Chapter 4

MATERIALS AND METHODS
4. MATERIALS AND METHODS:

4.1. & 4.2. Epidemiological pattern of chronic Hepatitis B virus infection and chronic hepatitis c virus infection in the adult and school children populations.

Design:

Proportionate to population size based community cluster survey. The basic unit of cluster is households in the target population.

Population:

Adults aged 15-45 years living in the target households. The study was conducted representing the entire state of Tamilnadu. The sample was stratified on the basis of three regions from Tamilnadu. Administrative districts from these three regions were listed and three districts were selected randomly. Thirty random clusters of urban and rural areas were made from the census data from these districts. Fifteen households were randomly selected from each of these households. Both men and women in the sexually active age group of 15 to 45 years residing in these houses were eligible for screening in the study.

Sample size:

The prevalence of HBsAg in the community, from literature review was thought to be about 5%. In order to estimate this with 95% CI and with the
Map of Tamilnadu showing the three districts where the study was conducted

Districts of Tamilnadu

- State Capital
- State Boundary
- District Boundary
The precision of 2%, the sample size needed was nearly 460. The number needed to be screened adjusting for drop out rate of 10% was about 600. Therefore the sample size needed for 3 districts was about 2000. These 2000 adults were from 90 clusters (village or ward) from 45 rural and 45 urban camps of three districts, Thanjavur, Dindigul and Ramanathapuram. Each cluster provided 15 households with an average of 1.5 adults per household. From every randomly selected individual, a sample of blood was collected to test for the presence of HBsAg, anti HBs and anti HCV.

<table>
<thead>
<tr>
<th>HBV Markers</th>
<th>No. of samples tested</th>
<th>Kit used</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg</td>
<td>1856</td>
<td>Hepanostika, uniform II, Netherlands</td>
</tr>
<tr>
<td>Anti HBs</td>
<td>1853</td>
<td>Hepanostika Anti HBs, Netherlands</td>
</tr>
<tr>
<td>Anti HCV</td>
<td>1851</td>
<td>UBI Diagnostics, USA.</td>
</tr>
</tbody>
</table>

Collection, Storage and Transport of Clinical Specimens

For laboratory diagnosis of Hepatitis B (HBsAg, anti HBs) and anti HCV, 5 to 10 ml of blood was aseptically collected using vacutainer system and serum was separated. The plasma/serum samples in storage vials were sealed by parafilm and aluminium tin foil. They were put into plastic bags (Ziploc) tied firmly & suspended in Liquid Nitrogen cylinders. After proper sealing, they were transported to Chennai by rail or road along with a courier. On receipt at Dept. of
Plate 2  :  Research team starting from MMHRC, to the campsite

Plate 3  :  Campsite: Enrolment of participants for the study
Microbiology, Dr. ALM PGIBMS, the specimens were checked for leakage etc. and stored frozen at -20°C until tested.

856 samples were collected from the different schools in Chennai as mentioned below:

<table>
<thead>
<tr>
<th>School</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sri Venkatramana Primary School, Abhirampuram, Chennai</td>
<td>171</td>
</tr>
<tr>
<td>Reserve Bank of India Hr. Sec. School, Besant Nagar, Chennai</td>
<td>155</td>
</tr>
<tr>
<td>Jaigopal Garodia Hindu Vidyalaya Hr. Sec. School, West Mambalam, Chennai</td>
<td>415</td>
</tr>
<tr>
<td>Dried blood spots (Jai Gopal Garodia Hindu Vidyalaya, Hr. Sec. School, W. Mambalam Chennai)</td>
<td>115</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>856</strong></td>
</tr>
</tbody>
</table>

**Procedure for collection of Dried Blood spots:**

- Whatman's filter paper cut into rectangular strips (1.5"x3")
- Prick was made on index finger of subject with sterile lancet
- Blood collected (Diameter of 1")
- Filter paper dried, rolled and put into a serum vial with Code No. of patient
- Stored at 4°C until further processed
- 200 µl of PBS → Soaked for 10 min at RT
- Centrifuged at 2000 rpm for 5 min
- Eluate Collected and Tested.
4.3. Characterisation of persons with chronic hepatitis B virus infection:

558 persons with chronic Hepatitis B virus infection coming to the Dept. of Microbiology, Dr. ALM PGIBMS, Taramani, Chennai 600 113 referred by various Government and private blood banks, Government and Private hospitals, private practitioners in and around Chennai, and also from many parts of India from Dec.95 to Jan.2000 were studied for their HBV marker profile, namely HBsAg, quantitative HBsAg estimation, HBeAg/anti HBe, anti HBc (Total), anti HCV, anti HBs and HBV DNA by PCR.

Samples:

5-10 ml of blood was collected by venepuncture using sterile disposable needle and syringe, the serum was centrifuged and separated and stored at -20 °C until tested.

4.4. Family clustering studies of Hepatitis B virus infection.

264 Household members from 81 (indexed carrier) HBsAg positive carriers and 102 household members from 28 (index carrier negative) families
Plate 4: Medical examination by Doctors

Plate 5: Filling up of patient’s proforma
who served as controls were analysed for HBV markers comprising of HBsAg, anti HBs and anti HBc (Total) by ELISA.

4.5. Purification of Hepatitis B surface antigen (By Mishiro et al., 1980 with modifications)

I. Buffers and solutions:

a. Tris Buffer (50 mM Tris-HCl, pH = 7.5)
   Dissolve 121 g Tris in 1 lt. water, adjust pH to 7.5 with concentrated HCl.
   Dilute 1:20 before use.

b. KBr solutions:
   - Density 1.28 g/ml: Dissolve 363.3 g KBr in 620 ml Tris Buffer
   - Density 1.30 g/ml: Dissolve 88.62 g KBr in 200 ml Tris Buffer
   - Density 1.32 g/ml: Dissolve 112.5 g KBr in 200 ml Tris Buffer

c. Sucrose solutions
   - Stock solutions (66%): Dissolve 855 g sucrose in 450 ml Tris Buffer.
   - 15% Sucrose: Dilute 35 ml stock in 165 ml Tris
   - 30% Sucrose: Dilute 79 ml stock in 121 ml Tris
   - 40% Sucrose: Dilute 166.5 ml stock in 133.5 ml Tris.

d. Phosphate Buffered Saline PBS
   Dissolve 4.29 g Na₂HPO₄. 7 H₂O; 0.55 g NaH₂PO₄·H₂O and 17.4 g NaCl in 2 lt. water (10 mM Phosphate, 150 mM NaCl, pH=7.2)

e. Saline solution:
e. Saline solution:

Dissolve 9.9 g NaCl in 1 lt. water (150 mM NaCl)

f. All solutions for the gradients should be chilled to 4 °C and degassed.

II. Equipment required:

High Speed centrifuge.

Ultracentrifuge.

Beckman Ti 15 zonal rotor with Reorienting Gradient core.

Fraction collector and absorbance monitor.

Ultrafiltration cell (200 ml).

Amicon YM 100 and XM 300 membranes.

Nitrogen supply.

Refractometer.

Autoclave.

Spectrophotometer.

Equipment for EIA.

Purification method:

HBsAg is purified as per the method of Mishiro et al., (1980) with modifications. Gloves, laboratory coat and goggles should be worn to prevent accidental exposure to infectious virus. All discarded material and products should be autoclaved at 121 °C for 1 hour.
1. Choose a high HBsAg titre plasma (at least $10^5$ as determined by ELISA). Start with 1- 1.2 lts. Heat inactivate 1 hr at 60°C for HIV.

2. Reconstitute 5000 units of lyophilized thrombin by addition of 5 ml of saline and 20 µl of 1 M CaCl₂. Add 1000 units of thrombin for each 250 ml of plasma, mix and let stand for 2-3 hr at 4°C. Centrifuge at 5000 rpm 10 min at 4°C. Filter through cheesecloth.

3. Measure the volume of serum and bring it to 30% of Ammonium sulphate saturation by gentle addition of solid salt. (176 g per liter of serum) at room temperature with constant stirring. Stir for 30 min and centrifuge at 5000 g, 20 min at 4°C. Discard the pellet. Measure the volume of supernatant and bring it to 50% of ammonium sulphate saturation by addition of solid salt. (127 g per liter). Stir for 30 min and centrifuge in the same way. Discard the supernatant and re-suspend the pellet in the minimum volume of Tris Buffer. Dialyze against the same buffer.

4. Measure the volume of the dialysed sample that should be lesser than 220 ml and bring it to a density of 1.30 g/ml by addition of solid KBr. The amount of KBr that should be added to the sample is calculated from the following equation

5. Weight of KBr (g) = volume of sample (ml) x 34/66

Test that the density of sample is 1.30. At this point, the volume of the sample should be lesser or equal to 270 ml. Adjust the volume to 250 by addition of KBr solution (density 1.30 g/ml).
Plate 6  :  Laboratory setup at Campsite

Plate 7  :  Collection of Blood sample from participant
6. Prepare the ultracentrifuge and the zonal rotor with RG core following the instruction manual.

7. Load the rotor from the edge port with the following solutions at a flow rate 1500 ml/hr.

50 mM Tris buffer : 240 ml.
KBr (d = 1.28 g / ml) : 440 ml.
Sample (d = 1.3 g / ml) : 660 ml.
KBr (d = 1.32 g / ml) : \( \frac{60 + \text{ml}}{1400 \text{ml}} \)

Spin at 29000 rpm for 19 hr at 10 °C. Add until Tris emerges from centre port.

After centrifugation unload the rotor by pushing from the center port with cold distilled water (flow rate 155 ml/hr). Collect fractions of 18 ml and detect HBsAg by ELISA. Use samples dilute 1/500 and decrease incubation times as follows:

Sample incubation \( \text{30 min} \)
Conjugate incubation \( \text{15 min} \)
Substrate incubation \( \text{5 min} \)

Pool the fractions containing HBsAg.

8. Measure the refraction index of the sample and calculate its density from the following equation.

\[
\text{Density} = (6.4773 \times \text{RI}) - 7.6431
\]
Bring the sample to a density of 1.30 g/ml by addition of solid KBr. The amount of salt that should be added to the sample is calculated from the following equation:

\[
\frac{34}{66} = \frac{D \times V \times X}{W} - p
\]

Where:
- \( W \) = weight of KBr
- \( D \) = density of the sample
- \( V \) = volume of the sample
- \( p \) = percentage of KBr in the sample

Test that the density of the sample is 1.30, by measuring the refraction index and bring volume to 250 ml by addition of KBr solution, density 1.30. Load the zonal rotor, spin, unload and pool positive fractions as in step 6.

1. Using an Amicon ultrafiltration cell with YM 100 membrane dialyze/concentrate the sample to a final volume of 25 ml using Tris buffer as diluent. Add 1 ml of sucrose stock solution and load the zonal rotor from the edge port with the following solutions at 1500 ml/hr.

   - 250 ml 15% Sucrose solution
   - 450 ml 30% Sucrose solution
   - 650 ml 40% Sucrose solution

When the tubing coming from center port is completely filled with the first solution, load from the center port of the rotor at 750 ml/hr with:

   - 50 ml sample.
   - 100 ml Tris solution.
Plate 8 : Labelling of Samples
Spin at 26000 rpm for 19 hr at 10 °C. Unload the rotor by pushing from the center with distilled water 1500 ml/hr. Collect fractions and detect HBsAg.

2. Pool positive fractions and dialyze/concentrate using an Amicon with YM100 membrane. Use PBS as diluent. Measure absorbance at 280 nm and calculate the HBsAg concentration by the following equation.

\[
\text{Absorbance}_{280} = \frac{[\text{HBsAg}] \text{ (mg/ml)}}{3.726}
\]

Purity of the preparation should be checked by SDS-polyacrylamide electrophoresis and specific activity may be calculated as described by Fields et al., (1988).

Purification based on the method Govender et al., (1985) with modifications:

HBsAg was purified as per the procedure of Govender et al., (1985), HBsAg particles were precipitated from plasma (500 ml) by addition of PEG-6000 to a final concentration of 11% (w/v). The precipitate was collected by centrifugation at a speed of 40,000 rpm for 4 hours at 4°C using a fixed angle rotor- Beckman L8 ultracentrifuge. The precipitate was dissolved in 5ml of 0.05M NaCl Buffer pH 8.0.

Five different concentration ranges starting from 10% to 30% (w/v) sucrose solution was prepared by dissolving analytical grade sucrose (mol.wt.342.30) in sterile distilled water under aseptic conditions. Simultaneously they were loaded into polycarbonate centrifuge tubes in order of ascending density range. It was
allowed to form a linear gradient by keeping it at room temperature for three hours in standing position.

A minimum volume of 1.7 ml PEG precipitate suspension was layered over the linear gradient. The tubes were sealed and placed in Beckman L8 ultracentrifuge in a swing out rotor and centrifuged at 40,000 rpm at 4°C for 16 hours. Fraction were collected using a LKB Bromma Redirac 2112 model fraction collector. Fractions were checked for the presence of HBsAg using Hepanostika HBsAg ELISA kits. The fraction which had HBsAg was dialysed.

Dialysis

Dialysis sac with pore size (342.30) to elute sucrose from the gradient sample and about 2 cm length was first washed with normal saline (0.85%) followed by double distilled water. Subsequently it was dried and one end of the dialysis sac was made airtight by tying with twine thread. The HBsAg positive fractions were poured in the dialysis sac and the opening was tied with twine thread. The dialysis sac with the sample was immersed completely inside a glass beaker with 350 ml of Sodium hydrogen carbonate buffer (pH 9.6) and teflon coated magnetic stirrer at the bottom. The beaker was set at rest on a magnetic platform which in turn, was connected to a voltage regulator. Dialysis was stopped after 36 hours with 6 hour interval change of buffer. After dialysis the sample was collected in a sterile tube under aseptic conditions. The total protein content of the dialysed fraction was estimated by Lowry's method and also for the presence of
Plate 9 : Unloading of samples from liquid nitrogen cylinder at the laboratory

Plate 10 : Recording results by ELISA Reader
HBsAg titer by ELISA. The SDS PAGE analysis of the purified product was done and silver staining of the product was carried out. This purified HBsAg antigen was used for the production of antibody in rabbits and goats.

**SDS PAGE gel analysis of the purified product**

50 µl of sample was mixed with equal volume of sample buffer (10% glycerol; 2% SDS; 0.01% Bromophenol blue; 5% 2-mercaptoethanol in Tris- HCl (pH 6.8)). The mixture was boiled for 3 mins before loading onto the wells. Approximately 40µl of each sample was loaded and electrophoresis was performed at constant voltage of 10mA for 15 hours. The gel was silver stained as described. The gel was polymerised chemically by the addition of 0.25% (vol/vol) N, N, N’N’ - tetramethyl ethylene diamine (TEMED) and 0.05% (wt/vol) ammonium per sulphate. The stacking gel and separation gel buffer used was 0.5M Tris HCl and 1.5 M Tris-HCl with pH 6.8 and 8.8 respectively. The electrode buffer used was 0.025 M Tris and 0.25M Glycine with 0.1% SDS (pH 8.3).

**SILVER STAINING (Herring et al., 1982)**

The gels were silver stained by the method of Herring et al., (1982) with modifications. Briefly, the gels were washed twice with 10% ethanol – 0.5% acetic acid after 15 min incubation each and then soaked in 0.011 M silver nitrate solution for 20 min. The gel was then briefly rinsed in distilled water. The
reduction step was performed with a solution of 0.28M sodium carbonate containing 0.1M formaldehyde. The bands appeared at this stage, and the reduction was continued until the bands were clearly visible. The reaction was then stopped with 5% acetic acid and photographed under transilluminator.

Separation gel buffer (4X) 1.5 m Tris – HCL (pH – 8.8)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>- 18.17 g</td>
</tr>
<tr>
<td>D H2O</td>
<td>- 80 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>- 1ml</td>
</tr>
</tbody>
</table>

Adjust pH to 8.8 with 12 N. HCl

(2.75 ml made to 100 ml with distilled water)

Stacking gel buffer (4x) : 0.5 M Tris – HCl (pH – 6.8)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>- 6.06 g</td>
</tr>
<tr>
<td>D H2O</td>
<td>- 80 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>- 1ml</td>
</tr>
</tbody>
</table>

Adjust pH to 6.8 with 12 N. HCl

(3.95 ml) made upto 100 ml with D. H2O

Reservoir Buffer (5x)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>-15.1g</td>
</tr>
<tr>
<td>Glycine</td>
<td>-94 g</td>
</tr>
<tr>
<td>10% SDS (w/v)</td>
<td>-50 ml</td>
</tr>
</tbody>
</table>
Adjust pH to 8.3 with 12N.HCl made up to 1000ml with D. H₂O.

**Sample buffer (2x)**

- 10% Glycine
- 2% SDS
- 5% Mercaptoethanol
- 0.02% Bromophenol blue in Tris HCl (pH 6.8)

**Ammonium per sulphate (APS)**

Freshly made for every run

2% (20 mg/ml) – 300 μl

**Silver staining solution**

**Fixing solution**

- 10% Ethanol
- 5% Acetic acid

For 400 ml

- Ethanol – 40 ml
- Acetic acid – 20 ml
- D. H₂O – 340 ml

**Silver Nitrate solution (0.011M)**

- Silver Nitrate - 0.748 g
- D. H₂O - 400 ml
Developing solution (Reducing agent)

0.28 M Sodium Carbonate  
5.936 g

38% formaldehyde  
0.5 ml

D. H₂O  
made to 200 ml

Dissolve Na₂CO₃ first in Distilled water and then add formaldehyde.

Stop solution  
For 400 ml

5% Acetic acid  
Acetic acid  
– 20 ml

D. H₂O  
-- 380 ml

Polyacrylamide solution  
- 30%

Acrylamide  
- 30.0 g

Methylene bisacrylamide  
- 0.8 g

D. H₂O  
- 100 ml

Store at 4°C.

Calculation:

Specific activity (HBsAg U/ml) = \[
\frac{\text{End point titration}}{\text{Protein estimation by Lowry method}} \times 5
\]
Purification Index (fold) = \[
\frac{\text{SA for each purified preparation}}{\text{SA for starting material}}
\]

Yield = \[
\frac{\text{Total HBsAg units for each preparation}}{\text{Total HBsAg units of starting material}} \times 100
\]

4.5.1a Immunogenicity studies in Rabbits.

150 µg / 0.5 ml doses of purified product was injected subcutaneously in adult rabbits weighing 1 - 5 kgs on day 0, 7, 21 and 28 and final bleeding was done on the 35th day. Four animals were used for the study. Animal 1 (Recombinant Vaccine control containing 10 µg / ml), Animal 2 (purified HBsAg product 150 µg / 0.5 ml), Animal 3 (Plasma derived vaccine control 10 µg / ml), Animal 4 (Control PBS) was used. Anti HBs was checked in these samples using Hepanostika anti HBs ELISA kit.

4.5.1b Immunogenicity studies in Goats:

40 µg / ml doses of purified HBsAg product was injected intramuscularly in healthy goats weighing 8-10 kgs. Immunisation doses was given on day 0, day 14 and on day 25. The animal was test blood on the 32nd, 39th and 56th day. On the 77th and the 89th day further booster doses of 40 µg / ml were given and on the
105th day the animal was test blood to check for anti HBs titre. Anti HBs was checked in these samples using Hepanostika anti-HBs ELISA kits.

4.6. ELISA techniques adopted for the entire study:

Hepatitis B surface antigen (HBsAg) (Wellcozyme, 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.

**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.

**Principle of the test:**

Wellcozyme 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 antibody on the solid phase and the conjugate creating and antibody-antigen-antibody ‘sandwich’. After washing, a sensitive peroxidase substrate was added which produces colour 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 HBcAg 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.

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.

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

<table>
<thead>
<tr>
<th>ROW</th>
<th>COLUMN 1 (anti-HBe test)</th>
<th>COLUMN 2 (HBeAg test)</th>
</tr>
</thead>
<tbody>
<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>
</tr>
<tr>
<td>C</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>D</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>E</td>
<td>Sample 1</td>
<td>Sample 1</td>
</tr>
<tr>
<td>F</td>
<td>Sample 2</td>
<td>Sample 2</td>
</tr>
</tbody>
</table>
2. 50 μl of Neutralising antigen was added into the wells of column 1 (i.e., the anti-HBe Tests).

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

4. The plate was incubated in a water bath at 45 to 50°C for 1 hour.

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

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

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

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

9. The absorbance was read at 450 nm.

Calculation of Results:

1. Anti-HBe assay (Column 1)

   (a) The mean absorbance of the two negative control values and the two anti-HBe positive control 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)

(a). The mean absorbance of the two negative control values were calculated.

(b). The cut-off value was calculated by adding 0.1 to the mean of the negative control values.

   \[ \text{Cut-off} = \text{Mean of Negative control} + 0.1 \]

(c). Samples giving an absorbance equal or greater than the cut-off were considered positive for HBeAg.

Anti HBc ELISA (Wellcozyme) (Murex VK 26/28)

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 200 µl 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 37º C for two hours.
4. Wash the wells for three or four washes with a forty five second soak time.
5. Add 200 µl of conjugate to each well, cover the wells with a lid and incubate for 60 min at 37ºC.
6. At the end of incubation, wash four times.
7. Add 200 µl of substrate solution to each well.
8. Incubate for 30 min at room temperature.
9. Add 50 µl stop solution (2 M 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.
Anti HBs (Hepanostika, Netherlands)

**Principle of the test:**

The test is an enzyme immunoassay based on a "sandwich principle". The wells of polystyrene microelisa strips have been coated with hepatitis B surface antigen, which constitutes the solid-phase antigen. The test sample is incubated in such a well; anti HBs, if present in the sample, will bind to the solid phase antigen. Subsequently, HBsAg which has been labeled with the enzyme horseradish peroxidase (HRP) is added.

With a positive reaction, this labeled antigen becomes bound to solid phase antigen / anti HBs complexes previously formed. Incubation with enzyme substrate produces a blue colour in the test well, which turns into yellow when the reaction is stopped with sulphuric acid.

If the sample contains no anti HBs then the labeled antigen cannot be bound specifically and only a low background colour develops.

**Qualitative test:**

The test may be performed with two different procedures.

1. Take out the required number of strips from the sachet and place in the strip holder

2. Pipette 100 µl of test sample into the wells
3. Include one negative control, one low positive control and one high positive control, if more than one strips is used, include two or more negative controls, two or more high positive controls in each strip holder.

4. Incubate at 37°C for one hour

5. Wash well four times

6. Pipette 100 µl of conjugate solution to each well.

7. Incubate at 37°C for 60 mins

8. Wash four times and then add 100 µl of substrate solution into each well.

9. Incubate at 20 to 25°C for 30 mins.

10. Stop the reaction by addition of 100 µl of 1 Mol sulphuric acid.

11. Read the absorbance at 450 nm.

**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', 5,5'-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.
4.7. PCR Methods

PCR for HBV-DNA

DNA Extraction from serum.
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.

The method followed for the HBV-DNA extraction has been adapted from Cheung

PCR Reaction mix

The reaction mix was constituted in the following manner:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Taq buffer</td>
<td>10 µl</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>2 µl</td>
</tr>
<tr>
<td>Sense primer</td>
<td>10 µl  (100 pg)</td>
</tr>
</tbody>
</table>
Antisense primer 10 µl (100 pg)

Taq polymerase (5U/µl) 0.5 µl (2.5 Units)

Water 58 µl

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
- 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 575 bp product was compared with molecular weight marker.

Primers used:

- Final product - 575 bp product.

- Sense primer: 1679 F (5'- GGG TGG AGC CCT CAG GCT CAG GGC A- 3')

- Antisense primer: 2254 R (5'- GAA GAT GAG GCA TAG CAG CAG GAT- 3')
4.8. Reproducibility study on treatment efficacy of *Phyllanthus amarus* for chronic Hepatitis B virus infection:

69 Chronic carriers of Hepatitis B virus who were HBsAg positive for more than a period of six months were selected as volunteers after obtaining written consent from them. The whole plant of *Phyllanthus amarus* in crude powder form was prepared in the form of 500 mg capsules after testing the preparation for biological activity like HBsAg binding and HBV DNA polymerase inhibition, sterility and stability they were given thrice daily for a period of six months. HBV markers namely HBsAg, HBsAg titer, HBeAg/anti HBe by ELISA were screened for the follow up samples during the six month treatment period. HBV DNA was also done for the follow up samples who showed HBeAg seroconversion and HBsAg clearance.

**Invitro HBsAg binding studies;**

Extract of *Phyllanthus amarus* powder was prepared in a standard concentration of 5mg/ml in PBS and used for this study.

**Principle:**

The test is an enzyme immunoassay based on the sandwich principle. HBsAg in the plasma is neutralized or bound by preincubation with anti HBs like substance and hence no longer reacts with the antibody coated in the wells. The
presence of HBsAg binding activity in an extract/compound is demonstrated by reduction of colour or a negative ELISA result. The presence of unbound HBsAg in a test sample is demonstrated by an increase in colour or a positive ELISA result.

Requirements:

1. ELISA kit (Hepanostika HBsAg kit Uniform II HBsAg kit) - Organon Technika
2. ELISA reader and washer - Biotek Instruments, Winooski, USA.

Procedure:

Preincubation with HBsAg plasma: Equal volume of pretitrated HBsAg positive plasma and 5mg/ml concentration of the extract was mixed and incubated at 37°C for 5 days. The mixture was assayed daily for the presence of bound/unbound HBsAg using Hepanostika ELISA Kit. Control tubes containing solvent (PBS) and plasma (Solvent control) and P. amarus and plasma (Positive control) were set up in each batch. The assay was conducted as per the procedure of Thyagarajan et al., (1982).

ELISA was performed as per the instructions of the manufacturer as follows. The conjugate was already supplied in the wells in a pearl shaped form, this conjugate dissolved on addition of sample/controls.
1. 100 μl of each sample was transferred from the pre-incubation tube to HBsAg micro ELISA strips.

2. The strips were covered and agitated for 15 seconds using a microshaker and then incubated at 37°C for one hour.

3. Each well was washed four times with PBS Tween 20.

4. 100 μl substrate solution was added to each well and incubated in dark for 30 minutes at 20 to 25°C.

5. The reaction was stopped by adding 100 μl of 2 N H₂SO₄ to each well.

6. Reading was taken at 450 nm in Biotek ELISA reader.

**Calculation:**

**Cut-off value:**

1. The cut-off value was calculated by the formula = Mean of negative control + 0.05.

2. Values equal to or greater than the cut-off should be taken as positive.

**HBV DNA polymerase inhibition assay**

**Principle:**

Replication of hepadna viruses involves a viral DNA polymerase which is a potential target for chemotherapy against HBV. In the presence of HBV-DNA polymerase, complimentary bases are added to the template (HBV-DNA), the
addition of which are quantitated with the help of tritiated thymidine triphosphate.
Reduction in the count of 50% or more in the test is noted as inhibitory activity.

Requirements:

1. Tris HCl
2. MgCl₂
3. KCl
4. dNTPs (dATP, dGTP, dTTP and dCTP)
5. ³H d TTP
6. HBsAg positive serum
7. HBeAg positive serum
8. NP 40
9. Mercaptoethanol
10. EDTA

All the chemicals were procured from Sigma Chemicals, St. Louis, USA.

Equipment required

1. Ultra centrifuge - Sorvall, UK (OTD Combi)
2. β Scintillation counter - Kontron Instruments, Milano Italy. (Betamatic - IV)
3. Water Bath
Virus preparation:

Pre titrated HBsAg and HBeAg positive serum was centrifuged at 35,000 rpm for 3 hours using SW41 rotor. The pellet was washed in PBS and again centrifuged at 35,000 rpm. The pellet got in this was dissolved in PBS and stored at -20°C.

Procedure:

The procedure followed was as described by Lofgren et al., (1989). Prior to the assay, the virus preparation was pretreated with 1/8th volume of 2% mercaptoethanol and 10% NP40 for 15-30 min. at room temperature. Aliquots of 25 µl were incubated at 37°C for 3 hours together with 25µl reaction mixture containing Tris HCl (pH 8.00 100 mM, MgCl₂ 20 mM, KCl 200 mM, dNTPs 10 mM each and ³H d TTP 10mM and 25µl of DNase and RNase free water, or a solution of the substance to be studied. After incubation, to 50µl of reaction mixture 10 µl of 0.2 M EDTA was added and spotted onto a Whatman DE81 filter paper discs and processed for a radioactivity measurement as explained earlier.

Quantitative HBV DNA estimation (Amplicor, Roche Diagnostics, USA).

HBV DNA was quantitated in pre / post P. amarus treatment samples by the following procedure:
Amplicor HBV Monitor™ test:

The AMPLICOR HBV MONITOR™ Test is an invtro test that utilizes Polymerase Chain reaction (PCR) nucleic acid amplification and DNA hybridization for the detection and quantitative measurement of Hepatitis B viral DNA in human serum or plasma.

Principle of the Procedure:

The AMPLICOR HBV MONITOR™ Test is based on four major processes: specimen preparation; PCR amplification of target DNA using HBV specific complimentary primers; hybridization of the amplified products to oligonucleotide probes specific to the target(s); and detection of the amplified products by colorimetric determination. In the AMPLICOR HBV MONITOR™ Test the amplification of HBV target and Internal Standard (IS) DNA occur simultaneously. The master Mix reagent contains a primer pair specific for HBV and IS target nucleic acid.

Specimen Preparation

The specimen is prepared by

(1) Centrifugation of the specimen to form a pellet

(2) Lysis of the pelleted virus
(3) Addition of a neutralization reagent to provide appropriate buffering conditions for PCR amplification.

PCR Amplification;

Target Selection

Amplification target for the AMPLICOR HBV MONITOR™ Test is a portion of the partly single stranded, circular DNA genome of HBV, defined by the primers HBV-104 UB and HBV-104 D. These primers amplify a 104 base sequence in the pre-Core/Core region of the HBV genome. DNA is amplified using one biotinylated and one non-biotinylated oligonucleotide primer.

Target Amplification:

Processed specimens are added to the amplification mixture in amplification tubes in which PCR takes place. The reaction mixture is heated to denature the double stranded DNA helix and expose the primer target sequences. As the mixture cools, the primers anneal to the target DNA. Thermus aquaticus DNA polymerase (Taq polymerase), in the presence of excess dNTPs, extends the annealed primers along the target template to produce a 104 base pair sequence termed an amplicon. This process is repeated for a number of cycles, each cycle effectively doubling the amount of amplicon. The entire HBV genome is not amplified.
Internal Standard Amplification:

In enzyme based amplification processes such as PCR, efficiency can be reduced by inhibitors that may be present in the clinical specimen. The HBV internal standard (IS) is added to detect substances that may interfere with PCR amplification. The HBV IS (Internal standard) is a synthetic double stranded DNA molecule with primer binding sites identical to the HBV target sequence, a randomized internal sequence of similar length and base composition as the HBV target sequence, and a unique probe binding region that differentiated IS from the target amplicon. The HBV IS is introduced into each amplification reaction with the master mix reagent and is co-amplified with target DNA from the standard or specimen.

Selective amplification:

Selective amplification of target nucleic acid from the clinical specimen in the AMPLICOR HBV MONITOR™ is achieved by the use of AmpErase and deoxyuridine triphosphate (dUTP). AmpErase (uracil-N-glycosylase, UNG) recognizes and catalyzes the destruction of DNA strands containing deoxyuridine but not DNA containing thymidine. Deoxyuridine is not present in naturally occurring DNA, but is always present in amplicon due to the use of deoxyuridine triphosphate as one of the dNTPs in the Master Mix reagent; therefore, only amplicon contain deoxyuridine. Amp Erase which is included in the Master Mix
reagent, catalyzes the cleavage of deoxyuridine containing DNA at deoxyuridine residues by opening the deoxyribose chain at the C-1 position. AmpErase in the AMPLICOR HBV MONITOR™ Test has been demonstrated to inactivate at least $10^3$ copies of deoxyuridine-containing HBV amplicon per PCR.

**Hybridization:**

Following PCR amplification, the HBV amplicon are immobilized onto a streptavidin coated microwell plate. Following hybridization, the double stranded amplicon are chemically denatured by the addition of HBV MONITOR eluent to the microwell late. The unbound, non-biotinylated strand is removed by washing to leave a single stranded DNA bound to the microwell plate.

**Detection:**

Following denaturation of the amplicon and removal of the unbound DNA strand, dinitrophenyl (DNP)-labeled oligonucleotide probes, one specific for HBV are allowed to hybridize with the immobilised amplicon. This specific hybridization of the probes to the bound amplicon increases the specificity of the test. Unbound probe is removed by washing and then an anti-DNP-alkaline phosphatase conjugate is added to the wells. The anti-DNP portion of the conjugate binds to the DNP moiety of the bound probes to immobilize the alkaline phosphatase. A substrate containing para-nitro-phenylphosphate is added and the
alkaline phosphatase catalyzes the formation of a colored complex. The intensity of the color reaction is measured at 405 nm using an automated microwell plate reader.

**HBV DNA Quantitation:**

The AMPLICOR HBV MONITOR™ Test quantitates virus titers from $4 \times 10^2$ to $4 \times 10^7$ viral particles per ml. A fixed amount of HBV IS, which is included in the Master Mix, is added to each of the tubes containing the processed HBV sample or HBV Standard DNA. HBV DNA and HBV IS DNA are co amplified with the similar efficiency. The ratio of HBV OD signal to HBV IS OD signal is calculated to correct for any differences in amplification from tube to tube. The HBV/IS ratio for each specimen is then compared to a standard curve generated from DNA standards run with each amplification and the number of HBV viral genomes present in each specimen is determined from the standard curve.

**Results:**

**Manual calculation of results**

1. The blank absorbance at each time point (4, 10 and 30 minutes) is calculated by averaging the OD$_{405}$ values from the two blank wells for each time point. Record this value as the blank OD$_{405}$ for that time point.
2. Calculate the HBV total OD\textsubscript{405} and the IS Total OD\textsubscript{405} for each specimen and standard as follows:

a. Select the HBV and IS OD signal for each specimen and standard as follows. The HBV and IS OD signal for a specimen or standard may be read from different stand points.

b. For specimens or standards which have OD\textsubscript{405} < 2.0 at the 30-minute reading, record the OD\textsubscript{405} value. Record the blank OD\textsubscript{405} for the 30-minute time point. Record the time factor as 1. If the OD\textsubscript{405} is > 2.0 at 30 minutes, go to the 10-minute reading.

c. For specimens or standards which have an OD\textsubscript{405} < 2.0 at the 10 minute reading, record the OD\textsubscript{405} value. Record the blank OD\textsubscript{405} for the 10-minute time point. Record the time factor as 3. If the OD\textsubscript{405} is >2.0 at 10 minutes go to the 4-minute reading.

d. For specimens and/or standards which have an OD\textsubscript{405} > 2.0 at 10 minutes, use the 4-minute reading. Record the OD\textsubscript{405} value. Record the blank OD\textsubscript{405} for the 4-minute time point. Record the time factor as 7.5.

e. Calculate the HBV and IS Total OD\textsubscript{405} for each specimen and standard by the following equation:

\[
(HBV \text{ or IS OD}_{405} - \text{Blank OD}_{405}) \times \text{Time factor} = \text{Total HBV or IS OD}_{405}
\]
3. For each standard and patient specimen, calculate the HBV/IS Ratio using the following formula:

\[
\text{HBV Total OD}_{405} \quad \frac{\text{HBV Total OD}_{405}}{\text{IS Total OD}_{405}} = \text{HBV/IS Ratio}
\]

4. a. Check the Total OD_{405} for the HBV 0 copy (Negative) and the 10 copy standards against the specifications listed in the quality control section. If the OD values are acceptable, proceed. If either OD value is unacceptable, the entire run is invalid and the amplification and the detection reactions must be repeated.

   b. Compare the HBV/IS ratio for all standards to the ranges listed on the HBV monitor data card provided. If all the ratio values are acceptable, proceed. If any standard ratio value falls outside the acceptance criteria, the entire assay is invalid and must be repeated.

5. Prepare a standard curve on log-log paper or use a spread sheet to plot the copy number of each standard (0 to \(10^6\)) on the X-axis and the corresponding HBV/IS ratio on the y-axis.

6. Using the HBV/IS ratio calculated for each patient specimen, determine the HBV copy number per reaction by reading from the standard curve the copy number that corresponds to the HBV/IS ratio.

7. For each patient specimen, multiply the HBV copy number/reaction obtained from the standard curve by 40 to determine the HBV copy number/ml.

   \[
   \text{Copy number/reaction} \times 40 = \text{Copy number/ml}
   \]

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