Materials & Methods
4. MATERIALS AND METHODS

4.1 ETHICAL CLEARANCE

The study was approved by the institutional review committee of Dr. ALM.PG.IBMS, University of Madras and the study design was prepared and monitored by National Institute of Epidemiology, (ICMR), Chetpet, Chennai. Informed written consent was obtained from each antenatal woman and from the husband / relative accompanying the pregnant women to the hospital prior to their registration in the study after explaining fully about the study and possible consequences of HCV infection.

Demographic data and clinical details of the antenatal women were collected using stratified proforma developed by the National Institute of Epidemiology.

4.1.1 Subjects

The materials of the study included a total of 3115 asymptomatic healthy pregnant women attending during the period of June 2000 to September 2002 from various hospitals listed below:

- Institute of Obstetrics and Gynaecology for Women and children, Egmore, Chennai.
- Nagamani Nursing Home, Royapuram, Chennai
- Kasthurbha Gandhi Hospital Triplicane, Chennai.
BREAK-UP SOURCE OF ASYMPTOMATIC PREGNANT WOMEN
ANALYSED IN THE STUDY (n =3115)

<table>
<thead>
<tr>
<th>Year</th>
<th>Institutions</th>
<th>Socio-economic status</th>
<th>Total no screened</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000-2002</td>
<td>Institute of Obstetrics &amp; Gynaecology for women and children, Egmore.</td>
<td>Lower socio-economic status</td>
<td>2379</td>
</tr>
<tr>
<td>2000-2002</td>
<td>Nagamani Nursing Home, Royapuram.</td>
<td>Upper middle class</td>
<td>24</td>
</tr>
<tr>
<td>2000-2002</td>
<td>Kasthurbha Gandhi Hospital, Triplicane.</td>
<td>Lower socio-economic status</td>
<td>712</td>
</tr>
</tbody>
</table>

4.1.2 Registration of asymptomatic pregnant women

All asymptomatic pregnant women enrolled in our study were given 2 coloured registered forms for identification by the doctors and the staff in the hospital. One was attached to the patients case sheet, and the other was given to the patient and asked to show to the doctors at the time of admission for delivery and at the time of follow-up. The coloured forms indicate the difference immediately from normal case sheet forms for easy identification of HCV positive cases and to contact us in time for collecting samples from newborns.

4.1.3 Follow – up Sample collection from Mother and Infant

Follow up of anti-HCV positive mothers in both the groups was done at 3 monthly intervals. A detailed information was collected using the proforma at the period of antenatal check-up, at the time of delivery and on follow-up of mother and child.
5 ml of blood samples were collected from all asymptomatic antenatal women. The serum was separated and stored at \(-70^\circ\)C until tested. After delivery the mother and child pairs were followed accordingly, if the mother is positive for anti-HCV.

Anti-HCV screening for antenatal women was done by two commercially available anti-HCV ELISA kits, namely

1. Anti-HCV by Murex Diagnostics
2. Anti-HCV by Xcyton Diagnostics

The follow-up visits, were scheduled at 3 monthly intervals until the infants were 12 months old. Strict adherence to the follow-up schedule was maintained and every mother was asked to return within 3 days of the target date and those who did not, were visited at their homes by Field visitors, Microbiologist and Clinician.

4.2 METHODS

4.2.2 Demographic and clinical

Clinical status of the asymptomatic antenatal women was assessed using a stratified proforma developed by the National Institute of Epidemiology (ICMR), Chennai (Appendix I-VI).

4.2.3 Biochemical Tests

HCV positive asymptomatic antenatal women were assessed for the biochemical parameters such as serum alanine transaminase (ALT/SGOT),
serum aspartate transaminase (AST/SGPT), serum alkaline phosphatase (SAP) and serum bilirubin including fractionation (Reitman et al., 1957)

4.2.3.1 Serum Bilirubin

Principle

Bilirubin reacts with diazo reagent to form azobilirubin a purple coloured compound that can be estimated colorimetrically.

Requirements

Sulphanillic acid
HCl
Sodium nitrite 20gm/dl
Methanol
Bilirubin

Sulphanillic acid 1 gm/dl (diazoblank)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulphanillic acid</td>
<td>10gm/500mDW</td>
</tr>
<tr>
<td>Conc. HCl</td>
<td>20ml</td>
</tr>
<tr>
<td>Make up to 1000ml</td>
<td></td>
</tr>
</tbody>
</table>

Diazoreagent

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazoblank</td>
<td>10ml</td>
</tr>
<tr>
<td>0.5 gm/dl sodium nitrite</td>
<td>0.3ml</td>
</tr>
</tbody>
</table>

Bilirubin Standard

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin</td>
<td>10mg</td>
</tr>
<tr>
<td>Chloroform</td>
<td>100ml</td>
</tr>
</tbody>
</table>

Test Procedure
Total bilirubin and direct bilirubin were estimated according to the procedure given below.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Tube 1</th>
<th>Tube 2</th>
<th>Tube 3</th>
<th>Tube 4</th>
<th>Blank</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>D.H₂O</td>
<td>6.2</td>
<td>6.2</td>
<td>2.7</td>
<td>2.7</td>
<td>2.7</td>
<td>-</td>
</tr>
<tr>
<td>Serum</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bilirubin std</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.7</td>
</tr>
<tr>
<td>Diazo blank</td>
<td>-</td>
<td>0.7</td>
<td>-</td>
<td>0.7</td>
<td>-</td>
<td>0.7</td>
</tr>
<tr>
<td>Diazo reagent</td>
<td>-</td>
<td>0.7</td>
<td>-</td>
<td>0.7</td>
<td>-</td>
<td>0.7</td>
</tr>
<tr>
<td>Methanol</td>
<td>-</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>13</td>
</tr>
</tbody>
</table>

The contents in the tubes were mixed and incubated for 10 minutes. The OD readings were taken at 540 nm.

**Calculation**

\[
\text{Direct bilirubin} = \frac{\text{OD} \ (T_2) - \text{OD} \ (T_1)}{\text{OD} \ (\text{Std}) - \text{OD} \ (\text{Blank})} \times 10 \times 10.5 \text{ mg/dl}
\]

\[
\text{Total bilirubin} = \frac{\text{OD} \ (T_3) - \text{OD} \ (T_1)}{\text{OD} \ (\text{Std}) - \text{OD} \ (\text{Blank})}
\]

**Interpretation**

Normal is less than 1 mg/dl.
4.2.3.2 Determination of Aspartate transaminase (ALT/SGOT), Alanine transaminase (AST/SGPT) and Serum alkaline phosphatase (SAP)

Principle

The enzyme AST takes part in the following reaction.

\[
\text{AST} \quad \text{Aspartic acid} + \alpha - \text{keto glutaric acid} \longrightarrow \text{glutamic acid} + \text{oxaloacetic acid.}
\]

The oxaloacetic acid is converted into pyruvic acid and the pyruvate is made to react with dinitro phenyl hydrazine. The hydrazine formed is coupled to sodium hydroxide to give brown colour and read colorimetrically.

The enzyme ALT takes part in the following reactions.

\[
\text{ALT} \quad \text{Alanine} + \alpha - \text{keto glutaric acid} \longrightarrow \text{glutamic acid} + \text{pyruvic acid.}
\]

Requirements

- dl - Aspartic acid AR
- dl - Alanine AR
- \(\alpha - \text{keto glutaric acid}\) AR
- Pyruvic acid AR (sodium salt)
- Sodium hydroxide AR
- Disodium hydrogen phosphate
- Dinitrophenyl hydrazine AR
- Chloroform AR
- Potassium hydrogen phosphate
- Potassium dihydrogen phosphate AK
- Phosphate buffer pH 7.4
**AST buffered substrate**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>dl Aspartic acid</td>
<td>13.3 g</td>
</tr>
<tr>
<td>IN NaOH</td>
<td>90 ml</td>
</tr>
<tr>
<td>α - ketoglutaric acid</td>
<td>0.146 g</td>
</tr>
<tr>
<td>IN NaOH</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

Adjust pH to 7.4 and make up to 500 ml with phosphate buffer.

**ALT – buffered substrate**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>200 mM</td>
</tr>
<tr>
<td>Alanine</td>
<td>9 g</td>
</tr>
<tr>
<td>D.H₂O</td>
<td>90 ml</td>
</tr>
<tr>
<td>IN NaOH</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>α - ketoglutaric acid</td>
<td>0.146 g</td>
</tr>
<tr>
<td>IN NaOH</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

Adjust pH to 7.4 and make up to 500 ml with phosphate buffer.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dinitrophenyl hydrazine</td>
<td>99 mg</td>
</tr>
<tr>
<td>Conc. HCl</td>
<td>50 ml</td>
</tr>
<tr>
<td>D.H₂O</td>
<td>500 ml</td>
</tr>
<tr>
<td>0.4 N NaOH</td>
<td></td>
</tr>
<tr>
<td>NaOH</td>
<td>15 g</td>
</tr>
<tr>
<td>D.H₂O</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Standardize using 0.1 M potassium hydrogen phthalate.

**Stock pyruvate standard 20 mM**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium pyruvate</td>
<td>20 mg</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td>100 ml</td>
</tr>
</tbody>
</table>
Working pyruvate is prepared by diluting the stock 1/10 with phosphate buffer.

**Calibration curve for AST and ALT**

<table>
<thead>
<tr>
<th>Tube No</th>
<th>Pyruvate working activity (ml)</th>
<th>AST Substrate (ml)</th>
<th>ALT Substrate (ml)</th>
<th>Water (ml)</th>
<th>AST in IU</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.2</td>
<td>0.0</td>
</tr>
<tr>
<td>2.</td>
<td>0.1</td>
<td>0.1</td>
<td>0.9</td>
<td>0.2</td>
<td>16.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>33.3</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>0.2</td>
<td>0.8</td>
<td>0.8</td>
<td>0.2</td>
<td>33.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>66.7</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>0.3</td>
<td>0.7</td>
<td>0.7</td>
<td>0.2</td>
<td>50.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>0.4</td>
<td>0.6</td>
<td>0.6</td>
<td>0.2</td>
<td>66.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>133.0</td>
<td></td>
</tr>
</tbody>
</table>

To all the above tubes, 1ml of dinitrophenyl hydrazine was added and left for 20 min at RT. 10ml of 0.4 N NaOH was added and incubated for 10 minutes.

Readings were taken at 505 nm using water as blank and the graph was plotted.
Test Procedure

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank (ml)</th>
<th>AST</th>
<th>Blank (ml)</th>
<th>ALT</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST substrate</td>
<td>0.5</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ALT substrate</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Keep in 37°C water bath for 2-3 minutes

Serum

The mixture was incubated for 60 minutes for AST and 30 minutes for ALT. 0.5 ml of dinitrophenyl hydrazine was added, mixed well and incubated for 20 minutes at RT. Then, 5 ml of 0.4 N NaOH was added and left for 10 min. Readings were taken against water blank at 505 nm the values of AST and ALT read from the graph.

Interpretation

Normal values
AST 2-40 IU
ALT 2-35 IU

Assay of alkaline phosphatase

The activity of alkaline phosphatase was determined as follows:

Reagents

1. 0.1 M Carbonate buffer, pH 10.0
2. 0.1 M Disodium phenyl phosphate
3. 0.1 M Magnesium chloride
4. 15% Sodium carbonate
5. Folin's phenol reagent (commercial reagent, 1:2 dilution)
6. Phenol
The reaction mixture containing 1.5 ml of carbonate buffer, 1 ml of disodium phenyl phosphate, 0.1 ml of magnesium chloride and 0.1 ml of serum was incubated at 37°C for 15 min. The reaction was arrested by the addition of Folin's phenol reagent. Control tubes were also incubated in the same way but the serum was added after the addition of Folin's phenol reagent. Then 1 ml of sodium carbonate was added. After 10 min the colour developed was read at 640 nm.

The activity of alkaline phosphatase was expressed as IU/ml.

4.2.4 Viral Serology

4.2.4.1 Hepatitis B surface antigen (HBsAg)

HBsAg is a rapid and sensitive enzyme immunoassay for the detection of hepatitis B surface antigen in human serum or plasma using the ELISA kit manufactured by Wellcozyme, Murex Diagnostics, UK.

Principle of the Procedure

In the Murex 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 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

STEP 1: The conjugate was reconstituted with conjugate diluent.

STEP 2: Wells were set up.

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

STEP 4: 75µl 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: 50µl of the conjugate was added to all wells.

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

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

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

STEP 9: Plate was covered and incubated for 30 minutes at 37°C.

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

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

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.
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',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide. A purple color will develop in the wells, which contain anti-HCV positive samples. The enzyme reaction is stopped with sulphuric acid.

Procedure

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

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

STEP 3: 20μl of samples of 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, 100μl 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, 100µl of substrate solution was added to each well.

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

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

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

STEP 13: The mean of the three negative control wells and 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.

4.2.4.3 Anti-HCV ELISA – HEP-Chex C (Xcyton Diagnostics ltd)

Principle

HEP-Chex C uses as antigens, synthetic peptides that represent the immunodominant epitopes of structural and non-structural proteins of HCV. These peptides are coated onto microtiter wells. Diluted serum/plasma is
reacted with the peptides in the wells. If the corresponding specific antibodies are present in the sample, they bind to these peptides.

Unbound excess serum is washed off and wells are incubated with a rabbit antihuman IgG-horse radish peroxidase conjugate. This conjugate binds to the antigen-antibody complex, if any, in the well. A substrate is added to the well after washing off the excess conjugate. A colour reaction occurs. After 10 minutes this reaction is stopped by the addition of stop solution and the intensity of the colour is read at 450 nm using an ELISA reader.

Sample dilution

1. Take a 96 well dilution plate and pipette 250μl of sample diluent into each well.
2. Pipette 5μl of negative control serum into B1, C1 and D1.
3. Pipette 5μl of positive control serum into E1 and F1.
4. Pipette 5μl of each test sample into each well and mark the sample number of the corresponding well on the template card.
5. Mix the sample thoroughly in the dilution plate.

Procedure

STEP 1 : Remove reagent bottles from the kit and bring them to room temperature (15-30°C) approximately 30 minutes prior to the beginning of the procedure. Invert reagents gently several times.

STEP 2 : Whenever each assay, apply one substrate blank, three negative controls and two positive controls in each plate. In
addition to above control number, determine the number of test specimens and assemble the strips into the holder as well. Unused wells should be stored at 2-8°C in the supplied aluminum foil pouch with desiccant, tightly sealed.

**STEP 3**: Arrange the assay control wells so that well 1A is substrate blank. The well site for three negative controls and two positive controls is followed by the substrate blank (1A).

**STEP 4**: Dispense 100μl of each of the pre-diluted samples, controls and blank from the dilution plate to the corresponding numbered wells in the ELISA plate.

**STEP 5**: After mixing gently, cover the microwell strip holder with a plate sealer and incubate at room temperature for 30 minutes.

**STEP 6**: After incubation, remove the plate sealer in the microwell strip holder and wash 5 times with the 1X wash buffer using washer device. As the washing method, first step is aspirating contents of all wells. Next step involves filling completely with 1X wash buffer and then aspirating. After washing 5 times, completely aspirate wells and invert the plate and firmly tap on a clean paper towel to remove excess wash buffer.

**STEP 7**: Dispense 100μl of conjugate solution to all the well.

**STEP 8**: Cover the microwell strip holder with a plate sealer and incubate at room temperature for 30 minutes. Prepare substrate solution about 10 minutes prior to the end of this reaction.

**STEP 9**: Wash the wells as described in STEP 6.

**STEP 10**: Dispense 100μl of substrate solution to all wells, including substrate blank (1A).

**STEP 11**: Incubate at 15 to 30°C in the dark for 10 minutes.
STEP 12: Dispense 50μl of 4N sulphuric acid to all wells,
STEP 13: After removing the moisture of bottom of the microwell strips, read the well at a wavelength of 450nm using ELISA reader. For dual wavelength reader, set the reference wavelength at 630nm. Blank the reader on well 1A.
STEP 14: 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 0.15 to the mean of the negative control replicates.

4.2.5 RT-PCR FOR HCV RNA

4.2.5.1 RNA-Extraction from serum

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 Guanidium isothiocyanate, 25mM Sodium citrate, 0.5% Sarcosyl, 0.1 M B-mercaptoethanol)
2. 50 μl of 2 M Sodium acetate (pH4.0)
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 at 4°C for 20 minutes.

7. The aqueous 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 at 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 and was stored at -20°C until further use.

4.2.5.2 RT-PCR Reaction Mixture.

The reaction mix was constituted in the following way:

- 10 X Taq buffer: 10 µl
- 50 mM MgCl₂: 5 µl
- 2.5 mM primers (10pm/ µl): 8 µl
- HCV Primers (10pm/ µl) # 1: 5 µl
- HCV Primers (10pm/ µl) # 3: 5 µl
- RNAsin (50U/ µl): 1 µl
- Reverse transcriptase (200 U / µl): 1 µl
- Taq polymerase (5U/ µl): 0.5 µl
- Water: 59.5 µl
- Template: 5 µl
The template was denatured at 65°C for 10 minutes and then added to the RT-PCR mix.

4.2.5.3 Thermocycling

The reaction mix was kept at 38°C for 1 hour for reverse transcription. Then it was subjected to the following 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 a final extension of 5 min at 73°C (PTC-100, MJ Research, Watertown, MA, USA).

4.2.5.4 Nested PCR

Nested polymerase chain reaction was carried out with an 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 stained with ethidium bromide. The gel was visualized under UV transilluminator and the 162 bp product was compared with molecular weight marker (100bp ladder, MBI Fermentas, Amherst, NY, USA). Primers used were:

**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 TTA GTA TGA GTG TC-3'
HCV RT-PCR was standardized "in house" as per methods of Panigrahi et al., (1994).

4.3 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 PCR assays.

4.4 HCV GENOTYPING

4.4.1 INNO-LIPA™ HCV II Amplification

The reagents are used to reverse transcriptase and nucleic acid amplification reaction of the 5' untranslated region (UR) of the hepatitis C virus (HCV). The existence of variations in the 5' UR of the different HCV genotypes enables the use of these sequences to determine 6 HCV genotypes and their subtypes. This kit makes use of biotinylated oligonucleotide primers to generate biotinylated amplified target material, to be used in the INNO-LiPA HCV II test for genotyping the HCV virus.
The purified RNA together with random primers is denatured and mixed with human placental ribonuclease inhibitor, deoxynucleotide 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 based on the 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) amplify the 5' UR 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, and 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 the exception that the cDNA is replaced by 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.

4.4.1.1 Sample preparation

1. 50 μl of serum was added to 150 μl of TRizol LS reagent. Cells were analysed 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 15 minutes at room temperature.
5. Centrifuged for 15 minutes at max. 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 max 12000 g in an eppendorf centrifuge at 4°C.
9. The supernatant was removed, and 200 μl of ice cold 75% ethanol was added then centrifuged at max 7500 g for 5 minutes.
10. The supernatant was removed and the RNA pellet was briefly dried and stored at −70°C until use.

4.4.1.2 cDNA synthesis

1. The reaction mixture was prepared as follows,
   10 μl of DEPC treated water.
2 μl of 150 ng / μl random primers pd (N)₈
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 procedure.
   25 U HPRI (human placental ribonuclease inhibitor)
   4 μl of 5x AMV-RT buffer
   1 μl of 10 mM dNTPs
   8 U of AMV-RT
   Final 8 μl reaction volume was adjusted with H₂O
   8 μl of cDNA reaction mixture was added with the 12 μl of RNA / random primers.

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

6. The converted cDNA was frozen at −20°C until tested for the first round DNA PCR.

4.4.1.3 First round amplification:

1. Master Mix was prepared as per the instruction manual
   4.5 μl 10X Taq buffer without MgCl₂
   10 μl outer HCV primers
   0.75 μl of 10 mM dNTPs
   1 U Taq polymerase
   1.5 mM MgCl₂

2. 0.5 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 the 2% gel

4.4.1.4 Second (nested) amplification round:

1. Master Mix was prepared as per the instruction manual
   5 μl 10X Taq buffer without MgCl2
   10 μl outer HCV primers
   1 μl of 10 mM dNTPs
   1 U Taq polymerase
   3 μl of 25 mM MgCl2

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) 94°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 the 2% gel

5. After verification the products were subjected for the INNO-LiPA HCV II test.
4.4.1.5 Quality control

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

4.4.2 Gel extraction and purification

Products of positive PCR reaction were purified using QIA Quick gel extraction kit (QIAGEN, USA).

Principle

The QIA Quick system combines the convenience of spin – column technology with the 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 water.

Procedure

1. The DNA fragment was excised from the agarose gel with a clean, 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) was added.
3. The mixture was incubated at 50°C for 10 min (or until the gel slice has completely dissolved). The tube was mixed by vortexing the tube every 2-3 min during the incubation.

4. After the sliced gel has dissolved completely, checked that the color of the mixture was yellow (similar to Buffer QG without dissolved agarose).

5. 1 gel volume of isopropanol to the sample was added and mixed thoroughly.

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

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

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

9. 0.5 ml of Buffer QG was added to QIAquick column and centrifuged for 1 min.

10. To wash, 0.75 ml of Buffer PE was added to QIAquick column and centrifuged for 1 min.

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

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

13. 50 µl of Buffer EB (10 mM Tris.HCl, pH 8.5) or water was added to the center of the QIAquick membrane and centrifuged the column for 1 min at 12000 rpm to elute DNA. Alternatively, for increased DNA concentration, 30 µl of elution buffer was added to the center of the QIAquick membrane, let the column stand for 1 min, and then the column was centrifuged for 1 min.
4.4.3 INNOLiPA HCV II (Genotyping kit for the hepatitis C virus)

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 the 6 major HCV types and their most common subtypes.

**Test principle**

The INNO-LiPA HCV II allows an easy and fast determination of 6 HCV genotypes and their subtypes. 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-labelled 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 (or line) that gives a perfect sequence match, allowing stringent discrimination at the subtype level. Such a high specificity can be obtained when very stringent hybridization conditions are used (50°C±0.5°C).

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 or positive line will occur only when there is a perfect match between the probe and the biotinylated PCR products.
The genotype can be deduced 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 of 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.

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 number 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 2ml to the denatured 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 were completely submerged in the solution.

8. All the trays were kept in the shaking water bath (at 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. 2 ml of prewarmed ready for use stringent wash solution (SW) were added into each trough and were 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 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 + 0.5°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. (10 minutes before the conjugate incubation, the substrate was prepared according to the instruction manual)

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. 2ml 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 each.

17. Finally by using tweezers, all the strips were removed and were kept on an absorbant 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.

Collect all line numbers, which are positive on the INNO-LiPA HCV II strip and deduce the genotype by using the INNO-LiPA HCV II interpretation chart.
4.5  SEQUENCING METHOD

4.5.1  HCV Cloning

Sequencing by cloning was done to confirm the Genotypes present in the mother and baby paired samples. The INNOLiPA Amplified products were cloned with the pMOS Blue vector (pMOS cloning kit for sequencing PCR product, Amersham, Life Sciences).

Procedure

1. Amplified products were ligated (Ligation mix & competent cells).
2. Plated onto LB plates containing IPTG (Iso propyl thio β- d galactoside) for transformation.
3. Blue colonies were picked from the plates and then inoculated into LB broth and kept for overnight incubation.
4. After incubation, the broth containing the transformed colonies were subjected for plasmid extraction.

4.5.1.1  Plasmid Extraction

Plasmid extraction was done as per the methods of Maniatis et al., 1989.

1. The broth containing transformed colonies were spun at 13,000 rpm for 10 minutes at 4°C.
2. The supernatant was removed and the pellet was air dried for 10-15 minutes.
3. 100 μl of solution I (ice cold) was added and vortexed.
4. 150 μl of solution II was immediately added and shaken thoroughly by repeated inversions.
5. 150 μl of solution III was then added and shaken thoroughly.
6. Centrifugation at 13,000 rpm for 20 minutes at room temperature was done.

7. The aqueous phase was collected and an equal volume of isopropanol was added and kept at -20°C for 10-15 minutes for precipitation.

8. Spun at 12,000 rpm for 10-15 minutes at 4°C

9. The pellet was collected and washed with 70% ethanol.

10. The pellet was redissolved in 20 μl of RNase free water.

11. The plasmid product was electrophoresed on a 2% agarose gel and stained with ethidium bromide (0.5 μg/ml concentration).

12. The gel was visualised under UV transillumination.

Note:

A. Solution I

<table>
<thead>
<tr>
<th>Required concentration</th>
<th>Volume needed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(200 ml)</td>
</tr>
<tr>
<td>1. Glucose 50 mM</td>
<td>0.2 gm</td>
</tr>
<tr>
<td>2. 1M Tris-HCl (pH-8)</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>3. 0.5M EDTA (pH-8)</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>4. RNase free water</td>
<td>50.0 ml</td>
</tr>
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</table>

B. Solution II

<table>
<thead>
<tr>
<th>Required concentration</th>
<th>Volume needed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(100 ml)</td>
</tr>
<tr>
<td>1. 10N NaOH 0.2N</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>2. 20% SDS 1%</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>3. RNase free water</td>
<td>93.0 ml</td>
</tr>
</tbody>
</table>

76
C. Solution III

<table>
<thead>
<tr>
<th>Required concentration</th>
<th>Volume needed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 5M potassium acetate, 0.2N</td>
<td>120.0 ml (200 ml)</td>
</tr>
<tr>
<td>(pH-5.5)</td>
<td></td>
</tr>
<tr>
<td>2. Glacial acetic acid</td>
<td>23.0 ml</td>
</tr>
<tr>
<td>3. RNase free water</td>
<td>57.0 ml</td>
</tr>
<tr>
<td></td>
<td>200.0 ml</td>
</tr>
</tbody>
</table>

4.5.2 Sequencing the cloned product

4.5.2.1 Reaction mix

A master mix was prepared in a 1.5 ml tube and then equally dispensed into MicroAmp 96 well plate.

<table>
<thead>
<tr>
<th>PCR reaction mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Requirement</strong></td>
</tr>
<tr>
<td><strong>Volume (μl)</strong></td>
</tr>
<tr>
<td>Big Dye™</td>
</tr>
<tr>
<td>Primer F</td>
</tr>
<tr>
<td>DDW</td>
</tr>
<tr>
<td>Template (10ng/μl)</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Thermocycling Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Steps</strong></td>
</tr>
<tr>
<td><strong>Conditions</strong></td>
</tr>
<tr>
<td>1</td>
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<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
</tbody>
</table>
The PCR of the MicroAmp plates containing the reaction mixture was carried out in the GeneAmp 9600 thermalcycler (Perkin-Elmer). The PCR was carried for 3 hours, after that the plate was processed.

4.5.2.2 Processing

1. 3 ml absolute alcohol was added to 120 µl of 3N sodium acetate in a fresh tube. 25 µl of the above mixture was added in each well of the plate.
2. The plate was centrifuged at 4000 rpm for 25 min.
3. The plate was inverted and supernatent removed.
4. 100 µl of fresh 70% ethanol was added to each well and centrifuged at 4000 rpm for 15 min.
5. Again the plate was inverted and alcohol was removed and spun for few seconds at 500 rpm. The plate was properly covered with foil.
6. 10 µl of Big Dye™ formamide was added to all the wells at the time of sequencing.
7. The sample plates was kept and run in the ABI prism 3700 DNA Analyzer (for sequencing).

4.6 COMPUTER ASSISTED SEQUENCE ANALYSIS

- Nucleotide sequences obtained were initially analysed using "Gene auto assembler software".
- The mother/child pairs sequenced were then compared with the BLAST tool programme in the EMBL database. (European Molecular Biology Laboratories).
The aligned sequence data were later analysed using the following multiple sequence alignment softwares namely, *Clustal W*, (EMBL – EBI).
COLLECTION OF DEMOGRAPHIC DATA AND CLINICAL STATUS OF THE ASYMPTOMATIC PREGNANT WOMEN.

BLOOD SAMPLES TAKEN FROM ASYMPTOMATIC PREGNANT WOMEN FOR SCREENING
BLOOD SAMPLES TAKEN FROM BABIES BORN TO HCV POSITIVE MOTHERS
THE BABIES WEIGHT WERE TAKEN DURING THE STUDY PERIOD.

NATIONAL INSTITUTE OF EPIDEMIOLOGY (ICMR) PROVIDED VEHICLE FOR FIELD VISITS.
CLINICAL ASSESSMENT AND FOLLOW-UP CARE BY THE GENERAL PEDIATRICIAN FOR BABIES BORN TO HCV POSITIVE MOTHERS