Materials and Methods
4. MATERIALS AND METHODS

4.1 STUDY MATERIALS

The materials of the study included 2523 serum samples from the different categories as enumerated in the table below:

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>NUMBER</th>
<th>SOURCE *</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sporadic AVH</td>
<td>'297'</td>
<td>GH,SMC,DDHD</td>
</tr>
<tr>
<td>2. Post-transfusion AVH</td>
<td>59</td>
<td>GH,SMC,DDHD</td>
</tr>
<tr>
<td>3. Fulminant hepatic failure (FHF)</td>
<td>77</td>
<td>GH,SMC,DDHD</td>
</tr>
<tr>
<td>4. Subacute hepatic failure (SAHF)</td>
<td>16</td>
<td>GH,SMC,DDHD</td>
</tr>
<tr>
<td>5. CLD</td>
<td>539</td>
<td>GH,SMC,DDHD,MMC</td>
</tr>
<tr>
<td>6. HCC</td>
<td>79</td>
<td>GH,SMC,DDHD</td>
</tr>
<tr>
<td>7. IVDU</td>
<td>177</td>
<td>IMH</td>
</tr>
<tr>
<td>8. CRF</td>
<td>165</td>
<td>GH, Apollo Hosp.</td>
</tr>
<tr>
<td>9. Health care workers</td>
<td>78</td>
<td>GH,SMC,PGIBMS</td>
</tr>
<tr>
<td>10. Voluntary blood donors</td>
<td>1036</td>
<td>VHS, Red Cross, GH,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MCRL.</td>
</tr>
</tbody>
</table>

* Abbreviations expanded in the text.

4.1.1 Liver disease group

Serum samples from AVH, SAHF and FHF and histopathologically-proven CLD and HCC cases were collected from patients attending the Gastroenterology unit of Government General Hospital (GH), Stanley Medical
College and Hospital (SMC), Department of Digestive Health and Diseases (DDHD), AnnaNagar Peripheral Hospital, Madras and Madurai Medical College and Hospital (MMC), Madurai and included in the study. Samples were collected over a period of 5 years (February 1992 to April 1997). Patient details like age, sex, history of jaundice/ surgery/ transfusion etc., liver function tests and virological markers were recorded in the prescribed proforma (Annexure I).

4.1.1.1 Acute Viral hepatitis (AVH)

a) Sporadic AVH

297 serum samples from AVH were collected and tested for anti-HAV IgM, HBsAg, anti-HBc IgM, anti-HCV, anti-delta and anti-HEV IgM by the commercially available ELISAs.

PCR was performed for HBV-DNA and HCV-RNA in the 59 cases negative for hepatitis A-E by the ELISA.

b) Transfusion-associated AVH

Serum samples from 78 patients who had post-transfusion hepatitis were collected and tested for viral markers. HBsAg and anti-HBc IgM testing was performed using the commercially available ELISA. Anti-HCV testing was carried out using the III Gen.ELISA and RIBA 3.0. HBV-DNA and HCV-RNA were detected by PCR.
4.1.1.2 Subacute hepatic failure and fulminant hepatic failure

Serum from 16 cases of SAHF and 77 FHF cases were collected and screened for the viral markers. Testing was performed for anti-HAV IgM, HBsAg, anti-HBc IgM, anti-HCV, anti-delta and anti-HEV IgM by the commercially available ELISAs. Anti-HCV was also tested by RIBA 3.0.

PCR was performed for HBV-DNA and HCV-RNA in the ELISA negative cases.

4.1.1.3 Chronic liver diseases

618 serum samples from histopathologically proven CLD cases (29-CPH, 38-CAH, 472-Cirrhosis and 79-HCC) were collected and tested for hepatitis viral markers.

Viral markers included HBsAg, anti-HBc IgM, anti-delta and anti-HCV were performed on the CLD samples by the commercially available ELISAs. Anti-HCV status was also tested by the RIBA.

HBV-DNA was tested by PCR and nested RT-PCR was performed for HCV-RNA.
4.1.2 High-risk groups

4.1.2.1 Chronic Renal Failure group

165 serum samples from patients with CRF undergoing haemodialysis/transfusion/transplantation were collected from patients attending the Nephrology unit of Government General Hospital and Apollo Hospitals, Madras.

Viral markers like HBsAg, anti-HBc IgM, anti-delta and anti-HCV were performed on the CLD samples by the commercially available ELISAs. Anti-HCV status was also tested by the RIBA 3.0. HBV-DNA was tested by PCR and nested RT-PCR was performed for HCV-RNA.

4.1.2.2 Intravenous drug users

Serum samples for the IVDU study were collected from 177 IVDUs at the Institute of Mental Health, Madras.

Viral markers like HBsAg, anti-HBc IgM, anti-delta and anti-HCV were performed on the CLD samples by the commercially available ELISAs. Anti-HCV status was also tested by the RIBA 3.0. HBV-DNA was tested by PCR and nested RT-PCR was performed for HCV-RNA.

4.1.2.3 Health Care Workers

Serum samples from 78 health care workers consisting of Doctors, Nurses, Technicians, Lab.attenders and students of the Government General hospital,
Stanley Medical college and hospital, Dr.ALM.PGIBMS and other private hospitals and clinics.

Viral markers like HBsAg and anti-HCV were performed on the samples by the commercially available ELISAs. Anti-HCV status was also tested by the RIBA 3.0.

HBV-DNA was tested in the HBsAg positive cases by PCR and nested RT-PCR was performed for HCV-RNA in the anti-HCV positive cases.

4.1.3. Baseline data

4.1.3.1. Healthy population

1036 Serum samples from voluntary blood donors were collected from the blood banks of Voluntary Health Services, Government General hospital, The Indian Red Cross Society, Lion’s blood bank and Madras clinical Research Laboratories, Madras.

Testing: Samples were tested for HBsAg and anti-HCV by the commercially available ELISA.

HBV-DNA was tested by PCR and nested RT-PCR was performed for HCV-RNA in all the ELISA positive cases.
4.1.3.2 Risk factors

All the HCV positive cases (positivity either by anti-HCV or HCV-RNA) formed the material for this study.

4.2 METHODOLOGIES

4.2.1 ELISA TECHNIQUES

4.2.1.1 Anti-HAV IgM: (Wellcozyme, UK)

Wellcozyme anti-HAV IgM is an enzymeimmunoassay for the detection of IgM antibody to Hepatitis A virus (anti-HAV IgM) in human serum or plasma.

Principle of the Test

To measure anti-HAV IgM, the test specimen is diluted before being incubated in a well coated with a specific mouse monoclonal anti-IgM. The well is washed to remove unbound material and the HAV antigen solution and anti-HAV conjugate are added together. The HAV antigen forms a bridge between conjugate and anti-HAV IgM captured by the coating antibody. After a second wash step, the bound conjugate is visualised using 3,3' 5,5" tetramethylbenzidine (TMB) and hydrogen peroxide which gives a yellow colour after the termination of the reaction with sulphuric acid. The presence of anti-HAV IgM in a sample is indicated by the production of the yellow cloured reaction product in the test well.
Method

Step 1: The wells were set up.

Step 2: 1/400 dilution of samples (not controls) were prepared.

Step 3: 10 μl of each Negative control (A1,B1,C1), Positive Control (D1,E1) and Samples (1/400 diluted) were added.

Step 4: 100 μl of sample diluent was added to all wells.

Step 5: Strips incubated at 37°C for 45 minutes.

Step 6: The wells were washed four times with the diluted wash buffer.

Step 7: 50 μl of hepatitis A antigen solution was added.

Step 8: 50 μl of conjugate was added.

Step 9: Incubated at 37°C for 60 minutes.

Step 10: The substrate solution was prepared 5 minutes before use.

Step 11: The wells were washed five times with the diluted wash buffer.

Step 12: 100 μl of substrate solution was added.

Step 13: Incubated at room temperature for 15 minutes in dark.

Step 14: 100 μl of the stop solution was added.

Step 15: Read at 450 nm (A 450) within 15 minutes.
Calculation of Results

1. Negative Control

The mean absorbance of the three replicates of the negative control (NCx) was calculated.

2. Positive Control

The mean absorbance of the two replicates of the positive control (PCx) was calculated.

3. Cut-off value

The cut-off value was calculated by adding one tenth of the mean PCx to the mean NCx value.

\[
\text{Cut-off} = \frac{\text{PCx}}{10} + \text{NCx}
\]

Interpretation of Results

1. Non-reactive Result

Specimens with absorbance values less than the cut-off value are presumed non-reactive (negative) for anti-HAV IgM by the Wellcozyme anti-HAV IgM test.
2. **Reactive Result**

Specimens with absorbance values equal to or greater than the cut-off value are considered reactive in the assay.

4.2.1.2 *Hepatitis B surface antigen (HBsAg)*

(Murex Diagnostics, UK)

Murex HBsAg is a rapid and sensitive enzyme immunoassay for the detection of hepatitis B surface antigen in human serum or plasma.

**Principle of the Procedure**

In the Murex HBsAg assay, microwells 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 colour which can be determined photometrically and is directly proportional to the amount of conjugate bound and hence the concentration of HBsAg in the sample.

**Procedure**

1. The conjugate was reconstituted with conjugate diluent.
2. Wells were set up.
3. 25 µl of sample diluent was added to each well.
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.

5. 50 μl of the conjugate was added to all the wells.

6. The plate was shaken for 10 seconds and incubated for 90 minutes at 37°C under humid conditions.

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

8. 100 μl of the substrate solution was added to each well.

9. Plate was covered and incubated for 30 minutes at 37°C under humid conditions in the dark. A purple colour appeared in wells containing reactive samples.

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

11. The absorbance was read at 450 nm.

Results

1. Negative Control

The mean absorbance of the replicates of the negative control were calculated.

2. Cut-off value

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

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

4.2.1.3. Anti-HBc IgM: (Wellcozyme, UK)

The IgM class antibody to hepatitis B core antigen (anti-HBc IgM) in serum or plasma was detected using the commercially available Wellcozyme kit.

Principle of the Test

In the Wellcozyme test, anti-HBc IgM is identified in the sample after a reaction with a monoclonal antibody to IgM bound to a plastic surface, followed by reaction with HBcAg conjugated to an enzyme. The bound enzyme is reacted with substrate to give a coloured product.

Procedure

1. 1 in 101 dilutions of each specimen was prepared by adding 10 μl of sample to a test tube and then adding 1 ml of sample diluent. It was mixed well.
2. 150 μl of assay buffer was pipetted into as many wells as there are samples and controls.
3. 150 μl of positive control was pipetted into 3 wells and 50 μl of negative control into 2 wells followed by 50 μl of diluted samples into the remaining wells.
4. The wells were covered with a lid and incubated in a water bath at 37°C for 30 min.
5. At the end of the incubation period the wells were washed using the wash fluid.
6. 200 µl of conjugate was added to all the wells.
7. The wells were covered with the lid and incubated in a water bath at 37°C for 30 min.
8. At the end of the incubation period, the wells are washed again.
9. 200 µl of the substrate solution was added to each well.
10. The wells were covered with the lid and incubated in at room temperature for 30 min in dark.
11. 50 µl of stop solution was added to all wells.
12. The absorbance of each well was read at 450 nm within 15 min.

Calculation of Results

1. The mean absorbance of the three positive control values and the two negative control values was calculated.
2. Cut-off = (0.4 x Mean A450 positive control) + Mean A450 Negative control.
3. Any sample which gives an A450 greater than the cut-off should be considered positive for anti-HBc IgM.
4. Any sample which gives an A450 within 10 percent of the cut-off value should be considered borderline and a repeat sample taken.
4.2.1.4. Anti-HCV ELISA: II Gen.(UBI HCV EIA, USA)

UBI HCV EIA is a qualitative enzyme immunoassay for the detection of antibodies to hepatitis C virus in human serum or plasma. It is intended as a screen for donated blood to prevent transmission of HCV to recipients of cellular blood components and as an aid in clinical diagnosis of HCV related infections.

Principle of the test

The UBI HCV EIA employs an immunosorbent, which consists of a mixture of synthetic HCV peptides which correspond to highly antigenic segments of both the structural and non-structural portions of the hepatitis C virus, bound to the microplate wells. During the course of the assay, diluent, controls and the diluted specimens are added to the microplate wells and incubated. HCV specific antibodies, if present, will bind to the immunosorbent. After a thorough washing of the wells to remove unbound antibodies and other serum components, a standardized preparation of horse-radish peroxidase conjugated goat antibodies specific for human IgG is added to each well. This enzyme-conjugate preparation is then allowed to react with antibodies which bind to the assay wells on the basis of their specificity for antigenic determinants present within the HCV immunosorbent.

After another thorough washing of the wells to remove unbound horse-radish peroxidase conjugated antibodies, a substrate solution containing hydrogen peroxide and 0-phenylene diamine (OPD) is added to each well. A
yellow-orange colour develops in proportion to the amount of HCV specific antibodies present, if any, in the serum or plasma samples tested.

**Assay Procedure**

1) 15 µl of the sample was diluted in 300 µl of specimen diluent (1:21) in the dilution plate. From this 200 µl of the diluted sample was added to the reaction microplate.

200 µl of the Blank, Non-reactive control, Weakly reactive control and Strongly reactive control was added in duplicates without diluting.

2) The plate was covered and incubated at 37°C for 15 minutes.

3) After 15 minutes the reaction plate was washed with diluted wash buffer which was prepared by mixing 1 volume of Wash buffer concentrate in 9 volumes of reagent grade water.

4) The working conjugate solution was prepared by diluting the horse-radish peroxidase conjugated goat anti-human IgG 1:101 with the conjugate diluent. 100 µl of the diluted conjugate was then added to all the wells.

5) The plate was covered and incubated for 15 minutes at 37°C.

6) The washing procedure was repeated.

7) During the incubation step the substrate solution is prepared. One OPD tablet was dissolved in 3 ml of the substrate diluent. 100 µl of this working substrate solution was added to all the wells.

8) The plate was covered and incubated for 15 minutes at 37°C.
9) 100 µl of the stop solution was added to each well of the reaction microplate.

10) The absorbance was read at 490 nm.

Calculations

The cut-off value was calculated by multiplying the absorbance of the strongly reactive control with 0.15.

\[ \text{Cut-off value} = 0.15 \times \text{SRC} \]

Interpretation of the Results

1) Specimens with absorbance values less than the cut-off value are considered not reactive by the criteria of the UBI HCV EIA and were considered negative for antibodies to HCV. Further testing was not required.

2) Specimens with absorbance values greater than or equal to the cut-off value were considered reactive. These samples (using the original samples) were retested in duplicate before final confirmation of the result.

3) Initially reactive samples which did not react in either of the repeat tests were considered negative for antibodies to HCV. Further testing was not required.

4) Specimens which have been found repeatedly reactive are interpreted to be positive for the presence of antibodies to HCV.
4.2.1.5. Anti-HCV ELISA: III Gen.(Murex VK 48)

**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 immobilised antigens. Following washing to remove unbound material, the captured anti-HCV antibodies are incubated with peroxidase conjugated monoclonal anti-human IgG. During the course of the second incubation an antigen-human 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 colour will develop in the wells which contain anti-HCV positive samples. The enzyme reaction is terminated with sulphuric acid to give an orange colour which is read photometrically. The amount of conjugate bound, and hence colour, in the wells, is directly related to the concentration of antibody in the sample.

**Procedure**

1) The conjugate was reconstituted with conjugate diluent and the substrate solution was prepared according to the manufacturer's instruction.

2) 180 µl of sample diluent was added into each well.
3) 20 μl of samples or controls was added into each well.

4) The wells were covered with the lid and incubated for 1 hour at 37°C under humid conditions.

5) At the end of the incubation period the plate was washed as described under wash procedures.

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

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

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

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

10) The wells were covered with the lid and incubated for exactly 30 minutes at 18-25°C while colour developed. A purple colour developed in wells with positive samples.

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

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

13) The mean A 450 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.

4.2.1.6. Anti-HD(Total) : (Wellcozyme, 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 the 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 visualised using TMB and hydrogen peroxide to give a yellow colour after termination of the enzymic reaction with sulphuric acid.

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

Procedure

1) The required number of wells were set up.
2) 100 μl of negative control, positive control and the samples were added into each well.
3) The wells were covered with the lid and incubated for 2 hour at 37°C under humid conditions.
4) At the end of the incubation period the plate was washed as described under wash procedures.

5) Immediately after washing the plate, 100 µl of conjugate was added to each well.

6) The wells were covered with the lid and incubated for 60 minutes at 37°C under humid conditions.

7) At the end of the incubation period the plate was washed as described under wash procedures.

8) Immediately after washing the plate, 100 µl of substrate solution was added to each well.

9) The wells were covered with the lid and incubated for exactly 30 minutes at 18-25°C while colour developed. A purple colour developed in wells with positive samples.

10) 100 µl of stop solution (2M sulphuric acid) was added to each well.

11) Within 15 minutes, the absorbance was read of each well at 450 nm using a microwell plate reader. the instrument was blanked on air.

12) The mean A 450 of the three negative control wells and of the two positive control wells was calculated.

Calculation of Cut-off value

The mean absorbance of the three negative control (NC) values and the two positive control (PC) values were calculated. The cut-off value was calculated using the following formula:

\[
\text{Cut-off value} = 0.5 \times (\text{Mean OD NC} + \text{Mean OD PC})
\]
Samples with absorbance less than or equal to the cut-off value are presumed positive for antibody to delta antigen.

4.2.1.7. Anti-HEV IgM : (Genelabs, USA)

The Genelabs diagnostics (GLD) HEV IgM ELISA is an enzyme-linked immunosorbent assay intended for the detection of IgM antibodies to Hepatitis E virus in human serum or plasma.

The wells of the polystyrene microplate strips are coated with three recombinant HEV antigens which correspond to the structural regions of the Hepatitis E virus. Human serum or plasma, diluted in diluent buffer, are incubated in these coated wells. HEV specific antibodies, if present, will bind to the solid phase HEV antigens. The wells are thoroughly washed to remove unbound materials and a mouse monoclonal anti-human IgM labelled with horseradish peroxidase is added to the wells. This labelled antibody will bind to any antigen-antibody complexes previously formed and excess unbound labelled antibodies are removed by washing. A substrate solution is then added to each well. The presence of specific antibodies is indicated by the presence of a yellow-orange colour after substrate addition.

Procedure

1. The wells were set up.
2. 200 µl of the diluent was added to all the wells.
3. 10 µl of the specimen are added to the wells which will give a final specimen dilution of 1:21. Specimens were not added to the "blanks". Instead 10 µl of diluent was added.
4. 10 μl of each of reactive control and non-reactive control was added to the assigned wells.
5. The microplate was covered with the plate cover provided and incubated for 60 minutes at 37°C in an incubator.
6. A 1:50 dilution of the conjugate with the diluent was prepared.
7. The wells were washed with the diluted wash buffer with at least 300 μl/well and aspirated immediately in the same order. This cycle was performed six times.
8. 100 μl of working conjugate was added to all wells.
9. The microplate was incubated for 30 minutes at 37°C.
10. The working substrate solution was prepared by adding one tablet to 5 ml of substrate buffer.
11. The wells were washed 5 times with the diluted wash buffer.
12. 100 μl of working substrate solution was added to each well.
13. Incubated for 15 minutes in the dark at room temperature (25±3°C).
14. 50 μl of the stop solution was added to all wells.
15. The absorbance for each well was determined at 492 nm.

Quality Control

1. The blank and non-reactive control should be assayed in duplicate and the reactive control in triplicate on each plate with each run of specimens.
2. Blank values must have an absorbance of < 0.100.
3. Non-reactive control values must have an absorbance of > 0 and < 0.100 after subtracting the blank.
4. At least 2 of 3 reactive control values must have absorbance of > 0.700 and < 1.800 after subtracting the blanks.

5. If two or more reactive control values deviate by > 30% from their respective control mean, the run is invalid and should be repeated.

6. For the assay to be valid, the difference between the mean absorbances of the reactive control and the non-reactive control (RCx-NRCx) should be 0.600 or greater. If not, technique may be suspected and the assay must be repeated. If RCx-NRCx is consistently low, deterioration of reagents may be suspected.

**Results**

The mean absorbance values of the blank controls must be subtracted from both the controls and the specimens absorbance values before interpretation of the results. The cut-off value for GLD HEV IgM ELISA is calculated as 0.400 + the Mean absorbance of the Non-reactive control.

**4.2.1.8. Anti-HIV ELISA : (Sanofi Pasteur, France)**

Genelavia Mixt is an indirect enzyme immunoassay for the detection of the various antibodies associated to the HIV 1 and/or HIV 2 in the human serum or plasma.

Genelavia is based upon the use of a solid phase coated with purified antigens (GP160 recombinant protein and peptides mimicking the immunodominant epitopes of the HIV 1 and HIV 2 envelope glycoproteins) and of peroxidase labelled anti-human IgG and IgM goat antibodies.
Procedure

1. 80 µl of the sample diluent was added to each well.
2. 20 µl of negative control, positive control, cut-off control serum and the samples were added to the assigned wells.
3. The microplate was covered with adhesive film and incubated at 40°C in a water bath for 30 min.
4. The plate was washed 3 times with the wash buffer.
5. 100 µl of conjugate was added to all the wells, sealed and incubated at 40°C for 30 min. in a water bath.
6. The plate was washed 3 times with the wash buffer.
7. 100 µl of the substrate solution was added to all the wells and incubated in the dark for 30 min. at 18-25°C.
8. 50 µl of stopping solution was added to all the wells.
9. The OD of the wells were read at 490nm and the cut-off value was calculated.

Calculation of cut-off value

The mean absorbance of the cut-off control serum (ODR4) is calculated and the cut-off value is calculated by the following formula:

\[
\text{Cut-off value} = \frac{\text{ODR4}}{10}
\]

Samples with absorbance values less than the cut-off value are considered to be negative by the Genelavia Mixt test.
4.2.2. RIBA for anti-HCV : III Gen. (RIBA 3.0, SIA)

The Chiron RIBA HCV SIA system 3.0 is an invitro qualitative enzyme immunoassay for the detection of antibody to hepatitis C virus encoded antigens/peptides (anti-HCV) in human serum plasma or plasma.

The RIBA HCV Test system 3.0 is a nitrocellulose based test. This test system utilizes three recombinant antigens and two synthetic peptide bands. The three recombinant antigens are c33c produced in E.coli, in addition to NS5 and SOD produced in Yeast. The two synthetic peptide bands contain peptides derived from the nucleocapsid (c22) and c100 region of the HCV genome.

Principle of the assay

In the RIBA HCV SIA test system 3.0, HCV recombinant antigens and peptides are immobilised as individual bands on nitrocellulose strips, the same matrix as is used in Western blots. During the incubation of the strips with serum or plasma specimens and the appropriate controls, antibodies to HCV, if present, will react to the corresponding recombinant antigen and/or peptide bands on the nitrocellulose strips. After the removal of nonspecific antibodies by aspiration and washing to remove excess conjugate, a solution containing hydrogen peroxide and 4-chloro 1-naphthol is added. Band patterns develop on each strip; the intensities of the blue-black coloured bands are proportional to the amount of specific antibodies bound to each of the HCV recombinant antigens and/or peptides on the strips. After the development of colour on the strips, the reaction is stopped by decantation and washing. The reactivity of
specimens towards each recombinant antigen and/or peptide band is determined by visually comparing the intensity of the individual band with that of the low and high IgG internal control bands included on each strip.

**Procedure**

1) The required number of strips in their respective tubes were removed from the sealed pouches and placed in the assay reack. One tube per specimen and one tube for each of the positive and negative controls are required.

2) 1 ml of the working specimen diluent was added to all the tubes. The working specimen diluent was prepared by adding 0.6 gm of Non-fat dry milk to 12 ml of the specimen diluent per 10 strips.

3) 20 μl of the specimen and the controls were added to the correspeonding labelled tube.

4) The strips were incubated at room temperature on a rocker for 4 hours.

5) The tubes were uncapped and the liquid aspirated into a waste container. 1 ml of Wash buffer was added to each tube and then the liquid was again aspirated.

6) To each tube 1 ml of the wash buffer was again added and then the liquid and the strips were poured into a wash vessel containing 30 ml of wash buffer (per vessel only 20 strips could be used).

7) The wash vessels were completely filled with the buffer and then immediately decanted making sure the strips are retained.
8) 60 ml of the wash buffer was added and immediately decanted, retaining the strips. This step was repeated once again.

9) 1 ml of conjugate per strip was added to each vessel (minimum 10 ml per wash vessel).

10) The wash vessels were rotated on a rotary shaker at 110 ± 5 rpm for 10 minutes at room temperature.

11) The conjugate was decanted and the strips were washed for 3 times with 60 ml of the wash buffer.

12) 1 ml of the working substrate per strip was added to each vessel (minimum 10 ml per wash vessel).

13) The wash vessel were kept on a rotary shaker at 110 ± 5 rpm for 15 minutes at room temperature.

14) The working Substrate was decanted and the strips were washed two times with 60 ml of distilled water.

15) Using forceps the strips were transferred to a absorbent paper and the excess water blotted. the strips were allowed to dry for 20 minutes at room temperature in the dark.

**Interpretation**

1) The identity and location of the antigens coated on the strip is as shown below. Two levels of human IgG (level I, weak positive, and level II, moderate positive) are included on each strip as internal controls. the intensity of Level I is taken as 1+ reactivity and that of Level II as 2+.
2) The HCV antibody positive control and the Negative control was examined. For the test system to be valid the following criteria was met:

a) The HCV positive control strip must show a response of 2+ or greater for all HCV antigen bands.

b) The negative control must show visibly lower response to each of the antigen bands than for the IgG level I control.

3) For a test sample to be valid, the two control levels of the human IgG on the strip must be clearly distinguishable by the eye. The response to each antigen was determined and recorded by comparing the antigen band intensity to the level I and level II IgG strip controls.

4) Interpretation of Results:

<table>
<thead>
<tr>
<th>Antigen Band pattern</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>* No bands of 1+ or greater reactivity present.</td>
<td>NON-REACTIVE</td>
</tr>
<tr>
<td>* 1+ or greater reactivity to any 2 HCV recombinant antigens.</td>
<td>REACTIVE</td>
</tr>
<tr>
<td>* Bands of 1+ or greater reactivity present but pattern does not meet criteria for REACTIVE.</td>
<td>INDETERMINATE</td>
</tr>
</tbody>
</table>
* Reactivity to SOD band alone is considered NON-REACTIVE.
* 1+ or greater SOD reactivity in addition to antigen reactivity is considered indeterminate.

4.2.3. PCR Methods

4.2.3.1. PCR for HBV-DNA

a. DNA Extraction

The method followed for the HBV-DNA extraction has been adapted from 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. 50 µl of serum is 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 is 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 min. to inactivate the protease.
4. The solution was stored frozen and 25 µl was used for PCR reaction.

b. 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 (100 pg).
<p>| | |</p>
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Antisense primer</td>
<td>10 µl (100 pg)</td>
</tr>
<tr>
<td>Taq polymerase (5U/µl)</td>
<td>0.5 µl (2.5 Units)</td>
</tr>
<tr>
<td>Water</td>
<td>58 µl</td>
</tr>
<tr>
<td>Template</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

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

c. **Thermocycling profile**

The reaction mix was subjected to the following thermocycling profile:

- 94°C for 1 minute
- 55°C for 2 minutes
- 72°C for 3 minutes

For 35 cycles.

Final extension at 72°C for 7 minutes.

At the end of 35 cycles the PCR product was separated on a 2% agarose gel and stained with ethidium bromide (0.5 µg/ml concentration). The gel was visualised under UV transilluminator and the 721 bp product was compared with molecular weight marker.

**Primers used**

Final product - 721 bp product.

**Sense primer**

5'- ATG GAG AAC ATC ACA- 3'

**Antisense primer**

5'- TTA AAT GTA TAC CCA- 3'
4.2.3.2. RT-PCR for HCV-RNA

a. RNA-Extraction

The method followed was that of Chomczynski and Sacchi (1987)-The single-step method of RNA isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform extraction.

100 µl of serum was taken and to it the following reagents were added in the same order and mixing well after each addition.

1. 0.5 ml of solution D (4M Guanidinium isothiocyanate
   25mM Sodium citrate
   0.5% Sarcosyl
   0.1 M β-mercaptoethanol)
2. 50 µl 2M Sodium acetate (pH 4.0)
3. 0.5 ml Water-saturated phenol.
4. 0.2 ml Chloroform-isoamyl alcohol (49:1)
5. After all additions, the mixture was vortexed rapidly for 10 seconds and kept on ice for 15 minutes.
6. It was then centrifuged in a microfuge at full speed and 4°C for 20 minutes.
7. The aqueous (top) phase was harvested without disturbing the protein interphase. Equal volume of isopropanol was added.
8. This mixture was then kept at -20°C for 1 hour.
9. After 1 hour, it was centrifuged for 20 minutes at 4°C in full speed.
10. The liquid phase was decanted and the pellet was dissolved in 0.3 ml solution D and 1 ml ethanol.

11. This was then stored at -20°C for 4 hours and centrifuged.

12. The pellet was resuspended in 70% ethanol and centrifuged for 10 minutes.

13. The pellet was dried in vacuo and resuspended in 10 µl DEPC-treated water. It was stored at -20°C until further use.

The Reverse transcription-polymerase chain reaction (RT-PCR) was carried out in a single tube and the reaction mix was prepared in the following manner:

b. **RT-PCR Reaction mix** was carried out as per the method of Panigrahi et al (1994).

The reaction mix was constituted in the following way:

- 10X Taq buffer: 10 µl
- 50 mM MgCl: 5 µl
- 2.5 mM dNTPs: 8 µl
- HCV Primers (10pm/µl) # 1: 5 µl
- HCV Primers (10pm/µl) # 3: 5 µl
- RNAsin (50U/µl): 1 µl
- Reverse transcriptase (200U/µl): 1 µl
- Taq polymerase (5U/µl): 0.5 µl
- Water: 60 µl
- Template: 5 µl
The template was denatured at 65°C for 10 minutes and then added to the RT-PCR mix.

c. Thermocycling

The reaction mix was thermocycled in the following profile:

- Reverse Transcription : 38°C for 1 hour
- PCR thermocycling : 95°C - 1 min.
  - 50°C - 1 min. 30 sec.
  - 73°C - 1 min. 30 sec.
- Final extension : 73°C - 5 min.

d. Nested PCR

Nested polymerase chain reaction was carried out with the inner set of primers. Additional amplification was achieved by the nested PCR under the same reaction conditions using a 10% aliquot of the RT-PCR mixture. A positive, negative and distilled water control was included in both the runs.

At the end of 35 cycles the n-PCR product was separated on a 2% agarose gel and stained with ethidium bromide. The gel was visualised under UV transilluminator and the 162 bp product was compared with molecular weight marker.

Primers used : Final product - 162 bp product.
Primers were selected from the highly conserved 5' untranslated region (UTR) of the HCV genome.

**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'

**Inner primers**

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

### 4.3 EVALUATION OF DIAGNOSTIC SYSTEMS

#### 4.3.1 Evaluation of HBV diagnostics

200 serum samples (65 AVH, 65 CLD and 70 voluntary blood donors), consisting of 112 HBsAg positive sera (35 each from AVH and CLD and 42 from blood donors) and 88 HBsAg negative sera tested by Murex HBsAg ELISA (Murex diagnostics, UK), were included in the HBsAg ELISA evaluation study. These samples were tested by 10 different ELISA kits to check the sensitivity, specificity, positive and negative predictive value (PPV and NPV) of these assays keeping the Murex HBsAg ELISA as the "Gold standard". According to the Murex catalogue GE13/14/16 pp 11, the kit has been evaluated at six European blood transfusion centers by including 12,371 routine blood donor samples. The specificity of the Murex HBsAg has been reported to be 99.98%. It has also been shown that the kit detected up to 0.5 ng/ml of HBsAg when tested at different international institutions.

All the ELISAs were based on the "sandwich" principle with minor variations in the test procedure. The test procedures, as outlined in the kit
brochures provided by the respective manufacturers were strictly adhered to. The HBsAg detectable limit of each of the assays was tested using CDC control panel sera (CDC, Atlanta, USA) with HBsAg at varying concentrations (75ng/ml, 50ng/ml, 40ng/ml, 30ng/ml, 20ng/ml, 10ng/ml, 5ng/ml, 4ng/ml, 3ng/ml, 2ng/ml, 1ng/ml, 0.9ng/ml, 0.8ng/ml, 0.7ng/ml, 0.6ng/ml, 0.5ng/ml, 0.4ng/ml, 0.3ng/ml, 0.2ng/ml and 0.1ng/ml).

4.3.2 HCV diagnostic systems

4.3.2.1 Evaluation of HCV diagnostics

213 serum samples (18-AVH, 94-Cirrhosis, 14-HCC, 45-CRF and 42-IVDU) were included in the evaluation study. 108 cases (94-Cirrhosis and 14-HCC) were screened by the II and III Gen.ELISA, RIBA 3.0 and the RT-PCR. The rest 105 cases were tested by the III Gen.ELISA, III Gen.RIBA and the RT-PCR.

The samples were tested for anti-HCV by the II generation ELISA (UBI, USA) which detects antibodies against the structural and non-structural antigens of the virus, the III generation ELISA (Murex, UK) which detects antibodies against the core, NS3, NS4 and NS5 regions of the virus and the III generation RIBA 3.0 (Chiron Corp., USA) which detects antibodies against the c100, c33, c22 and NS5 regions of the virus. RT-PCR for HCV-RNA was performed on all the samples using the nested primers from the highly conserved 5'UTR as specified under 4.2.3.
4.3.2.2 RIBA seroreactive pattern vs HCV infection status

All the 272 anti-HCV positive cases by RIBA 3.0 were included in this study and the seroreactive pattern was analysed to see whether it could indicate the stage of HCV infection and the probable genotype.

4.3.2.3 RIBA Indeterminates

All the 58 RIBA indeterminate samples from the various groups were included in this study. These samples were tested for the presence of HCV-RNA by the RT-PCR and anti-HIV 1/2 antibody by the HIV1/2 ELISA.

4.3.2.4 Value of Surrogate markers in HCV infection

All the 317 HCV positive cases (both antibody positive and RNA positive) and 915 HCV negative cases were included in this study.

ALT levels of all these cases was analysed and anti-HBc IgM testing was performed on these samples using the ELISA kits as mentioned under 4.2.1.

4.4 HCV AND AUTOANTIBODIES

A total of 200 serum samples with 50 each from HBV-positive, HCV-positive, NBNC CLD cases and voluntary blood donors were included in this study. Almost equal number of males and females were included in each CLD group (17-CPH; 23-CAH and 110-Cirrhosis).
The minimum age found among the cases included was 24 years and the maximum, 59 years (Mean 42.8 ± 2.3).

All these serum samples were tested for anti-nuclear antibodies (ANA), anti-mitochondrial antibodies (AMA) and anti-smooth muscle antibodies (ASMA) using the in-house immunofluorescence technique.

4.4.1. Immunofluorescence (IF) technique for autoantibodies

4.4.1.1. Composite block preparation

Rat liver, kidney and stomach tissues were cryo-sectioned and immediately fixed in pre-cooled acetone. These sections were stored at -50°C until tested.

4.4.1.2. IF Methodology as per Bala et al (1977)

1. The slides were immersed in the pre-cooled acetone (-20°C) and left for 20 min.
2. The slides were allowed to dry briefly at room temperature.
3. Slides were immersed for 10 min in staining jar filled up with PBS at room temperature.
4. The slides were laid flat, section-side up, in a humid chamber.
5. 50 μl of controls/test sample was added over the sections on the slide.
6. The chamber was covered and the slides were left undisturbed at room temperature for 2 hours.
7. The slides were transferred to a staining jar filled with PBS at room temperature. The PBS was replaced at least twice at 5 minutes intervals.
8. Slides were wiped dry around the sections and laid flat in the humid chamber.

9. 50 µl of diluted conjugate was pipetted over each section.

10. The chamber was covered and the slides were left undisturbed at room temperature for 2 hours.

11. Slides were washed as in step 7 and wiped dry around the sections and viewed under a Nikon-optiphot fluorescence microscope.

4.5 FOLLOW-UP

27 HBV positive and 25 HCV positive liver disease cases were followed up for virological markers for a period of 6 months to 2 years.

4.6 STATISTICAL ANALYSIS

The values in each of the study groups were analysed statistically using the Chi-square test, McNemer's test, Fisher's exact test, Chi-square trend analysis and t/modified t test.