Chapter 4

Materials and Methods
4. MATERIALS & METHODS

4.1 MATERIALS

4.1.1 Ethical clearance

Institutional review committee approval was obtained for clearance of the project. Informed written consent was obtained from each study adult volunteers/parents or guardians of adolescent volunteers and husband or relative accompanying the pregnant women to the hospital prior to participation in the trial after explaining about the consequences of HBV infection / research study. The consent form was also made in the regional language (Appendix I & II).

4.1.2 EVALUATION OF IMMUNOGENICITY AND REACTOGENECITY OF A NEW RECOMBINANT HEPATITIS B VACCINE GENEVAC B IN HEALTHY ADULTS.

A total of 400 healthy adult volunteers of both the sexes were recruited into the study and were allocated into three groups; 240 volunteers for receiving Genevac B and 80 each for Engerix B and Shanvac B. Healthy adults (students and staff members) from the following institutions were studied,

a) University of Madras, Guindy and Taramani campuses.
b) Madras Medical College, Chennai.
c) Stanley Medical College, Chennai.
d) Murugappa Chettiar Research Centre, Taramani, Chennai.

All the volunteers were fully informed of the open labeled clinical trial and a written consent was obtained from all (Table:4.1.2 and 4.1.2a).

4.1.2.1 Clinical trial criteria

Internationally accepted criteria for vaccine adopted in the study including the following:
4.1.2.2 Inclusion criteria

The inclusion criteria of the volunteers into the study were healthy adults aged above 20 years and below 50 of both the sexes; signed informed consent; tested negative for HBs Ag & Anti-HBs, and Anti HBc IgM; without any previous history of hepatitis B vaccination in the past; no evidence of skin diseases or infection at any site.

4.1.2.3 Exclusion criteria

The exclusion criteria of the volunteers into the study were individuals aged below 20 years and above 50 years; HBs Ag and anti-HBs tested positive; subjects inability to come for follow up; subjects enrolled in another vaccination trial; active, moderate or severe illness; known history of Hepatitis B infection/carer state; subjects with hepatomegaly and/or splenomegaly; known allergy to aluminum, uncontrolled coagulopathy; known immunological deficiency including HIV infection; treatment with immunosuppressors including corticosteroids; previous administration of immunoglobulins and blood derived products in the last 6 months or planned to receive such products in the next 7 months; chronic illnesses like epilepsy; previous history of treatment with extracted growth hormone; evidence of skin diseases or infection at any site.
### Table 4.1.2 Demography data

The demographic data of patients were collected using standard proforma.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Age (years)</th>
<th>Weight (KG)</th>
<th>Height (CMS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum</td>
<td>50</td>
<td>98</td>
<td>186</td>
</tr>
<tr>
<td>Minimum</td>
<td>20</td>
<td>37</td>
<td>138</td>
</tr>
<tr>
<td>Mean</td>
<td>20.3</td>
<td>58.5</td>
<td>164.0</td>
</tr>
<tr>
<td>S.D</td>
<td>9.5</td>
<td>9.7</td>
<td>8.8</td>
</tr>
</tbody>
</table>

**Genevac B - Demography (N = 240)**

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Genevac – B</th>
<th>Engerix – B</th>
<th>Shanvac – B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name of the company</td>
<td>Serum Institute, Pune.</td>
<td>Smithkline Beecham, U. K.</td>
<td>Shantha Biotech, Hyderabad.</td>
</tr>
<tr>
<td>Yeast</td>
<td><em>Hanseneulla polymorpha</em></td>
<td><em>Saccharomyces cerevisiae</em></td>
<td><em>Pichia pastoris</em></td>
</tr>
<tr>
<td>Adsorbent used</td>
<td>Aluminum Hydroxide</td>
<td>Aluminum Hydroxide</td>
<td>Aluminum Hydroxide</td>
</tr>
<tr>
<td>Preservative used</td>
<td>Thiomersal (1: 20, 000)</td>
<td>Thiomersal (1: 20, 000)</td>
<td>Thiomersal (1: 20, 000)</td>
</tr>
<tr>
<td>Strength in 1 ml</td>
<td>20 μg.</td>
<td>20 μg.</td>
<td>20 μg.</td>
</tr>
</tbody>
</table>

**Table 4.1.2a Properties of the recombinant vaccines used**
4.1.2.4 Design of the clinical trials among adults

On fulfilling the inclusion criteria, all the volunteers were subjected to pre-vaccination clinical examination, screening for HBsAg, Anti-HBc IgM and anti-HBs, besides subjecting them for complete haemogram, liver function and renal function tests. The study population was vaccinated on a subsequent visit with the respective hepatitis B vaccines in 1 ml quantities following 0.1 and 2 months schedule by intra muscular route. Volunteers of the study were recruited one month after the first, second and the third doses. In all these regimens, the vaccinees were subjected to clinical examination, data collection and blood sample collection for hematological, biochemical and immunological studies. The clinical examination and data collection was done by using structured proforma to analyse any vaccine associated adverse events besides concurrent infections. All the samples from the vaccinees were analysed to quantitate the levels of anti-HBs using Monolisa anti-HBs 3.0 (Belgium) commercial kits, using Sanafi pastuer anti-HBs standards.

4.1.3 Evaluation of immunogenicity and safety of a new recombinant hepatitis B vaccine Genevac B in healthy adolescents.

237 normal healthy adolescents (11-19 yrs) of both the sexes were enrolled into the study. They were school children studying at CHS Kamaraj Avenue Corporation Higher Secondary School, Adyar, Chennai-600 020. The new genetic recombinant hepatitis B vaccine, Genevac B, was evaluated on volunteers who were recruited based on the inclusion and exclusion criteria. (Table 4.1.3)

4.1.3.1 Inclusion criteria

The inclusion criteria of the volunteers into the study were healthy adults aged above 11 years and below 19 of both the sexes; signed informed consent; tested negative for HBs Ag, Anti-HBs, and Anti-HBc IgM; without any previous history of hepatitis B vaccination in the past; no evidence of skin diseases or infection at any site.
4.1.3.2 Exclusion criteria

The exclusion criteria of the volunteers into the study were age below 11 years and above 19 years; HBs Ag test positive and/or anti-HBs positive; subjects inability to come for follow up; subjects enrolled in another vaccination trial; active, moderate or severe illness; known history of Hepatitis B infection/carrier state; subjects with hepatomegaly and/or splenomegaly; known allergy to aluminum, uncontrolled coagulopathy; known immunological deficiency including HIV infection; treatment with immunosuppressors including corticosteroids; previous administration of immunoglobulins and blood derived products in the last 6 months or planned to receive such products in the next 7 months; chronic illnesses like epilepsy; previous history of treatment with extracted growth hormone; evidence of skin diseases or infection at any site.

4.1.3.3 Demography data

Table 4.1.3 The demographic data of patients were collected using standard proforma

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Age (Years)</th>
<th>Weight (KG)</th>
<th>Height (CMS)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genevac B - Demography – 1 ml (N =100)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>19</td>
<td>85</td>
<td>180</td>
</tr>
<tr>
<td>Minimum</td>
<td>11</td>
<td>36</td>
<td>148</td>
</tr>
<tr>
<td>Mean</td>
<td>15</td>
<td>48</td>
<td>156</td>
</tr>
<tr>
<td>S.D</td>
<td>5.6</td>
<td>8.2</td>
<td>8.3</td>
</tr>
<tr>
<td><strong>Genevac B - Demography – 0.5 ml (N = 100)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>19</td>
<td>80</td>
<td>178</td>
</tr>
<tr>
<td>Minimum</td>
<td>11</td>
<td>40</td>
<td>140</td>
</tr>
<tr>
<td>Mean</td>
<td>15</td>
<td>47.5</td>
<td>145</td>
</tr>
<tr>
<td>S.D</td>
<td>5.4</td>
<td>8.0</td>
<td>8.4</td>
</tr>
</tbody>
</table>
Table 4.1.3a  Properties of the vaccine

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Genevac – B (1ml)</th>
<th>Genevac – B (0.5ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name of the company</td>
<td>Serum Institute, Pune.</td>
<td>Serum Institute, Pune.</td>
</tr>
<tr>
<td>Yeast</td>
<td><em>Hanseneuilla polymorpha</em></td>
<td><em>Hanseneuilla polymorpha</em></td>
</tr>
<tr>
<td>Adsorbent used</td>
<td>Aluminum Hydroxide</td>
<td>Aluminum Hydroxide</td>
</tr>
<tr>
<td>Preservative used</td>
<td>Thiomersal (1: 20,000)</td>
<td>Thiomersal (1: 10,000)</td>
</tr>
<tr>
<td>Strength in 1 ml</td>
<td>20 µg</td>
<td>10 µg</td>
</tr>
</tbody>
</table>

4.1.3.4 Design of the clinical trials among adolescents

On fulfilling the clinical trial criteria, signed informed consent was obtained from the participants and their parents/guardians. 200 healthy adolescents were recruited into the study and were randomly allocated as 100 subjects for receiving 1ml (20µg) doses and 100 for receiving 0.5ml of (10µg) doses of Genevac B vaccine. The vaccine was administered in the deltoid muscle at 0, 1 and 6 months. Following administration of each dose of vaccine, volunteers were observed for any adverse events. Blood samples collected one month after each dose was subjected to clinical examination, data collection and for immunological studies.

4.1.4 Evaluation of the efficacy of HBV vaccination in the prevention of HBV transmission to children born to HBsAg positive mothers

Babies born to HBsAg positive mothers attending the following hospitals were studied they were

a) Institute of Obstetrics and Gynecology for women and children, Egmore, Chennai-600008.

b) Kasturibai Gandhi Hospital, Triplicane, Chennai-600005

c) Department of Obstetrics and Gynecology, R. Madras Medical College and Hospital, Chidambaram.
4.1.4.1 Design of the clinical trial among infants born to positive mothers

Consent was obtained from the pregnant women and the relatives accompanying them for the study after explaining the consequences of the study, consequences of the viral infection and benefits of vaccination to the babies born to them.

Accordingly, all the babies born to HBsAg positive mothers received three 0.5 ml doses of Hepatitis B vaccine each by intramuscular injection in the lateral aspect of the thigh. At birth as first dose, second dose at one month after birth and the third dose at six month after birth.

4.1.4.2 Follow up sample collection from Infants

A total of 158 babies born to HBsAg positive mothers were recruited and they were administered with the new recombinant hepatitis B vaccine, Genevac B and commercially available vaccine Shanvac B and Engerix B with 10µg of hepatitis B surface antigen per 0.5ml of dose. The vaccine was administered intramuscularly on the lateral aspect of the thigh immediately after birth within 24 hours. The study population was vaccinated on a subsequent visit with the hepatitis B vaccines following 0, 1 and 6 months schedule.

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; a detailed information was collected using the proforma at every follow up (Appendix IV); every mother was asked to return within 3 days of the target date and those who did not, were visited at their homes by the field visit.

The infants were bled using scalp vein bleeding needle from the cubital vein at 0 month if they were more than 2 kg. (0 ± 7 days), 1 month (30 ± 7 days), 2 month (60 ± 7 days), 6 month (185 ± 10 days), 9 month (279 ± 12 days) and 12 month (370 ± 12 days) 3 ml of blood samples were collected with infant vaccination.
The serum was separated and stored at -70°C until tested. After delivery the mother and child pairs were followed up accordingly (Table 4.1.4).

Table 4.1.4 Follow up analysis of baby born to HBsAg positive mothers

<table>
<thead>
<tr>
<th>Current follow up</th>
<th>Number of baby sample analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delivery</td>
<td>158</td>
</tr>
<tr>
<td>2 month after birth</td>
<td>158</td>
</tr>
<tr>
<td>3 month after birth</td>
<td>149</td>
</tr>
<tr>
<td>6 month after birth</td>
<td>118</td>
</tr>
<tr>
<td>9 month after birth</td>
<td>91</td>
</tr>
<tr>
<td>12 month after birth</td>
<td>68</td>
</tr>
</tbody>
</table>

4.1.5 Prospective follow up study of vaccinees involved in the Genevac B clinical trials

Adults involved in the hepatitis B vaccine clinical trials were followed up for three years after primary vaccination regimens. Adolescents and babies born to hepatitis B surface antigen positive mothers involved in the hepatitis B vaccine clinical trials were followed up for one year after primary vaccination regimens. Follow-up blood samples were assessed for anti-HBs levels for seroconversion, seroprotection and for the Geometric mean titer.

4.1.6 Analysis of cell mediated immunity markers response in vaccinees involved in the clinical trials

Vaccine non responders, hypo responders and high responders from the adults and adolescents clinical trials were selected based on their anti-HBs levels and were enrolled in the fourth part of the study (Table-4.1.6).
Table 4.1.6  Vaccine responders / Non responders

<table>
<thead>
<tr>
<th>Vaccinees</th>
<th>Adults</th>
<th>Adolescents</th>
<th>Infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non responders</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hypo responders</td>
<td>15</td>
<td>7</td>
<td>15 (5 – for each group)</td>
</tr>
<tr>
<td>High responders</td>
<td>15 (5 – for each group)</td>
<td>14 (7 – for each group)</td>
<td>15 (5 – for each group)</td>
</tr>
</tbody>
</table>

4.1.6.1 Design of the study

Serum samples from vaccine non-responders, hypo responders and high responders were quantified for IL-2, IL-12, IFN-γ and TNF-α. In order to observe the specificity of the cytokines, invitro peripheral blood mononuclear cells (PBMCs) were isolated from non-responder, hypo responder and high responder adults and adolescents and babies. The cells were stimulated invitro with recombinant HBsAg and PHA mitogen with Rosewell Park Memorial Institute Medium (RPMI). The culture supernatants were quantified for IL-2, IL-12, IFN-γ and TNF-α by using commercially available BIOSOURCE kit.

4.2 METHODS

4.2.1 Clinical: Clinical examination of the study volunteers was assessed using a stratified proforma.

4.2.1.1 Biochemical

The biochemical parameter includes:
4.2.2 Hematology

4.2.2.1 Haemoglobin

N/10 Hydrochloric acid up to mark 20 in the graduated tube. 20 c.mm of blood (0.02) was added with the help of Haemoglobinometer pipette. The pipette was rinsed two to three times, mix well and allow to stand for five minutes till the solution becomes dark brownish in colour. The solution is diluted drop by drop, each time mixing the solution with a stirring rod until it matches the standard. Read the result from the scale on the graduated tube by observing graduation mark at a lower edge of the meniscus at the top of the liquid column. If the final reading is not made within ten minutes after mixing the blood with hydrochloric acid N/10, 2% should be deducted from the results obtained. In every anemic case, the quality of blood and also hydrochloric acid N/10 should be doubled and the result divided by two. It is best to match in the daylight.

4.2.2.2 Total RBC count

Haemocytometer

The haemocytometer Neubauer counting chamber has a total ruled area of 9 sq.mm. It consists of a centrally heavy ruled area of 1 sq.mm in size of four others of the same size in each corner. The central area is divided into 25 squares and each square is subdivided into 16 squares. For total counts, count one each at the corner and the centre (that is 80 small squares). The four outer 1 sq.mm area are divided into 16 squares. The squares are used for total WBC counts.

The mature erythrocyte is a biconcave disk, circular shape, centrally unstained and periphery stained, pink in colour, size: 7.2 microns in average diameter. It contains haemoglobin.

Therefore, the volume of a small square is 1/400 X 1/10 = 1/4000 c.mm.
The dilution of the blood is 1/200.

Total RBC's = 435/80 X 4000/1 X 1/200 = 50000
Total WBC count

WBC diluting fluid, Truck's:

- Acetic Acid (glacial) 3 ml
- Distilled water 97 ml

Add Gentian violet to give a pale violet colour.

Draw the blood up to 0.5 marks in WBC pipette marked RBC counting and fill the counting chamber in the same manner. Allow 3 minutes for cells to settle. If the Neubauer counting chamber is used, count the cells in the four corner blocks. Each of these 4 - square millimeter area is subdivided into 16 squares, by using the low power objective and a medium occular. In counting the cells include those cells touching on the inner lines on the right and top, but do not count the cells touching the lines on the left and bottom. The difference between the two square millimeter areas should not be more than 10 WBC's.

Calculation

Total no of cells in 4 squares/2 and add two zeros.

E.g.

No. of cells counted=120/2 = 60 and 2 zeros, that is = 6000 per c.mm.

To get WBC's per c.mm:

- No. of WBC's counted in four corners : 120 cells
- The volume of a square is : 1/10 c.mm.
- The blood is diluted to : 1/20

Therefore, the number of cells per c.mm of undiluted blood

\[ = \frac{120 \times 10 \times 20}{\frac{1}{20}} = 6000 \text{ per c.mm} \]
4.2.2.3 Differential leucocytes count (D.L.C)

(Leishman’s method)

Take a few perfectly clean grease-free slides. Clean the figure or ear lobe and prick as described for the collection of capillary blood. Touch the end of a slide to a large drop of blood and then spread the drop with a second slide. As soon as the blood has spread entirely across the end of the spreader slide (the spreader must be held at an angle of about 45 degree), with a quick movement push the spreader towards the other end of the under slide. The blood film should not be too thin or too thick. Allow the blood to dry.

Leishman’s method

Leishman’s stain poured into the blood smear, which should be evenly distributed over the entire slide. At the end of one minute, double the quantity of buffer solution or distilled water carefully added and mixed with, the stain for seven or eight minutes and excess stain is removed by washing with the distilled water for two minutes. The water is then washed off with distilled water. Dry the film in air. When the film is dried examine microscopically.

Count about 200 to 500 cells and take an average percent of cells counted. The best place to count DLC is the ideal thickness, which is the third part of the blood film from the head of smear. The following are the varieties of leucocytes found in the normal blood.

Polymorphonuclear neutrophils

The polymorphonuclear neutrophil shows a faintly pinkish tinged cytoplasm filled with nearly uniform, fine granules, which take a pink colour. The nucleus is usually divided irregularly into two to five lobes, which are connected by fine bands. It is a round cell with a distinct nuclear membrane. There are no nuclei, size 10-20 microns, Function; Phagocytosis; Life 5 days.
**Lymphocytes**

Round, deeply staining nucleus, which almost fills the cell leaving a rim of strongly basophilic cytoplasm. Size 8-10 microns. Function; it produces antibody. It has antitoxic property. Life: less than 24 hours.

**Eosinophiles**

It is distinguished by compact coarse granules with eosin colour, circular in shape. Bilobed nucleus look like spectacles. Size: 10-12 microns. Function; It contains histamine. Present in large numbering allergic infection. Life; 8-12 days.

**Basophiles**

Contain purplish or bluish-black granules, which are usually intermediate in size between those of the preceding types of cells and are less refractile than the eosinophil granules, they tend to vary in size and depth of staining and are often sparse, the nucleus stains more faintly and lobulation is often indistinct. Function: It produces histamine, serotomine and a large amount of heparine; Life: 8-12 days.

**Monocytes**

It is larger than a lymphocyte, the nucleus which appears like a kidney twisted. The cytoplasm has a frosty appearance and has fine granules. Size: 18 microns. Function: Phagocytes increase in chronic infection.

### 4.2.2.4 Erythrocyte sedimentation rate

*(Westergren's method)*

Take 0.4 ml of 3.8% sodium citrate in a tube. Withdraw 2 ml of venous blood in a dry sterile syringe and place exactly 1.6 ml of blood in the tube containing sodium citrate solution. Invert the tube 2-3 times to mix the blood with the citrate solution. Fill the Westergren's E.S.R. tube exactly to the mark and place it in stand. The tube must be held firmly at an angle.

...
made at 5 minutes interval over period of 1 hour, or one reading may be made at the end of half-an-hour and another at the end of one hour and two hours.

4.2.3 Liver function test

4.2.3.1 Serum bilirubin

Principle

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

Requirements

Sulphanilic acid

HCl
Sodium nitrite 20 gm/dl
Methanol
Bilirubin

Sulphanilic acid 1 gm/d (diazoblank)

Sulphanilic acid - 10 gm / 50 mDW
Conc. HCl - 20 ml
Make up to 1000 ml

Diazo reagent

Diazo blank - 10 ml
0.5 gm/dl sodium nitrite - 0.3 ml

Bilirubin standard

Bilirubin - 10 mg
Chloroform -
Test procedure

Total bilirubin and direct bilirubin were estimated.

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

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

\[
\text{OD}(T_4) - \text{OD}(T_3)
\]

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

Interpretation

Normal is less than 1 mg/dl.

4.2.3.2 Determination of Aspartate transaminase (AST/ SGOT) and Alanine transaminase (ALT) SGPT

Principle

The enzyme AST takes part in the reaction...
Aspartic acid + α-ketoglutaric acid → glutamic acid + 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 the brown colour and red colorimetrically.

The enzyme ALT takes part in the following reactions.

Alt
Alanine + α-ketoglutaric acid → glutamic acid + pyruvic acid.

Requirements

dl-Aspartic acid AR
dl Alanine AR
α-ketoglutaric acid AR
Pyruvic acid AR (sodium salt)
Sodium hydroxide AR
Na₂HPO₄
Dinitrophenyl hydrazine AR
Chloroform AR
Potassium hydrogen phosphate
Potassium dihydrogen phosphate AK
Phosphate buffer pH 7.4

AST buffered substrate

dl Aspartic acid - 13.3 g
1N NaOH - 90 ml
α-ketoglutaric acid - 0.146 g
1N NaOH - 5 ml

Adjust pH to 7.4 and make up to 50 mL.
ALT-buffered substrate

Alanine 200 mM

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>9 g</td>
</tr>
<tr>
<td>D.H₂O</td>
<td>90 ml</td>
</tr>
<tr>
<td>1N NaOH</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>α-ketoglutaric acid</td>
<td>0.146 g</td>
</tr>
<tr>
<td>1N 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>Ingredient</th>
<th>Amount</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>NaOH</td>
<td>0.4 N</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>Ingredient</th>
<th>Amount</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 ALT working activity (ml) in IU</th>
<th>AST working activity (ml)</th>
<th>ALT substrate (ml)</th>
<th>Water substrate (ml)</th>
<th>AST (ml) 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>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>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>5.</td>
<td>0.4</td>
<td>0.6</td>
<td>0.6</td>
<td>0.2</td>
<td>66.7</td>
</tr>
</tbody>
</table>

To all the above tubes, 1 ml of dinitrophenyl hydrazine was added and left for 20 min at RT. 10 ml 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 (ml)</th>
<th>Blank (ml)</th>
<th>ALT (ml)</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 and left at room temperature for 20 minutes. Then, 5 ml of 0.4 N NaOH was added for 10 minutes. Readings were taken against water blank in the graph and ALT read from the graph.
4.2.3.3 Serum Alkaline Phosphatase

Method
PNP KINETIC

Mode
Kinetic

Reagent
4 parts of R1 + 1 part of R2
R1 = Reagent solution
R2 = Start solution

Procedure
Sample solution : 10 μl (10)
Reagent solution : 500 μl

Mix, after 1 minute, measure the increase in absorbance for every 3 minutes.

Calculation

Enzyme activity (U/L) = (Δ A/ min) X 2754
Wavelength of filter = 405 nm
Delay = 60 sec
Measurement = 180 seconds.

4.2.4 Renal function test

4.2.4.1 Serum Creatinine

Principal

The method commonly used for the estimation of Creatinine makes use of the Jaff's reaction. The production of a red colour with an alkaline picric solution.

Reagents

1. Saturated picric acid solution 1%
2. Creatinine stock standard solution (1ml=1mg)
   Dissolve 1gm of pure dry creatinine in 0.1 N hydrochloric acid and make up to a liter with the acid.
3. 10% Sodium tungstate W/V.
4. 2/3 N Sulphuric acid
5. 10% Sodium hydroxide solution
Technique

Preparation of protein free filtrate

Into a suitable test tube measure 2.0 ml serum accurately. Add 4 ml of distilled water 1 ml of 10% sodium tungstate and 1 ml of 2/3 N Sulphuric acid mixing well after each addition.

Test

Take two test tubes labeled as blank and test. In blank take 4 ml of distilled water and in test take 4 ml of protein free serum filtrate or diluted urine sample. Add 2 ml of alkaline picrate solution (prepared freshly by mixing 5 volume of saturated picric acid with 1 volume of 10% Sodium hydroxide). Mix and allow to stand for 15 minutes. Measure the optical density with green filter or at wavelength 520 mμ against blank set at zero.

Note

1. Prepare alkaline solution before use.
2. If colour is too deep, repeat the determination with a smaller amount of filtrate and dilution made up to 4 ml with distilled water and multiply result accordingly.

Calculation

For blood serum calculate m.g% Creatinine from factor, graph or curve as established by standardization. For urine calculate as for as blood and then multiply by 25. Standard curve can be prepared as follows:

Take 1 ml of Creatinine standard solution (1ml/1mmol) in to a 100 ml volumetric flask and make up to the mark with distilled water four tubes as 1,2,3 and 4 and add as follows.
Add 2 ml of alkaline picric reagent to all the tubes and allow them to stand for 15 minutes and measure optical density at wavelength 520 nm or with a Green filter against blank set at zero. Plot a graph of O.D against mg.% of Creatinine or calculate level by taking the nearest equivalent standard tube from the above standardization, as follows.

\[
\text{Reading of unknown} \\
\text{Creatinine level} = \frac{\text{Reading of unknown}}{\text{Concentration of standard}} \\
= \text{mg. Creatinine per 100 ml, reading in unknown.}
\]

4.2.4.2 Urea

Deacetylmonoxime thio semicorbazide method

Principle

Urea reacts with diacetyl monoxime and thiosemi corbazide in presence of Fe+++ ions to give pink colour which is measured colorimetrically.

Reagents

1. **DAM- TSC reagent**

   - Diacetyl monoxime : 1 gm
   - Thiosemicorbazide : 200mg
   - Sodium chloride : 9 gms
   - Distilled water : 100 ml
(Dissolve 1 gm of diacetyl monoxime in about 500 ml distilled water.
Add 200 mg of thiosemicorbazide to the above solution and dissolvent. Then add
9 gm of sodium chlororide and dissolve, dilute to 1000 ml).

2. **Urea acid reagent**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orthophosphoric acid</td>
<td>10 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulphuric acid</td>
<td>60 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferric chloride (aqueous) 10% sol</td>
<td>1 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td></td>
<td>to 1000 ml</td>
<td></td>
</tr>
</tbody>
</table>

(Into 800 ml of distilled water, 10 ml of orthophosphoric acid and 60 ml of Sulphuric acid are added. After cooling, add 1ml of 10% ferric chloride (aqueous); make up to 1000 ml).

3. **Stock urea standard**: 1 gm % (1gm of dry urea is dissolved to 100 ml with distilled water)

4. **Working standard**: 50 mg % (5 ml of stock standard is diluted to 100 ml with distilled water).

**Procedure**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>3.6 ml</td>
<td>3.5 ml</td>
<td>3.5 ml</td>
</tr>
<tr>
<td>Blood</td>
<td>-</td>
<td>-</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Working Std 50 mg%</td>
<td>-</td>
<td>0.1 ml</td>
<td>-</td>
</tr>
<tr>
<td>Sodium tungstate 10%</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Acid Sulphuric 2/3 N</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
</tr>
</tbody>
</table>

Mix well and stand 5 minutes. Into pipette out as follows.
Mix and plug with cotton and place it in a boiling water bath for exactly 15 minutes; cool well. Take reading at 520 nm or by green filter.

**Calculation**

\[
\text{O. D test - Blank} \\
\text{-----------------} \\
\text{X 5 = mg%}.
\]

\[
\text{O.D Standard - O.D Blank}
\]

**4.2.4.3 Uric acid**

**Principal**

Uric acid reduces phosphotungstic acid in the presence of sodium carbonate to give blue colour, which can be measured calorimetrically.

**Reagents**

1. **Sodium tungstate 10%**: Dissolve 10 gram of sodium tungstate in distilled water and make up to 100 ml.

2. **Sulphuric acid 2/3 N**

   Dilute 2 ml of Sulphuric acid concentrated in distilled water and make up to 100 ml and standardize against a known sodium hydroxide solution.

3. **Sodium carbonate 10%**

   Dissolve 10 gm of sodium hydroxide in distilled water and make up to 100 ml.
4. **Phosphotungstic acid**

- Sodium tungstate : 100 gm
- Disodium hydrogen phosphate (anhydrous) : 20 gm
- Sulphuric acid (Conc) : 25 ml.
- Distilled water : to 1000 ml.

Weigh 100 gm of sodium tungstate and 20 gm of anhydrous disodium hydrogen phosphate and place in a flask. Dissolve in about 20 ml of water mix 25 ml of conc. Sulphuric acid with about 75 ml of water and pour the solution slowly into the flask, mix well. Boil gently for 1 hour under a reflux condenser. Cool and make up to 100 ml with distilled water, store in a brown bottle.

5. **Stock uric acid standard 100 mg%**

- Lithium carbonate : 60 mg
- Uric acid : 100 mg
- Formaldehyde : 2 ml.
- Acetic acid 50% : 1 ml.
- Distilled water : to 1000 ml

Dissolve 60 mg of lithium carbonate in about 25 ml of distilled water in a small beaker. Add 100 mg of uric acid and dissolve by gently warming, cool and add 2 ml of formaldehyde and 1 ml of 50% acetic acid and make up to 100 ml with distilled water; store in a brown bottle.

6. **Working uric acid standard 5 mg%**

Dilute 5 ml of stock to 100 ml with distilled water.
Mix well and stand 5 minutes, centrifuge, into 3 big test tubes pipette out as follows.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>4 ml</td>
<td>3.5 ml</td>
<td>3.5 ml</td>
</tr>
<tr>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>3.5 ml</td>
</tr>
<tr>
<td>Working Std 5 mg%</td>
<td>-</td>
<td>0.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>Sodium tungstate 10%</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Acid Sulphuric 2/3 N</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

Mix well. Take reading at 660 nm or by red filter.

Calculation

\[
\text{OD test} - \text{Blank} \times 5 = \text{mg%}. \\
\text{OD Standard} - \text{OD Blank}
\]

4.3 SEROLOGICAL ANALYSIS

Serological tests to detect the standard markers of HBV were done for all the volunteers.

Screening of hepatitis B viral markers were all analysed using the commercial ELISA kits mentioned below:
4.3.1 HBsAg: Hepatitis B surface antigen Bio-Rad Laboratories, Belgium

Principle of the test

The discovery of the Australian Antigen by Blumberg, et al., and its subsequent identification as the surface antigen of Hepatitis B Virus (HBsAg) represents a significant breakthrough in the understanding of viral hepatitis. It is known that the screening of blood donors and donated blood products for the presence of this antigen in serum has significantly reduced the occurrence of hepatitis B infection in blood transfusion recipients.

The chemical composition of the HBsAg consists of lipid, carbohydrate and protein. The protein moiety of HBsAg contains several polypeptides ranging in molecular weight from 23000 to 97000. The antigenic determinants on the protein moiety of HBsAg determine the specific characteristics of the different serotypes of the virus and are the basis of the immunoassay.

Other particles have been observed called Dane particles, which have two different antigenic sites: a superficial one, identifiable as HBsAg and an inner one identifiable as the core. It has also been suggested that HBsAg is a fragment of the viral lipoprotein capsid and the Dane particle could be the real virus.

Bio-Rad NovaPath™ HBsAg EIA is a solid phase sandwich immunoassay which employs monoclonal and polyclonal antibodies specific for HBsAg. A serum specimen is added to the antibody coated microtiter wells together with enzyme-conjugated polyclonal antibodies. HBsAg, if present, will form an antibody HBsAg antibody/enzyme complex. The plate is then washed to remove unbound materials. Finally, a solution of substrate is added to the wells and incubated. A blue color develops in proportion to the amount of HBsAg present in the specimen. The enzyme substrate reaction is then stopped and the absorbance is measured in a microplate reader at a wavelength of...
Requirements

1. HBs Ag detection kit (BIORAD).
2. ELISA reader and washer: Biotek instruments.

Procedure

1. Fix the required number of strips to the microtitre frame.
2. Dilute Conjugate Concentrate 1:26 with Conjugate Diluent.
3. Pipette 100μl of each of wells 1A, 1B and 1C and 100μl of Positive Control into wells 1D and 1E, respectively. Then pipette 100μl of each specimen into the remaining wells.
4. Pipette 25μl of prepared working Conjugate (from assay step 2) into each well and tap the frame gently to mix completely. Then incubate at 37± 1°C for 90 minutes.
5. Five to ten minutes before the end of the incubation, Dilute Substrate concentrate 1:101 with substrate buffer.
6. Aspirate the contents from all wells and wash each well 5 times with at least 300μl prepared Wash Solution per well for each wash.
7. Invert the place and tap it dry on absorbent paper. Pipette 100μl of prepared working Substrate (from assay step 5) into each well and incubate for 30 minutes at room temperature after mixing with a gentle tap.
8. Pipette 100μl of Stop Solution into each well and shake until the blue color in each well turns yellow.
9. Within 30 minutes, read the absorbance of Negative and Positive controls, plus specimens at 450 nm (wavelength 620 nm) against an air blank.
ASSAY VALIDATION

A run is considered valid if

1. At least two of three Negative Controls should be less than or equal to 0.200 OD. Also the Negative Control mean (NCX) should be greater than or equal to 0.000 and less than or equal to 0.200 OD.

2. All the Positive Controls should be greater than or equal to 0.800 OD.

CALCULATIONS

Calculation of the cut-off value

1. Calculate the Negative Control mean (NCX)
   
   Negative Control 1 absorbance 0.031
   Negative Control 2 absorbance 0.033
   Negative Control 3 absorbance 0.032

   NCX = (0.031 + 0.033 + 0.032) / 3 = 0.032

2. Calculate the cut-off value

   Cut-off value = NCX + 0.05 = 0.032 + 0.05 = 0.082

4.3.2 Anti-HBS

Elisa (Monolisa Anti-HBs-BIO RAD Belgium)

Monolisa Anti-HBs 3.0 is an immunoenzymatic method, which employs the two stage sandwich technique; it uses a mixture of HBs Ag (Hepatitis B surface antigen) of the ad subtype and ady subtype of human origin. The solid phase consists of strips of 8 polyesterene wells sensitized with a mixture of purified HBsAg. The conjugate uses the amplifying effect of the STREPTAVIDINE-BIOTIN system, the purified HBsAg mixture being linked with biotin. The peroxidase with streptavidine.

The assay includes the following steps...
1) Incubation of control and serum samples in the presence of HBsAg mixture immobilized on the solid phase.

2) Washing, then incubation with the mixture of HBsAg–Biotin and the streptavidine–Preoxidase conjugate.

3) Washing, then detection of enzyme activity bound to the solid phase by the addition of substrate.

4) Stopping of the reaction, then reading of the optical densities at 450/620 nm and interpretation of the results.

Requirements

2. ELISA reader and washer: Biotek instruments.

Quantitative determination

For accurate determination is based on a standard curve obtained using standards provided in the Monolisa anti-HBs standard calibration kit.

The negative control, standards, pure samples and samples diluted in negative control solution are distributed in to the corresponding wells.

1. Prepare the washing solution
2. Take the support frame and required number of strips out of the protective wrapping.
3. Add directly without prior washing of the plate, depending on the method selected
   a. Wells A1, B1, C1, D1, were added with 10 mIU/ml, 50 mIU/ml, 100 mIU/ml, and 150 mIU/ml, standards respectively
   b. Well E1, F1, G1 were added with kit positive control; Well H1, with kit positive control
   c. Add serum samples
4. Cover with adhesive film and then allow to incubate 60±5 minutes at 37± 1°C or 40± 1°C.

5. Prepare the conjugate solution

6. Remove the adhesive film, empty the contents of each well by suction in to the waste container, and then wash 4 times.

7. Add 100µl of conjugate solution to all the wells

8. Cover with adhesive film and allow to incubate for 60±5 minutes at 37± 1°C or 40± 1°C.

9. Remove the adhesive film, empty the contents of each well by suction into the waste container, then wash 5 times.

10. Prepare the colour development solution.

11. Distribute 200µl of the detection solution into each well and put the plate in the dark at room temperature (18-25°C for 30± 5°C).

12. Add 100µl of reaction–stopping solution into each well.

13. Carefully wipe the underside of the plate and read the optical density at 450/620 nm within 20 minutes of stopping the reaction.

Calculation and interpretation of results

Calculation of the cut-off value

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 0.075 to the mean of the Negative Control were calculated.

3. Positive control : Samples giving absorbance equal to or more than the cut-off value were considered positive in the test.
4. **Calculate the Negative Control mean (NCX)**

   **Negative Control 1** absorbance 0.031
   **Negative Control 2** absorbance 0.040
   **Negative Control 3** absorbance 0.037

   \[
   \text{NCX} = \frac{(0.031 + 0.040 + 0.037)}{3} = 0.036
   \]

The **positive standard**

**OD positive standard**

   **Positive control 1** absorbance 0.925
   **Positive Control 2** absorbance 0.961

   \[
   \text{PCX} = \frac{(0.925 + 0.961)}{2} = 0.943
   \]

5. Calculate the cut-off value

   \[
   \text{Cut-off value} = \text{NCX} + 0.075 = 0.036 + 0.075 = 0.111.
   \]

6. Using the graph paper, plot the optical densities recorded for the negative control and for each standard in the MONOLISA anti-HBs “Standards” calibration kit versus anti HB antibody concentration (abscissa) and draw the standard curve.

7. The determinations of mIU/ml of each sample are performed automatically on the spectrophotometer equipped with Hepatitis B cassette available through our technical services.

**Draw the standard curve**

The graph was plotted based on the optical densities recorded for the negative control and for each standard in the "Standard calibration kit" (ordinate) versus the anti-HBs antibody concentration (abscissa) and draw the standard curve.
Test validation conditions

a. Validation conditions are flows for screening and plasma and selection.
   1. All negative control values must be below 0.150 optical density units.
   2. All positive control values must be above 0.500 optical density units.

b. Validation conditions are as follows for the quantitative determination:
   a. All negative control values must be below 0.150 optical density units. The optical density recorded for the 150 mIU/ml standard must be greater than 0.750 optical density units.
   b. The difference in optical density between the 10 mIU/ml standard and the negative control mean must be greater than 0.075 optical density units.
   c. The ratios OD standard 50 mIU/ml to OD 10 mIU/ml and OD standards 100 mIU/ml to OD 50 mIU/ml must be as follows.

\[
\frac{\text{OD of standard 50 mIU/ml}}{\text{OD of standard 10 mIU/ml}} > 2.5
\]
\[
\frac{\text{OD of standard 100 mIU/ml}}{\text{OD of standard 50 mIU/ml}} > 1.2
\]

Interpretation of the results

Quantitative determination

Plot the optical densities recorded for assays of undiluted and 1/10 dilutions of the samples on a standard curve.

Assays of undiluted samples with an OD equal to the cutoff value will be considered negative.
For example, showing an OD within the range of cut-off value and the 150 mIU/ml standard (assayed undiluted or at 1/10 dilutions), the HBs antibody concentration is being determined from the standard curve.

In case of diluted samples, the results must be multiplied by the dilution factor to obtain the final concentration.

4.3.3 Anti-HBs Standards

Monolisa Anti-HBs Standards.

Principle

The reagents contained in the MONOLISA anti-HBs "standards" calibration kit are intended for use with the MONOLISA anti-HBs 3.0 test kit to allow the quantitative determination of antiHBs antibodies in human serum or plasma.

Samples

1. The assays are performed on serum or plasma. Each assay is to be carried out on pure serum, and serum diluted 1:10 using reagent R3 of the MONOLISA anti-HBs 3.0 test kit (20µl of serum + 180µl of negative control).

2. Specimens assayed after being diluted 1:10 and exhibiting an OD value higher than that recorded with the 150 mIU/ml standard should be retested at the final dilution of 1:100 (20µl of serum diluted 1:10 + 180µl of negative control.)

Procedure

1. The test procedures is as per the method of the MONOLISA antiHBs 3.0 test instructions.
2. The negative control in triplicate, the antiHBs standards and the unknown specimens (pure and diluted 1:10) must be run in parallel during each assay.

**Calculation and interpretation of the results**

Example of calibration curve obtained from the OD values recorded for the negative control and for each antiHBs standard.

On the calibration curve, plot the OD values recorded for the specimens assayed pure or diluted 1:10

Specimens assayed pure and exhibiting an OD value less than the cutoff value shall be considered negative. Specimens assayed after being diluted 1:10 and exhibiting an OD value higher than that recorded with the 150 mIU/ml standard should be retested at a final dilution of 1:100.

**4.3.4 Anti HBc IgM**

The IgM antibodies against hepatitis B core antigen (anti-HBc IgM) in serum was detected using Wellcozyme, Murex Diagnostics Ltd., UK.

**Summary and Explanation of the Test**

The presence of high concentrations of IgM class antibody to HBcAg has been shown to be an indicator of acute Hepatitis B infection. The level of anti-HBc IgM gradually decreases throughout the course of the disease and therefore, through the use of suitable "cut-off", acute cases of Hepatitis B can be identified. Anti-HBc IgM has been shown to be present in samples from patients with symptoms of acute illness when HBsAg is not detectable, and its measurement has assisted the correct diagnosis of patients who have multiple infections (HCV, HDV and HEV).

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

The HBsAg used to prepare the conjugate is genetically engineered. This ensures both continuity of supply and safety in handling since such HBcAg is non-infective.

The use of labelled HBsAg in the assay reduces the number of addition and incubation steps compared with the usual type of assay used for anti-HBc IgM and thus vastly reduces the total time required for the assay.

Principle of the Test

To measure the anti-HBc IgM, the test specimen is diluted before being incubated in a well, coated with a specific mouse monoclonal anti-IgM. After incubation, the well is washed to remove unbound material and the HBcAg conjugated to horseradish peroxidase is subsequently added. The conjugate binds to any anti-HBc IgM captured by the coating antibody and, after a final wash step, a sensitive peroxidase substrate is added which produces colour development.

Requirements

1. Anti HBC IgM detection kit (BIORAD).
2. ELISA reader and washer: Biotek instruments.

Procedure

Step 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. Mixed well.

Step 2 : The number of strips required for the test were used.

Step 3 : 150 μl of assay was placed into as many wells as there are samples and controls.
Step 4: 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.

Step 5: The wells were covered with a lid, and incubated in a water bath at 37°C for 30 min or at room temperature for 1 hour.

Step 6: At the end of the incubation period the wells were washed using the wash fluid.

Step 7: 200 µl conjugate was added to all wells.

Step 8: The wells were covered with a lid and incubated in a water bath at 37°C for 30 min or at room temperature for 45 min.

Step 9: Substrate solution is prepared ready.

Step 10: At the end of the incubation period, the wells were again washed.

Step 11: 200 µl of substrate solution was added to each well.

Step 12: Incubated for 30 min, at room temperature, in the dark.

Step 13: Added 50 µl of stop solution, to each well.

Step 14: The absorbance of each well was read at a wavelength of 450 nm, within 15 min, using a micro well plate reader.

Calculation of Results

1. The mean absorbance of the three positive control values and the two negative control values was calculated.

2. Cut-off = (0.4x Mean A₄₅₀ positive control) + Mean A₄₅₀ Negative control.

3. Any sample which gives absorbance greater than the cut-off should be considered positive to the IgM.

4. Any sample which gives absorbance % of the cut-off value should be considered and a follow-up taken.
Clinical Interpretation of Results

The detection of anti-HBc IgM in serum or plasma is used, in conjunction with the measurement of other Hepatitis B markers, to assess the status of known infected patients and those presenting with clinical symptoms of acute hepatitis.

In the acute stage of Hepatitis B, anti-HBc IgM is generally present in high titers, which decrease through the course of the illness. Thus, when HBsAg is detected in a sample, a positive anti-HBc IgM result usually indicates an acute infection while a negative result is associated with a chronic disease state. It is, however, important to note that a small minority of patients, particularly those who develop chronic aggressive hepatitis, have been shown to maintain appreciable titers of anti-HBc IgM for up to 2 years after infection.

It has been reported that anti-HBc IgM is sometimes the only specific marker in acute Hepatitis B. This situation occurs during early resolution of infection (the "window period") after clearance of HBsAg and HBeAg and before the appearance of anti-HBs.

4.4 ANALYSIS OF CMI MARKERS IN VACCINE RESPONDERS/NON RESPONDERS

4.4.1 In Vitro Cytokine assay

Requirements

EDTA

RPMI medium

Histopaque -1077 (Sigma chemical Co., St. Louis, MO, USA)

Preparation of medium for growth and maintenance of cells

1. RPMI -1640 with L-Glutamine (Hi media, India) - 10.4 g
2. Hapes (Hi media, India) - 5.96 g
3. Sodium bicarbonate (Hi media, India) - 2.1 g
4. Triple distilled water - 900 ml
5. New born calf serum
   (Sterile, Mycoplasma free: Hyclone, USA) - 100 ml

6. Antibiotics
   100 µg/ml Penicillin - 0.25ml
   50 µg/ml Gentamycin - 0.25ml

All the ingredients after aseptic addition were mixed together. The medium prepared was distributed in aliquots and stored at 4 °C till use. Sterility testing was done for each batch of medium.

Equipment used: Rota 4R-V/FM Plasto Crafts Refrigerated centrifuge

4.4.2 Isolation of PBMC

1. Add 10 ml of blood was drawn from a healthy donor to a tube containing EDTA.
2. Diluted with 10 ml RPMI prepared as per directions above.
3. This 20 ml of diluted blood was layered slowly over 10 ml of Histopaque-1077
4. Centrifugation was done at 2000 rpm for 20 minutes at 20°C.
5. The upper plasma layer was removed.
6. The PBMC layer seen as a buffy coat was pipetted out into a fresh tube.
7. 10 ml of fresh RPMI was added and PBMC were washed further to clear off the RBC contamination if any by centrifugation at 1800 rpm for 10 minutes at room temperature.
8. The supernatant was removed.
9. The pellet was resuspended in fresh RPMI and viable cells counted.
4.4.3 Determination of total cell count and viable cell number

Requirements

Haemocytometer

Trypan Blue

100 mg of Trypan blue dissolved in 100 ml phosphate buffered saline and filtered through Whatman filter No.4 was stored at 4°C till use.

Principle

Trypan blue is one of the several stains recommended for use in dye exclusion procedure for viable cell counting. This method is based on the principle that live cells do not take up dye when compared to dead cells.

Procedure

1. A cell suspension containing approximately $2-5 \times 10^5$ cells/ml was prepared in RPMI.

2. 0.5 ml of 0.4% trypan blue solution was transferred to a micro centrifuge tube.

3. 0.3 ml of RPMI and 0.2 ml of cell suspension (dilution factor 5) were added and mixed thoroughly and allowed to stand for 5 minutes.

4. The suspension was viewed in a haemocytometer and viable cells counted.

Cell enumeration was done as follows:

Cells per ml = Average count per square x dilution factor x $10^4$

Total cells = Cells per ml x Volume of which cell sample was removed
Cell viability = \[
\left\{ \frac{\text{Total viable cells (unstained)}}{\text{Total cells (stained and unstained)}} \right\} \times 100
\]

### 4.4.4 Plating of PBMC

#### Requirements

- 24 well TC plates (Nunc USA)
- Phytohemagglutinin (PHA) (GIBCO BRL, Gaithersburg, MD)
- RPMI 1640 (HI Media, India)

#### Procedure

1. **Step 1:** \(4 \times 10^6\) cells/ml/well were added to microtitre wells of the 24 well plates.
2. **Step 2:** PHA at a concentration of 50\(\mu\)g/ml was added to the control well.
3. **Step 3:** 20\(\mu\)g/ml of PHA (sub optimal concentration) was added to test wells.
4. **Step 4:** Different concentrations of test extracts in duplicates were added to appropriate wells.
5. **Step 5:** Incubation was carried out for 72 hrs. in a humidified 37°C, 5% CO2 incubator.

#### Control

- PBMC- Alone - Baseline
- PBMC + PHA - Positive Control

#### Test

PBMC + rDNA vaccine
Peripheral blood mononuclear cells PBMC were separated from 10 ml of heparinized blood drawn from healthy donor using histopaque – 1077.

Cells were counted, plated and incubated with different concentration of extracts dissolved in RPMI medium supplemented with antibiotics.

Control Test

<table>
<thead>
<tr>
<th>Condition</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC- Alone</td>
<td>Baseline</td>
</tr>
<tr>
<td>PBMC + PHA</td>
<td>Positive Control</td>
</tr>
<tr>
<td>PBMC + rDNA vaccine</td>
<td></td>
</tr>
</tbody>
</table>

The supernatant was removed from each well and stored at -70°C till EASIA was performed using the commercial kit procured from BIOSOURCE EUROPA S.A.

PBMCs were cultured by using commercially available RPMI medium.

4.5 EASIA BASED CYTOKINE QUANTITATION ASSAY USING COMMERCIAL KIT (BIOSOURCE EUROPA S.A.)

It is a solid phase Enzyme Amplified Sensitivity Immunoassay (EASIA) performed on microtitre plates. The assay is based on an oligoclonal system in which blends of monoclonal antibodies (MAbs) are directed against distinct epitopes of the cytokine under study. Antibody producing cells are immortalised using myeloma cell fusion to produce hybridoma cells. These cells secrete specific homogeneous antibodies, which can bind to a particular cytokine present in the test samples. The binding is detected using a second monoclonal antibody (MAb2) labeled with horseradish peroxidase (HRP). It is then incubated to form a sandwich of coated Mabs 1-Cytokine -MAB 2-HRP. Bound -labeled antibodies are
measured through a chromogenic reaction using chromogenic solution (TMB+H$_2$O$_2$).

4.5.1 Estimation of Interleukin -2 (IL-2) EASIA (BIOSOURCE EUROPE SA)

Principle of the Test

The BIOSOURCE IL-2 EASIA is a solid phase Enzyme Amplified Sensitivity Immunoassay (EASIA) performed on microtitre plate. The assay is based on an oligoclonal system in which blends of monoclonal antibodies (MAbs) directed against distinct epitopes of IL-2 are used. Standards or samples containing IL-2 react with capture monoclonal antibodies (MAbs 1) coated on the microtitre well and with a monoclonal antibody (MAbs 2) labelled with horseradish peroxidase (HRP). After an incubation period allowing the formation of a sandwich: coated MAbs 1 – IL-2 – Mabs2-HRP, the microtitre plate is washed to remove unbound enzyme labelled antibodies. Bound enzyme labelled antibodies is measured through a chromogenic reaction. The reaction is stopped with the addition of stop solution and the microtitre plate is then read at the appropriate wavelength. The amount of substrate turnover is determined calorimetrically by measuring the absorbance, which is proportional to the IL-2 concentration. A standard curve is plotted and IL-2 concentrations in a sample are determined by the interpolation from the standard curve.

Requirements

1. IL-2 detection kit (IL-2 EASIA, BIOSOURCE EUROPE SA).

2. ELISA reader and washer: Biotek instruments.

Procedure

1. Number of strips for the run was selected.

2. 100 µl of Solution B (diluent for plasma / serum samples) was pipetted into the appropriate well for the Standards and Controls.
3. 100 μl of each Standards (0 U/ml, 0.9 U/ml, 2.3 U/ml, 7.5 U/ml, 15 U/ml, 30 U/ml), Controls (Low Positive control and High Positive Control), or Sample were pipetted into appropriate wells.

4. 50 μl of anti-IL-2 Conjugate was pipetted into all the wells.

5. Incubated for 2 hours at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm.

6. The liquid from each well was aspirated.

7. The plate was washed three times by:
   a. Dispensing of 0.4 ml of Wash solution into each well.
   b. Aspirating the contents of each well.

8. 100μl of Chromogen was pipetted into each well within 15 minutes following the washing step.

9. The plate was incubated for 15 minutes at room temperature on horizontal shaker at 700 rpm ± 100 rpm, avoiding direct sunlight.

10. 200 μl of stop solution was pipetted into each well.

11. The absorbance at 450 nm (reference filter: 630 or 650nm) was read within 3 hours and the results were calculated.

**Calculation of Results**

Standard curve was constructed using all standard points for which absorbance are below the limit of linearity of reader use. The OD on the ordinate was plotted against the standard concentrations on the abscissa using either linear or semi-log graph paper and the curve was drawn by connecting the plotted points with straight lines. Then the IL-2 concentrations of samples or controls was determined for which absorbance of the last...
4.5.2 Estimation of Interleukin -12 (IL-12) EASIA (BIOSOURCE EUROPE SA)

Interleukin -12

Human Interleukin-12 (IL-12) is a 75 kDa lymphokine produced mainly by monocytes, macrophages, B-lymphocytes, and dendritic cells. IL-12 shows an unusual heterodimeric structure composed of one 40 kDa (p40) and of one 35 kDa (p35) subunits linked together by disulfide bonds. P35 subunit is distantly related to IL-6 and G-CSF while p40 shows homology to the extracellular domain of the α chain of the IL-6 receptor. This suggests that IL-12 may have evolved from a cytokine/soluble receptor complex.

P40 is secreted in large excess over the biologically active heterodimer. P40 is involved in receptor binding but p35 is necessary for signal transduction. Monomers and mainly homodimers of p40 show antagonist activity to IL-12.

In vivo, IL-12 appears to play a major role in auto-immune disease, in the resistance to bacterial and parasitic infections, in antiviral responses including HIV, in the promotion of antitumor immunity. IL-12 has been shown to be a powerful adjuvant in vaccination.

Principle of the Test

The BIOSOURCE IL-12 + p40, EASIA is a solid phase Enzyme Amplified Sensitivity Immunoassay (EASIA) performed on microtitre plate. The assay is based on an oligoclonal system in which a blend of monoclonal antibodies (Mabs) directed against distinct epitopes of IL-12 + p40 are used. Antibody producing cells are immortalized using the myeloma cell fusion method of Kohler and Milstein. A hybridoma cell is produced which secretes specific homogenous antibodies.

The use of a number of distinct Mabs avoids hyperspecificity and allows high sensitive assays with extended standard range and short incubation time. Standards or samples containing IL-12 + p40 coated with capture monoclonal antibodies (MAb1) coated on the bottom of an incubation, the occasional
excess of antigen is removed by washing. Mab 2, the horseradish peroxidase (HRP)-labelled-antibody, is then added. After an incubation period allowing the formation of a sandwich coated MAbs 1-IL-12 + p40-Mabs-HRP, the microtitre plate is washed to remove unbound enzyme labeled antibodies are measured through a chromogenic reaction. Chromogenic solution (TMB+H2O2) is added and incubated. The reaction is stopped with the addition of stop solution (H2SO4) and the microtitre plate is then read at the appropriate wavelength. The amount of substrate turnover is determined calorimetrically by measuring the absorbance, which is proportional to the IL-12 +p40 concentration. A standard curve is plotted and IL-12 + p40 concentrations in a sample is determined by interpolation from the standard curve. The use of the EASIA Reader (linearity up to 3 OD units) and a sophisticated data reduction method (polychromatic data reduction) result in high sensitivity in the low range and in an extended standard range.

Requirements

**IL-2 detection kit (IL-2 EASIA, BIOSOURCE EUROPE SA).**

ELISA reader and washer: Biotek instruments.

Procedure

1. Number of strips for the run was selected.

2. 100 μl of Solution B (diluent for plasma / serum samples) was pipetted into the appropriate wells foreseen for the Standards and Controls.

3. 100 μl of each Standards (0 pg/ml, 15 pg /ml, 35 pg /ml, 100 pg/ml, 400 pg/ml, 1300 pg/ml), Controls (Low Positive control and High Positive Control), or Sample were pipetted into appropriate wells.

4. Incubated for 2 hours room temperature on a horizontal shaker set at 700 rpm ± 10 rpm.

5. The liquid from was a.
6. The plate was washed three times by:

7. Dispensing of 0.4 ml of Wash solution into each well.

8. Aspirating the contents of each well.

9. Pipette 100μl of IL-12-P 40 conjugate into all wells.

10. Incubated for 2 hours at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm.

11. 200μl of Chromogen was pipetted into each well within 15 min following the washing step.

12. The plate was incubated for 15 min at room temperature on a horizontal shaker at 700 rpm ± 100 rpm, avoiding direct sunlight.

13. 50 μl of stop solution was pipetted into each well.

14. The absorbance at 450 nm (reference filter: 630 or 650nm) was read within 3 hours and the results were calculated.

**Calculation of Results**

Standard curve was constructed using all standard points for which absorbance were below the limit of linearity of reader use. The OD on the ordinate was plotted against the standard concentrations on the abscissa using either linear or semi-log graph paper and the curve was drawn by connecting the plotted points with straight lines. Then the IL-12 concentrations of samples or controls was determined for which absorbance of the last standard read at 450nm.

**4.5.3 Estimation of Interferon - (IFN-γ) by EASIA (BIOSOURCE EUROPE SA)**

**Principle of the Test**

The BIOSOURCE IASIA is a phase Enzyme Amplified Sensitivity Immunoassay (EASIA) plate. The assay is based on an oligoclonal system of monoclonal antibodies (MAbs).
directed against distinct epitopes of IFN-γ are used. Standards or samples containing IFN-γ react with capture monoclonal antibodies (MAbs 1) coated on the microtitre well and with a monoclonal antibody (MAbs 2) labelled with horseradish peroxidase (HRP). After an incubation period allowing the formation of a sandwich: coated MAbs 1 – IFN-γ– Mabs2-HRP, the microtitre plate is washed to remove unbound enzyme labelled antibodies. Bound enzyme labelled antibodies are measured through a chromogenic reaction. Chromogenic solution (TMB + H₂O₂) is added and incubated. The reaction is stopped with the addition of stop solution and the microtitre plate is then read at the appropriate wavelength. The amount of substrate turnover is determined calorimetrically by measuring the absorbance which is proportional to the IFN-γ concentration. A standard curve is plotted and IFN-γ concentrations in a sample are determined by the interpolation from the standard curve.

Requirements

1. IFN-γ detection kit (IFN-γ EASIA, BIOSOURCE EUROPE SA).
2. ELISA reader and washer: Biotek instruments.

Procedure

1. The number of strips for the run was selected.
2. 50 μl of each Standards (0 IU/ml, 1 IU/ml, 2 IU/ml, 5 IU/ml, 10 IU/ml, 30 IU/ml), Controls (Low Positive control and High Positive Control), or Sample were pipetted into appropriate wells.
3. 50 μl of anti- IFN-γ Conjugate was pipetted into all the wells.
4. Incubated for 2 hours at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm.
5. The liquid from each well was aspirated.
6. The plate was washed three times by:
15. Dispensing of 0.4 ml of Wash solution into each well.

16. Aspirating the contents of each well.

7. 200µl of Chromogen was pipetted into each well within 15 min following the washing step.

8. The plate was incubated for 15 min at room temperature on horizontal shaker at 700 rpm ± 100 rpm, avoiding direct sunlight.

9. 50 µl Stop solution was pipetted into each well.

10. The absorbance at 450 nm (reference filter: 630 or 650nm) was read within 3 hours and the results were calculated.

Calculation of Results

Standard curve was constructed using all standard points for which absorbance were below the limit of linearity of reader use. The OD on the ordinate was plotted against the standard concentrations on the abscissa using either linear or semi-log graph paper and the curve was drawn by connecting the plotted points with straight lines. Then the IFN-γ concentrations of samples or controls was determined for which absorbance of the last standard read at 450nm.

4.5.4 Estimation of Tumour Necrosis Factor - α (TNF- α)

Principle of the Test

The BIOSOURCE TNF- α EASIA is a solid phase Enzyme Amplified Sensitivity Immunoassay (EASIA) performed on microtitre plate. The assay is based on an oligoclonal system in which a blend of monoclonal antibodies (MAbs) directed against distinct epitopes of TNF- α are used. Standards or samples containing TNF- α react with capture monoclonal antibodies (MAbs 1) coated on the microtitre well and with a monoclonal antibody (MAbs 2) labelled with horseradish peroxidase (HRP). After an incubation period allowing the formation of a sandwich: coated MAbs 1 – TNF- α– Mabs2-HRP, the microtitre plate is washed to remove unbound enzyme labelled antibodies. Bound enzyme labelled antibodies
is measured through a chromogenic reaction. Chromogenic solution (TMB + H₂O₂) is added and incubated. The reaction is stopped with the addition of stop solution and the microtitre plate is then read at the appropriate wavelength. The amount of substrate turnover is determined calorimetrically by measuring the absorbance, which is proportional to the TNF-α concentration. A standard curve is plotted and TNF-α concentrations in a sample are determined by the interpolation from the standard curve.

Requirements

1. TNF-α detection kit (TNF-α EASIA, BIOSOURCE EUROPE SA).
2. ELISA reader and washer: Biotek instruments.

Procedure

1. The number of strips for the run was selected.
2. 50 μl of incubation buffer was pipetted into all wells.
3. 200 μl of each Standards (0 pg/ml, 15 pg/ml, 50 pg/ml, 150 pg/ml, 500 pg/ml, 1500 pg/ml), Controls (Low Positive control and High Positive Control), or Samples were pipetted into appropriate wells.
4. Incubated for 2 hours at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm.
5. The liquid from each well was aspirated.
6. The plate was washed three times by:
   a. Dispensing of 0.4 ml of Wash solution into each well.
   b. Aspirating the contents of each well.
7. 100 μl of standard 0 was pipetted into all wells.
8. 50 μl of anti-TNF-α conjugate was pipetted into all wells.
9. Incubate for 2 hours at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm.

10. The liquid from each well was aspirated.

11. The plate was washed three times by:
   a. Dispensing of 0.4 ml of Wash solution into each well.
   b. Aspirating the contents of each well.

12. 200μl of freshly prepared Chromogen was pipetted into each well within 15 minutes following the washing step.

13. The plate was incubated for 30 min at room temperature on a horizontal shaker at 700 rpm ± 100 rpm, avoiding direct sunlight.

14. 50 μl stop solution was pipetted into each well.

15. The absorbance was read at 450 nm (reference filter: 630 or 650nm) within 3 hours and the results were calculated.

Calculation of Results

Standard curve was constructed using all standard points for which absorbance is below the limit of linearity of reader use. The OD on the ordinate was plotted against the standard concentrations on the abscissa using either linear or semi-log graph paper and the curve was drawn by connecting the plotted points with straight lines. Then the TNF - α concentrations of samples or controls was determined for which absorbance of the last standard read at 450 nm.

Statistical analysis

All the results of the study were analysed by using statistical package SPSS version 4.0, In Institute for research medical statistics) and whenever applicable Levene's test and Man-Whitney was done at YRG Care, Chennai - 113.
## 4.6 REFERENCE VALUES FOR NORMAL HEALTHY VOLUNTEERS

### 4.6.1 Hematology

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RBC Count</td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>4.6 – 6.0 millions/cmm</td>
</tr>
<tr>
<td>Females</td>
<td>4.2 – 5.4 millions/cmm</td>
</tr>
<tr>
<td>Total WBC Count</td>
<td>4000 – 11000/ cu mm</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>13.5 – 18 g/dL</td>
</tr>
<tr>
<td>Females</td>
<td>12 – 16 g/dL</td>
</tr>
<tr>
<td>Neutrophiles</td>
<td>40 – 80%</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>20 – 40%</td>
</tr>
<tr>
<td>Eosinophiles</td>
<td>1 – 6%</td>
</tr>
<tr>
<td>Erythrocytes sedimentation rate</td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>4 – 15 mm</td>
</tr>
<tr>
<td>Females</td>
<td>4 – 20 mm</td>
</tr>
</tbody>
</table>

### 4.6.2 Liver functions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Bilirubin</td>
<td>Upto 1.0 mgs%</td>
</tr>
<tr>
<td>SGOT</td>
<td>5 – 42 IU/L</td>
</tr>
<tr>
<td>SGPT</td>
<td>5 – 42 IU/L</td>
</tr>
<tr>
<td>Serum Alkaline Phosphatase</td>
<td>37 – 147 IU/L</td>
</tr>
</tbody>
</table>

### 4.6.3 Renal functions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine</td>
<td>0.9 – 1.4 mg/dL</td>
</tr>
<tr>
<td>Urea</td>
<td>10 – 50 mg/dL</td>
</tr>
<tr>
<td>Uric acid</td>
<td>3 – 7 mg/dL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Males</th>
<th>3 – 7 mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td>2 – 5 mg/dL</td>
</tr>
</tbody>
</table>
4.7 CRITERIA FOR IMMUNOGENICITY

A titer of at least 1 mIU/ml or more was interpreted as seroconversion and a titer above 10 mIU/ml was considered as seroprotection. The anti-HBs values were converted as geometric mean titer (GMT) of anti-HBs and compared for statistical significance using Mann–Whitney test and Levenes test.
Vaccines used for the study
Blood collection from Adult volunteer
School involved in the Adolescent trial (Kamaraj Avenue Higher Secondary School)
Blood collection from Adolescent volunteers
Blood collection from babies born to HBsAg Positive mothers
Vaccination of Infants born to HBs Ag Positive mothers
Examination of Infants born to HBsAg Positive mothers for adverse events
Mother and Child Pairs at the time of follow up