streptavidin labeled with alkaline phosphatase is added and bound to any biotinylated hybrid previously formed. Incubation with BCIP/NBT chromogen results in a purple/brown precipitate. Consequently a purple/brown line will occur only when there is a perfect match between the probe and the biotinylated PCR products. A line is considered positive when a clear purple/brown band appears at the end of the test procedure.
Interpretation of Results

The conjugate control line, which should be lined up with conjugate control line on the plastic reading card. The second positive line (Amplification control on reading card) controls for the addition of amplified material for hybridization. This line should be positive if cDNA amplified product from HCV is present. All line numbers, which are positive on the INNO-LiPA HCV II strip, were collected and the genotype was detected using the INNO-LiPA HCV II interpretation chart.
HCV Genotyping by RFLP

Amplification of HCV 5' noncoding region (5' NCR) and HCV Typing by RFLP using Bst N1 and Bst U1

All samples positive for HCV RNA were considered for genotyping by RFLP using enzymes Bst U1 and Bst N1. RFLP was done as per the method of Thiers et al, (1997).

First round PCR for 5'NCR

Primers

A209 5' – ATA CTC GAG GTG CAC GGT CTA CGA GAC CT -3' (-349 to -321)
S939 5' – CTG TGA GGA ACT ACT GTC TT – 3'(-45 to -64)

The first round PCR was standardized with primers S939 and A209. The optimal conditions for various PCR parameters were 200uM dNTPs, 1.5mM MgCl₂, 20 pmoles of each primer and 2.5 U of Taq Polymerase.

Second round PCR

Primers

AS211 5' – CAC TCT CGA GCA CCC TAT CAG GCA GT -3' (-313 to -228)
S940 5' – TTC ACG CAG AAA GCG TCT AG – 3'(-63 to -82)

The second round PCR was standardized with primers S940 and AS211. The optimal PCR parameters were 200uM dNTPs, 1.5mM MgCl₂, 20 pmoles of each primer, 2.5 U Taq Polymerase and 1 uL of diluted first round amplicon (appr. 1ng) as template.

A final extension was carried out at 72°C for 7 min.
A 251 bp amplicon was obtained after 2nd round PCR. The amplified products were subsequently purified by using a commercial kit (Qiaquick Gel Extraction kit; Qiagen, USA) and subjected for RFLP using enzymes Bst U1 and Bst N1. Purified products were quantitated either on an agarose gel or by UV spectrophotometer. 500ng of each amplicon was put for digestion with each enzyme. A mixture of appropriate 1X restriction enzyme buffer, 5 Units of each enzyme, Bst N1 and Bst U1 (Fermentas Inc., Hanover, USA) and DNA were added to a 0.5ml tube and volume adjusted to 20μl. Restriction digestion was performed at 37°C overnight. A 4% agarose gel was prepared fresh and pre-run given at 80V for 2hrs after loading the wells with 2μl of 6X loading buffer. After ascertaining that the agarose gel is free of any air bubbles or blocks, entire digestion product mixture was loaded into the wells along with loading buffer. Electrophoresis was performed for about 1 hr at a constant voltage of 80V in running buffer containing 0.5X TBE and ethidium bromide (Sigma – Aldrich Chemical Pvt Ltd., Bangalore, India). After electrophoresis each gel was visualized using a gel documentation system (Chemi Imager 5.5, Alpha Innotech Inc., USA) and restriction pattern noted. Each restriction pattern was compared to genotype and subtype specific patterns predicted by in silico digestion by the software Clone manager.